Primäre und sekundäre Signalwege im AhR-ARNT-System: Definition der gewebsspezifischen Genexpressions-signatur in Zellen des Immunsystems

Inaugural-Dissertation

zur

Erlangung des Doktorgrades der

Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Markus Frericks

aus

Duisburg

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Inhaltsverzeichnis

Inhaltsverzeichnis		I
Abkürzungsverzeichnis		III
Zusammenfassung		V
1. Einleitung		1
1.1	Biologische Bedeutung der halogenierten aromatischen Hydrokarbone (HAKs)	2
1.2	Der Arylhydrokarbon Rezeptor (AhR) als Vermittler der HAK-Toxizität	3
1.3	Kontrolle des AhR-Signalwegs und Mechansimen der Spezifität	6
1.4	AhR-Toxizität mit Schwerpunkt auf dem Immunsystem	11
1.5	Mausmodelle des AhR	16
1.6	Das duale Modell des AhR der AhR-Aktivität	19
1.7	Toxicogenomic des AhR-Systems	21
1.8	Fragestellung	26
2. Ergebnisse		27
2.1	Veröffentlichungen/Manuskripte als Erstautor	28
2.1.1	- Transcriptional signatures of immune cells in aryl hydrocarbon receptor (AhR)- proficient and AhR-deficient mice	28
2.1.2	- Microarray analysis of the AhR system: tissue-specific flexibility in signal and target genes	30
2.1.3	- Transcription factor crosstalk controls transcriptional response to AhR-over-activation by TCDD in thymic epithelial cells (eingereicht)	32
2.2	Für diese Arbeit wichtige Veröffentlichungen als Koautor	35
2.2.1	- Role of the aryl hydrocarbon receptor in thymocyte emigration in vivo	35
2.2.2	- Detection of a novel population of fetal thymocytes characterized by preferential emigration and a TCR γδ+ T cell fate after dioxin exposure	36
2.2.3	- Impairment of maturational competence of cultured Langerhans cells from aryl hydrocarbon receptor (AhR) null mice and suppression of AhR signalling points to its role in LC tolerogenic strategy (eingereicht)	38

3. Diskussion 40
3.1 TCDD-induzierte Expressionsänderungen 40
3.2 Verminderte Genexpression nach AhR-Aktivierung 42
3.3 Identifikation einer AhR-Zielgenbatterie 44
3.4 Anwendung von Zielgenbatterien 46
3.5 Limitationen und Möglichkeiten der Datenbank NC-GED 48
3.6 Nutzung der Datenbank zur Vorhersage von AhR-vermittelten Effekten 49

4. Literaturverzeichnis 54

5. Anhang 67
A Veröffentlichungen A
B Inhalt der beigefügten DVD B
C Zusätzliche Dateien der Paper und die Datenbank NC-GED auf DVD C

Abkürzungsverzeichnis

TCDD	2,3,7,8-Tetrachlordibenzo-*p*-dioxin
HAKs	Halogenierte aromatische Kohlenwasserstoffe
AhR	Arylhydrokarbon Rezeptor
Arnt	AhR nukleärer Translokator
DRE	Dioxin-responsives Element
PCBs	Polychlorierte Biphenyle
PCFDs	Polychlorierte Dibenzofurane
PCDDs	Polychlorierte Dibenzodioxine
DZ	Dendritische Zelle
PBDEs	Polybromierte Diphenylether
Hsp90	Hitzeschock Protein 90
Aip	AhR interagierendes Protein
Rbp	Retinoblastomaprotein
TFBS	Transkriptionsfaktorbindestelle
PWM	Positions-Gewichtungs-Matrix
cAMP	Zyklisches AMP
LDL	Low density Lipoprotein
FICZ	6-formylindolo[3,2-b]carbazole
Hif1α	Hypoxie induzierbarer Factor 1 alpha
Hif1β	Hypoxie induzierbarer Factor 1 beta (Arnt)
ÖR	Östrogenrezeptor
AhRR	AhR Repressor
miRNA	Mikro RNA
APZ	Antigen präsentierende Zelle
TZR	T-Zell-Rezeptor
DN	Doppelt negativ
MHC	Haupthistokompatibilitätskomplex (Abkürzung *engl.* von Major Histocompatibility Complex)
TEZ	Thymusepithelzellen
B[a]P	Benzo[a]pyren
DMBA	Dimethylbenzo[a]anthrazen
IFNγ	Interferon gamma

IL-12	Interleukin 12
DEX	Dexamethasone
GRE	Glykokortikoid-responsives Element
HRE	Hypoxie-responsives Element
ÖRE	Östrogen-responsives Element
LZ	Langerhans Zellen
OVA	Ovalbumin
ConA	Concavalin A
P_{O2}	Sauerstoffpartialdruck
E2	17-β-Estradiol
bHLH-PAS	basic-Helix-Loop-Helix-Per-Arnt-Sim (bHLH-PAS)-Superfamilie
BPDE	7,8-dihydroxy-9,10-epoxy-7,8,9,10-benzo[a]pyren
GSEA	Gene set enrichment analysis

Zusammenfassung

Der Arylhydrokarbon Rezeptor (AhR), ist ein ligandeninduzierter Transkriptionsfaktor, der im Metabolismus und der Vermittlung der toxischen Effekte vieler Umweltschadstoffe eine wichtige Rolle spielt. Zu diesen Substanzen gehören polychlorierte aromatische Kohlenwasserstoffe (HAKs), deren giftigster Vertreter 2,3,7,8-Tetrachlordibenzo-*p*-dioxin (TCDD) unter anderem eine Suppression des Immunsystems auslöst. Nach Ligandenbindung bildet der AhR mit Arnt (AhR nuclear translocator) einen heterodimeren Komplex, der durch die Bindung an DNA-Elemente adverse Effekte hervorruft. Ob die regulierten Gene und somit induzierten Effektormechanismen für alle Zelltypen gleich sind, ist jedoch ungeklärt.

Die Untersuchung der TCDD-vermittelten Expressionsänderungen in Zellen des Immunsystems zeigen, dass es keine einheitlichen Zielgene und somit keinen einheitlichen Effektormechanismus gibt. Viele Gene werden verringert ausprägt, was durch den klassischen Weg der Geninduktion durch den AhR nicht erklärt wird. Die Identifizierung der direkten AhR-Zielgene wurde durch eine TCDD-vermittelte veränderte Reifung der belasteten Thymozyten und Thymusemigranten verhindert.

Die TCDD-induzierten Expressionsänderungen wiesen eine hohe Zelltyp- und Gewebsspezifität auf. Dennoch konnte eine Reihe von Genen identifiziert werden, die in mehreren Transkriptionsstudien AhR-abhängig reguliert waren. Mit diesen Genen wurde eine zu diesem Zweck aufgebaute, Genexpressionsdatenbank durchsucht, um weitere AhR-abhängige Prozesse (Modelle) zu identifizieren. Eines dieser Modelle war die Untersuchung der Kontrolle der Präsentation von Selbstantigenen in Thymusepithelzellen (TEZ), die eine wichtige Rolle bei der Differenzierung von Thymozyten spielt. Die Thymozytenreifung wird bei Belastung mit TCDD verändert. Um die TCDD-induzierten Effektormechanismen in TEZ zu untersuchen, wurde die zeitabhängige, TCDD-induzierte Transkriptionsänderung in einer kortikalen TEZ-Zelllinie charakterisiert. Eine Promoteranalyse der regulierten Gene zeigte eine signifikante Häufung von Bindestellen für HIF, dem Östrogenrezeptor (ÖR) sowie einiger weiterer Transkriptionsfaktoren. TCDD beeinflusste die HIF- oder ÖR-abhängigen Transkriptionsänderungen und umgekehrt. Das zeigt, dass der AhR mit anderen Signalwegen interagieren kann und dieses bei der Analyse der zellspezifischen TCDD-Effekte berücksichtigt werden muss.

Diese Resultate liefern neue Einblicke in die fremdstoffinduzierte Immunsuppression und bieten somit neue Ansatzpunkte für die therapeutische Manipulation der involvierten Prozesse.

1. Einleitung

Viele Chemikalien sind in der Lage mit dem Immunsystem zu interagieren. Dabei können sowohl Immunantworten ausgelöst als auch unterdrückt werden. Verstärkte Immunantworten können zu Allergien oder Autoimmunerkrankungen führen, während eine Unterdrückung der Immunantwort eine Immunsuppression zur Folge hat.

Die Immuntoxikologie untersucht die Auswirkungen und Wirkmechanismen von Fremdstoffen auf die Funktionen des Immunsystems und versucht aufzuklären, welcher Art die Beeinträchtigungen der partizipierenden physiologischen Prozesse sind. Eine immuntoxikologisch wirksame Substanzklasse sind die halogenierten aromatischen Kohlenwasserstoffe (HAKs), deren prominentester – und zugleich giftigster- Vertreter 2,3,7,8-Tetrachlordibenzo-*p*-dioxin ist, welches auch unter dem Namen „Seveso-Gift" oder Dioxin bekannt ist(146;149;153). Von einer Immunsuppression spricht man, wenn das Immunsystem aufgrund gegebener Umstände (z.B. γ-Strahlen oder immunsuppressive Substanzen) weniger effizient als gewöhnlich funktioniert. Dies äußert sich beispielsweise im vermehrten Auftreten von Infektionen oder der Entwicklung von Tumoren, kann sich aber auch in der Verlängerung von Krankheitssymptomen zeigen.

Viele Vertreter der HAKs, zu der unter anderem polychlorierte Biphenyle (PCBs), Dibenzofurane (PCDFs) und Dibenzodioxine (PCDDs) gehören, entfalten ihre toxische Wirkung als Liganden eines Transkriptionsfaktors, des Arylhydrokarbon Rezeptors (AhR). Dabei sind die Ausmaße und Art der toxischen Effekte sehr stark vom Liganden und der betroffenen Spezies abhängig. So reicht der LD_{50}-Wert von nur 0,6 µg/kg Körpergewicht in Meerschweinchen bis zu 5 mg/kg Körpergewicht im syrischen Goldhamster(75).

Die Bindung eines exogenen Liganden an den AhR und seine Aktivierung über das physiologische Niveau hinaus wird als AhR-Überaktivierung bezeichnet. Eine Überaktivierung durch TCDD zeigt in Mäusen teratogene, kanzerogene, kardiovaskuläre und hepatotoxische Effekte, die von einer allgemeinen Gewichtsabnahme, dem so genannten „Wasting-Syndrom", begleitet werden. Darüber hinaus kommt es zu einer massiven Immunsuppression, die mit einer Atrophie des Thymus einhergeht(52).

Zur Behandlung von nicht gewünschten Immunantworten, beispielsweise bei Allergien, Entzündungsreaktionen oder Transplatabstoßungsreaktionen ist die Unterdrückung einer Immunantwort oft therapeutisch wichtig. Daher ist ein Verständnis der zugrunde liegenden Prozesse von großem wissenschaftlichem Interesse.

Eine Immunsuppression wirkt sich oft unterschiedlich auf die verschiedenen Kompartimente des Immunsystems, also die verschiedenen Zelltypen und ihre Umgebung, aus. Die toxikologische Wirkung einer AhR-Überaktivierung durch TCDD betrifft dabei sowohl Komponenten der angeborenen, als auch der adaptiven Immunantwort(52;69;159). So kommt es unter anderem zu einer Reifungsstörung von antigenpräsentierenden Zellen, wie B-Zellen und dendritische Zellen. TCDD wirkt auf Makrophagen und hematopoetische Stammzellen(107;128), verändert die Antikörperproduktion(106), die Zytokin- und Interleukinausschüttung(81) und hat massive Auswirkungen auf die T-Zellentwicklung(33). Während die phänotypischen Veränderungen umfassend beschrieben wurden, sind die zugrunde liegenden Mechanismen, also die veränderte Genexpression, welche primär die Effekte hervorruft, noch nicht ausreichend geklärt.

1.1 Biologische Bedeutung der halogenierten aromatischen Kohlenwasserstoffen (HAKs)

Bei den HAKs handelt es sich um eine chemisch sehr diverse Substanzklasse. Sie umfasst die Substanzklassen der polychlorierten Biphenyle (PCBs), Dibenzofurane (PCFDs) und Dibenzodioxine (PCDD), sowie der in den letzten Jahren verstärkt als Flammschutzmittel eingesetzten polybromierten Diphenylether (PBDEs)(17;21;64). Neben ihrer beabsichtigten chemischen Produktion entstehen sie primär als Nebenprodukte in der Petrochemie, der chemischen Synthese, sowie bei unvollständigen Verbrennungen von langkettigen Kohlenwasserstoffen und bei erhöhten Temperaturen in elektrischen Geräten, wie z.B. in Umspannwerken(32;53). Viele HAKs sind hoch persistent und werden biologisch kaum abgebaut. So führte die Torfverbrennung im Süden Englands über die Jahrhunderte zu einer deutlich messbaren Akkumulation verschiedener HAKs(98).

HAKs sind stark lipophil, sammeln sich in fettreichem Gewebe an und werden so über die Nahrungskette angereichert und weitergegeben. So kommt es z.B. in der maritimen Nahrungskette zu einer Akkumulation, die sich dann bei Populationen mit sehr fischhaltiger Ernährung, wie beispielsweise den Inuit, in der Muttermilch nachweisen läßt(25). Obwohl die HAK-Freisetzung in den Industriestaaten durch den weit verbreiteten Einsatz von industriellen Filtertechniken stark zurückgegangen ist(32), ist in den nächsten Jahren gerade durch das klimatisch bedingte verstärkte Auftreten von Waldbränden(109), sowie einer erhöhten Freisetzung durch die aufstrebenden Schwellenländer, insbesondere China, mit einem erneuten Anstieg zu rechnen. Gerade in den nordchinesischen Industrieanlagen werden

ungefiltert stark Schwermetall- und HAK-haltige Abgase produziert(87). Durch Ausbreitung der Wüste Gobi kommt es vermehrt zu Sand- und Staubstürmen. Überqueren diese die nordchinesischen Industrieanlagen kommt es zu einer Beladung des Staubs mit diesen Industrieabfällen und einem signifikanten Eintrag dieser Bestandteile in den südostasiatischen Raum. Das so entstehende Gesundheitsproblem, auch „Toxic-Dust-Syndrom" genannt, fordert Schätzungen zur Folge allein in Südkorea jährlich 200 Tote und führt zu weit verbreiteten Gesundheitsproblemen(12). Die Effekte beruhen dabei wahrscheinlich auf einer kumulativen Wirkung von Partikeln, Schwermetallen und HAKs. Daher ist ein genaues Verständnis der HAK vermittelten Toxizität nach wie vor von großem gesundheitspolitischen und wirtschaftlichen Interesse.

Da viele HAKs metabolisch verändert werden, und die verschiedenen Metaboliten teilweise unterschiedlich zur Gesamttoxizität der Substanz beitragen, wurde in dieser Arbeit mit TCDD gearbeitet, welches nicht metabolisiert wird. Die chemische Struktur von TCDD ist in Abbildung 1 dargestellt.

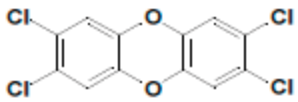


Abbildung 1: Chemische Struktur des AhR-Liganden 2,3,7,8-Tetrachlordibenzo-*p*-dioxin

1.2 Der Arylhydrokarbon Rezeptor (AhR) als Vermittler der HAK-Toxizität

Ein breites Spektrum der HAK-vermittelten Toxizität wird durch einen ligandeninduzierten Transkriptionsfaktor, den Arylhydrokarbon Rezeptor (AhR), vermittelt. So sind Mausstämme mit einer Deletion oder funktionellen Mutation dieses Rezeptors gegenüber den adversen Effekten einer TCDD-Exposition weitestgehend resistent(40;80;100;101). Der AhR gehört zur Familie der basic-Helix-Loop-Helix-Per-Amt-Sim (bHLH-PAS)-Superfamilie der DNA-bindenden Moleküle(10;100). Unter physiologischen Bedingungen liegt der AhR als Multiproteinkomplex im Zytosol vor. Er ist mit zwei Moleküle des Hitzeschockproteins 90 (Hsp90), sowie dem Co-Chaperon Aip (AhR interagierendes Protein) assoziiert(14;80;117).

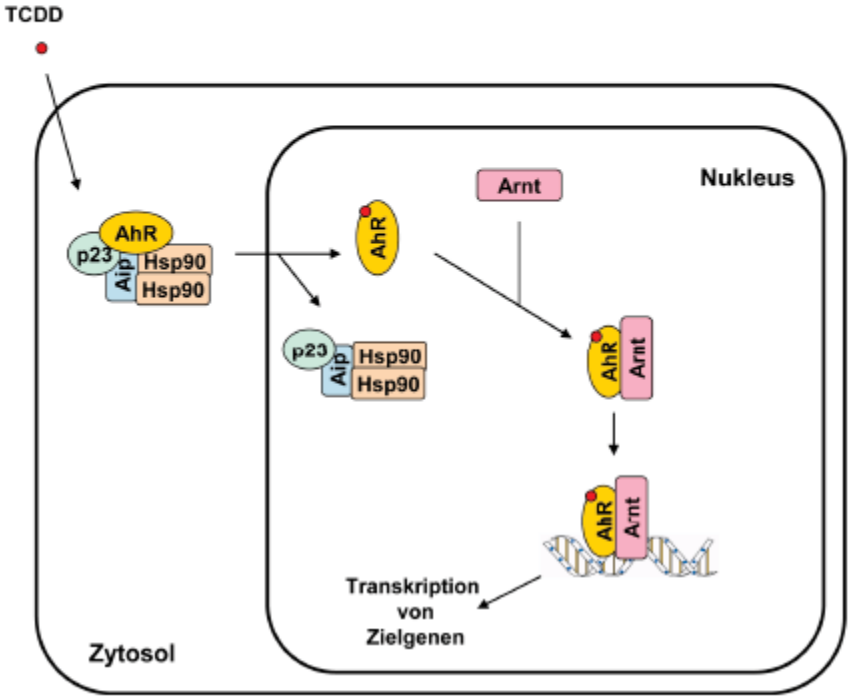


Abbildung 2: Schematisches Modell des AhR-Signalweges

AhR-Liganden, wie TCDD, binden den im Zytosol als Komplex mit Aip, Hsp90 und p23 vorliegenden AhR. Die Bindung induziert eine Translokation des AhR in den Nukleus, wo die Co-Chaperone abdissoziieren, und sich ein transkriptionell aktives Heterodimer aus AhR und Arnt bildet. Eine Bindung des AhR/Arnt-Heterodimers an dioxinresponsive Elemente führt zur veränderten Transkription von Zielgenen.

In Abhängigkeit vom untersuchten Zelltyp sind weitere Proteine mit dem AhR-Komplex aggregiert. Hierzu zählen das Retinoblastomaprotein (Rbp), Immunophilin p23, oder das Protoonkogen c-Src(19;27;28).

Bei einer Aktivierung des AhR durch einen Liganden kommt es zu einer Konformationsänderung und Translokation in den Zellkern, wo die zytosolischen Bindungspartner abdissoziieren. Durch eine Assoziation mit Arnt (AhR nuclear translocator) bildet sich ein heterodimerer Komplex aus, welcher an bestimmte DNA-Motive, so genannte dioxinresponsive Elemente (DREs), binden kann(63;92).

Die eigentliche Bindung an die DNA erfolgt sowohl mit dem AhR, als auch mit Arnt. Das DNA-Motiv ist ein nichtklassisches Mitglied einer weit verbreiteten Klasse von Transkriptionsfaktorbindestellen (TFBS), den E-Box-Motiven, wobei die Arnt-Halbseite, also der Bereich der Sequenz an den Arnt bindet, der klassischen E-Box-Sequenz entspricht(163). Der Signalweg ist schematisch in Abbildung 2 dargestellt.

A	7	21	43	21	0	0	21	0	0	0	0	0	43	21	64	57	29	7
C	21	43	14	14	29	7	7	0	100	0	0	0	21	29	7	7	43	29
G	31	36	36	43	79	71	7	100	0	100	0	100	0	50	7	71	79	71
T	14	0	7	14	21	72	64	0	0	0	100	0	0	0	21	14	0	43
Ci	31	34	26	23	36	53	38	100	100	100	100	100	58	36	38	41	33	23

Abbildung 3: Positions-Gewichtungs-Matrix für dioxinresponsive Elemente
DRE-Motive bestehen aus einer konservierten, für die AhR/Arnt-Bindung essentiellen Kernsequenz (5'-GCGTG-3') und teilweise konservierten flankierenden Regionen, deren Rolle bei der Geninduktion zurzeit nicht genau verstanden ist. (Modifiziert nach Lee et. al. 2006(86))

Experimentell konnte gezeigt werden, dass diese Bindestelle aus einer konservierten Kernregion mit der Sequenz 5'-GCGTG-3' sowie einer Reihe flankierender, bedingt konservierter Nukleotide besteht(91;96). Die positionelle Wahrscheinlichkeit für jedes Nukleotid wird dabei als Positions-Gewichtungs-Matrix (PWM) dargestellt, wie sie für das AhR/Arnt-Heterodimer in Abbildung 3 dargestellt ist(86). Lai et. al. haben die Kernsequenz in den regulatorischen Regionen einer Reihe von Genen identifiziert(83). In einer Erweiterung dieser Analyse wurden kürzlich die Frequenzen und Positionen der kompletten PWM für alle Promotoren des Menschen, der Maus und der Ratte veröffentlicht.(134).

1.3 Kontrolle des AhR-Signalwegs und Mechanismen der Spezifität

Wie oben bereits angedeutet, sind die Wirkungen einer Überaktivierung des AhR vom untersuchten Organ, Zelltyp, bzw. dessen Reifungsgrad und Aktivierungszustand abhängig. So ist zum Beispiel die AhR-abhängige Induktion von IL-2 in Milzzellen von der gleichzeitigen Stimulation des T-Zellrezeptors abhängig(62). Die hohe Diversität der Effekte zeigt sich auch in den unterschiedlichen Genen, die nach einer AhR-Aktivierung differentiell exprimiert werden. So werden z.B. in der Leber primär fremdstoffmetabolisierende Enzyme, wie Cyp1a1 reguliert, während diese in normalen humanen Fibroblasten nicht AhR-abhängig moduliert werden(42).

Es ist nicht klar, worauf dieses unterschiedliche Anfälligkeit und Reaktion verschiedener Zelltypen gegenüber TCDD beruht. Im Folgenden sollen einige mögliche Regulationsmechanismen vorgestellt werden.

Ein möglicher Faktor ist die Stärke der AhR-Expression. So besitzt die Leber als primäres Organ des Fremdstoffmetabolismus eine hohe AhR-Expression, während in Muskeln oder im Gehirn nur eine schwache Expression gezeigt werden konnte. Die letztgenannten Organe zeigen nach einer TCDD-Belastung wenig transkriptionelle und toxische Änderung, weshalb davon ausgegangen werden kann, dass sie keine primären Ziele einer TCDD-Belastung sind (13;34;35).

Verschiedene Liganden lösen unterschiedliche Effekte aus. Während die toxischen Effekte von TCDD primär auf der veränderten Genexpression beruhen, wird beispielsweise Benzo[a]pyren zu 7,8-dihydroxy-9,10-epoxy-7,8,9,10-benzo[a]pyren (BPDE), einem hochreaktiven, DNA-bindenden Metaboliten verstoffwechselt, der über diese Reaktivität Tumore induzieren kann(79). Diese kanzerogene Aktivität geht in AhR-defizienten Mäusen verloren(127). Die nach metabolischer Aktivierung gebildeten Metabolite können somit ihrerseits zu einer veränderten Genexpression führen, die nicht, oder nur schwer von der primär AhR-vermittelten Transkriptionsänderung zu unterscheiden ist.

Der AhR besitzt eine breite Spezifität für verschiedene Substanzklassen. Er gehört zur Klasse der so genannten „Waisenrezeptoren“ (engl.: Orphan receptors), deren physiologischer Ligand bisher nicht eindeutig charakterisiert werden konnte. Es wurden jedoch einige physiologische AhR Liganden, wie Indirubine, Indole, cAMP, low density Lipoproteine (LDL) sowie Intermediate des Tryptophanstoffwechsels charakterisiert(18;49;73;97;112). Neben den HAKs können noch eine Reihe weiterer Substanzklassen, wie z.B. Catechine oder Flavonoide, an den AhR binden (zusammengefasst von Denison et. al. 2002(21)). Die

parallele Exposition gegenüber einer Mischung von AhR-Liganden führt oft nicht zu additiven Effekten. Ein Modell hierfür stellt die Kompetition von Liganden niedriger und hoher Affinität um die Ligandenbindungsstelle des AhR dar, wenn dieser limitiert vorliegt(118). Ein solcher Mechanismus ist auch für die Verdrängung von physiologischen Liganden denkbar. Die stöchiometrischen und thermodynamischen Verhältnisse hierfür sind jedoch vollkommen unbekannt.

Ein weiterer potentieller Mechanismus für die unterschiedliche Genregulation ist in der direkten Interaktion des AhR mit anderen Proteinen zu sehen. So konnten Ge et. al. zeigen, dass die TCDD-vermittelte Unterdrückung der Leberregeneration zumindest teilweise von der direkten Interaktion des AhR mit dem Retinoblastomaprotein und damit der Kontrolle des Zellzyklus, abhängig ist(38).

In diesem Zusammenhang müssen auch die zytosolischen Bindungspartner des AhR, also Aip und Hsp90, aber auch c-Src und p23 diskutiert werden. Hsp90 und Aip sind als Co-Chaperone allein oder gemeinsam mit einigen weiteren Proteinkomplexen assoziiert. So bindet Hsp90 beispielsweise zytosolisch den Glucocorticoid-Rezeptor(20). Ein weiteres Beispiel ist der Transkriptionsfaktor PPARα (peroxisome proliferator-activated receptor alpha), der zytosolisch in einem Komplex mit Aip und Hsp90 vorliegt(133). Die Folgen der Freisetzung der zytosolischen Bindungspartner des AhR sind zurzeit nur wenig verstanden.

Eine weitere Ebene der Regulation bietet eine Kompetition des AhR um Arnt. Arnt, auch Hif1β genannt, bildet auch mit anderen Proteinen sowie mit sich selbst Transkriptionsfaktoren. Arnt ist insbesondere im hypoxischen Signalweg wichtig. Unter hypoxischen Bedingungen wird Hif1α (Hypoxie-induzierter Faktor alpha) stabilisiert und bildet mit Arnt einen Transkriptionsfaktor(154;155). Bei limitierender Arnt-Konzentration kann es also zu einer Kompetition um Arnt kommen. In verschiedenen Zellsystemen spielt eine solche Kompetition eine Rolle. So führt beispielsweise eine parallele Behandlung von Hepatozyten mit TCDD und $CoCl_2$, einer Modellsubstanz, die wie hypoxische Bedingungen Hif1α stabilisiert und damit eine Hif1a/Arnt-Heterodimerisierung und so eine vermehrte Kompetition um Arnt auslöst, zu einer Veränderung der TCDD-induzierten Genexpression(86). Viele Organe, wie auch der Thymus, sind unter physiologischen Bedingungen hypoxisch, so dass hier mit einer basalen Aktivität des hypoxischen Signalwegs und damit potentiell mit einem Einfluss auf die AhR-vermittelte Genexpression zu rechnen ist (44).

Eine weitere Stufe der Regulation ist die Kompetition um Transkriptionskofaktoren. Beischlag et. al. konnten zeigen, dass Src-1, NCoA-2, und p/CIP in TCDD-Abhängigkeit an

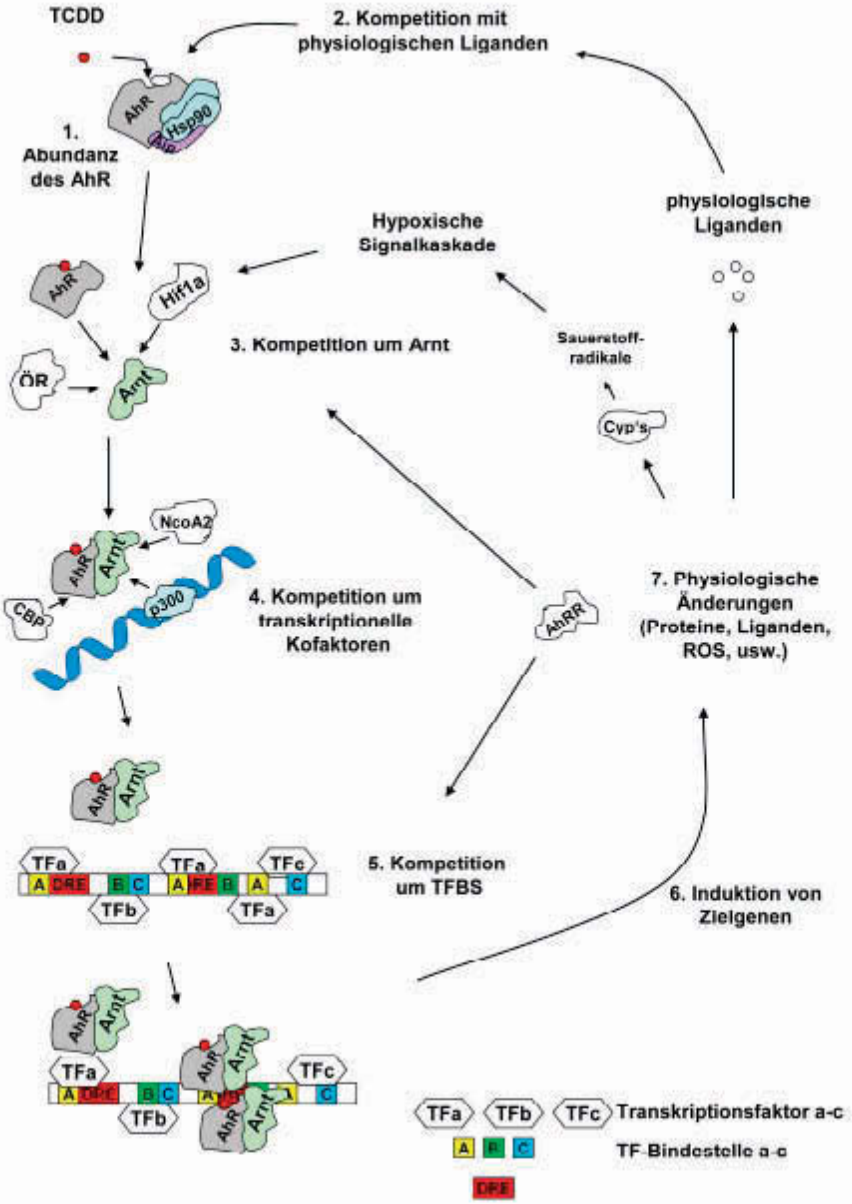

TCDD
2. Kompetition mit physiologischen Liganden
AhR
Hsp90
Asp
1. Abundanz des AhR
physiologische Liganden
Hypoxische Signalkaskade
AhR
Hif1a
ÖR
Arnt
3. Kompetition um Arnt
Sauerstoff-radikale
Cyp's
NcoA2
AhR
Arnt
CBP
p300
4. Kompetition um transkriptionelle Kofaktoren
AhRR
7. Physiologische Änderungen (Proteine, Liganden, ROS, usw.)
TFa
CRE
TFb
TFc
5. Kompetition um TFBS
6. Induktion von Zielgenen
TFa
TFb
TFc
Transkriptionsfaktor a-c
TF-Bindestelle a-c
DRE

Abbildung 4: Mögliche regulatorische Mechanismen des AhR-Signalwegs
Jeder Schritt des AhR-Signalwegs kann reguliert werden, oder regulatorisch wirken. Die Abundanz des AhR (1) ist ein wichtiger Faktor, der über den Umfang der AhR-vermittelten Transkriptionsänderung entscheidet. Desweiteren können hochaffine exogene Liganden, wie TCDD, ein intrinsisches Signal verändern, indem sie endogene Liganden von der AhR-Bindestelle verdrängen (2). Arnt ist neben seiner Rolle im AhR/Arnt-Komplex u.A. mit Hif1α oder dem Ostrogenrezeptor (ÖR) assoziiert. Daher kann eine Kompetition verschiedener Signalwege um Arnt die Transkription beeinflussen (3). Die Kompetition um für die Transkription notwendige Kofaktoren, wie CBP oder p300, kann ebenfalls die Transkription beeinflussen (4). Für die meisten transkriptionellen Effekte der AhR-Aktivierung ist eine Bindung an DREs erforderlich. Diese liegen auf der DNA nicht isoliert vor, sondern teilen ihre Bindesequenz mit anderen TFBS. Liegen andere TFBS in gebundener Form vor oder werden als Folge der AhR-Aktivierung an die DNA rekrutiert, so kann es zu Verdrängungsreaktionen kommen, welche die Transkription beeinflusst (5). AhR-vermittelte Transkriptveränderungen (6), beispielsweise die Induktion des AhRR, können zu negativen Rückkopplungsreaktionen, zum Beispiel durch Kompetition um Arnt oder TFBS führen. So führt eine Induktion von Cyp-Genen primär zur Verstoffwechslung des Liganden (nicht im Fall von TCDD), wodurch reaktive Spezies, wie Sauerstoffradikale oder andere DNA-/Protein-schädigende Intermediate entstehen können, also ein verändertes physiologisches Niveau entstehen können (7). Außerdem können weitere AhR-Liganden, wie cAMP, entstehen, so dass es zu einer erneuten Konkurrenz um die AhR-Bindestelle kommen kann. Diese Aufzählung soll nur einen Einblick in die Komplexität der Regulation des AhR-Signalwegs geben, und erhebt keinen Anspruch auf Vollständigkeit.

den Cyp1a1-Promotor binden(4). Die Rekrutierung und Kompetition um Transkriptionskofaktoren spielt physiologisch eine wichtige Rolle. So ist beispielsweise der Transkriptionskofaktor CBP an der transkriptionellen Aktivität von mehr als 200 Transkriptionsfaktoren, u.a. von Hif1α, beteiligt(3). Die Konzentration der einzelnen Transkriptionsfaktoren ist in der Regel groß genug, um Kompetitionsreaktionen auszugleichen. Bei Depletion eines Transkriptionskofaktors durch verstärkte Rekrutierung durch einen anderen Transkriptionsfaktor kann es jedoch zu Konkurrenzreaktionen kommen, wodurch eine Veränderung anderer Signalkaskaden auftritt(46;65;71). In Abhängigkeit des

physiologischen Niveaus sind solche Konkurrenzreaktionen also durchaus wahrscheinlich. Ein Beispiel hierfür wäre p300CBP, das sowohl mit dem hypoxischen Signalweg, als auch dem AhR-Signalweg assoziiert ist, und so eventuell unter hypoxischen Bedingungen limitierend wirken könnte(144).

Ein wichtiger regulatorische Mechanismus stellt die Kompetition um Transkriptionsfaktorbindestellen dar, der im Rahmen dieser Arbeit durch umfassende Promoterstudien untersucht wurde. Umfangreiche Studien des ENCODE-Konsortiums konnten unter Verwendung von Hochdurchsatz Koimmunnopräziptitationsassays (CHIP), wie Tilingarrays und Chip-Sequenzierung zeigen, dass viele Transkriptionsfaktoren permanent an Hunderte von Promotoren gebunden sind und große Teile des Transkriptoms auf diese Weise in unterschiedlicher Stärke immer transkribiert werden(5;22). Viele der AhR-vermittelten Effekte beruhen auf der Bindung des AhR/Arnt-Heterodimers an DREs. Diese überlappen oft mit den Bindestellen anderer Transkriptionsfaktoren, die im physiologischen Niveau besetzt sein können. Bei einer AhR-Überaktivierung können also je nach Bindung der DNA durch anderen Transkriptionsfaktoren andere Bindestellen erreichbar sein. Darüber hinaus kann das AhR/Arnt-Heterodimer bereits gebundene Transkriptionsfaktoren verdrängen, und so die Regulation ihrer Zielgene beeinflussen. Für den AhR wurden solche Wechselwirkungen unter anderem für die Transkriptionsfaktoren NFκB, Hif1α, den Östrogenrezeptor sowie für eine Reihe weitere Signalkaskaden nachgewiesen(110;122).

In Abhängigkeit des untersuchten Zelltyps kommt es nach TCDD-Behandlung zur raschen Aufregulation des AhR Repressors (AhRR). Dabei handelt es sich um ein weiteres bHLH-PAS Protein, welches eine sehr hohe Homologie zum AhR aufweist. Der AhRR besitzt keine transaktivierende Domäne. Der AhRR formt wie der AhR ein Heterodimer mit Arnt und kann an dieselben DREs binden wie der AhR/Arnt-Komplex. Durch diesen Mechanismus kann es sowohl zu einer Kompetition um Arnt, als auch um die DNA-Bindestellen kommen, so dass die verstärkte TCDD-induzierte AhRR-Expression ein negativen Rückkopplungs-mechanismus ist(43).

In den letzten Jahren wurde zusätzlich über eine Beteiligung von regulatorischen RNA-Molekülen, wie Mikro-RNAs diskutiert. Eine kürzlich veröffentlichte Arbeit zeigte jedoch, bei einer TCDD-Exposition nur wenige Veränderungen der miRNA-Konzentrationen, und diese von nur geringem Ausmaß aufwiesen. Daher scheint es unwahrscheinlich, dass in der Leber, als hochgradig TCDD-sensiblen System, miRNAs eine essentielle Rolle spielen. Bedenkt man jedoch, dass sich die miRNA-Expression zwischen Zelltypen stark

unterscheidet, kann ein solcher Einfluss für andere Systeme nicht ausgeschlossen werden(104).

Für keinen der beschriebenen Mechanismen ist die Stöchiometrie und Thermodynamik bekannt. Berücksichtigt man all diese Mechanismen, so ist es sehr wahrscheinlich, dass eine genaue Trennung der einzelnen Komponenten nur schwer möglich ist. Diese potentiellen Mechanismen sind schematisch in Abbildung 4 zusammengefasst.

1.4 AhR-Toxizität mit Schwerpunkt auf dem Immunsystem

Eine Belastung mit TCDD führt zu sehr stark spezies-abhängigen Symptomen. Dabei kann man klar zwischen Spezies mit hoher Sensitivität, wie dem Meerschweinchen, welches schon bei geringen Dosen massive Schädigungen zeigten, und solche, die nahezu resistent sind, z.B. Hamster, unterscheiden.

Der Mensch ist auch hohen Dosen gegenüber relativ unempfindlich. Insbesondere treten Hyperkeratosen, Chlorakne, aber auch sehr langfristige, wenn auch kleine Veränderungen des Immunsystems auf. So zeigten z.B. TCDD-belastete Arbeiter noch 20 Jahre nach Belastung eine Einschränkung ihrer T-Helferzellfunktion(145). Die TCDD-Effekte im Menschen wurden kürzlich von Pelclová et al. umfangreich diskutiert(116).

In der Maus und der Ratte sind die adversen Effekte deutlich ausgeprägter. Die Anfälligkeit ist stark abhängig vom Mausstamm. So hatte TCDD in einer 30 Tage-LD_{50} –Studie in C57BL/6-Mäusen eine LD_{50}=182 µg/kg, in B6D2F1/J (B6D) von 296 µg/kg und in DBA/2J (DBA)-Mäusen von 2570 µg/kg Körpergewicht(15). Zu den beobachteten Effekten gehören Kanzerogenität, Teratogenität, Kardio- und Hepatotoxizität, Ausbildung einer Hasenscharte, sowie eine starke Gewichtsabnahme, das so genannte „Wasting-Syndrom“. Ein Merkmal, das auch schon bei sehr niedrigen Dosen auftritt, ist eine systemische Immunsuppression, die mit einer Atrophie des Thymus einhergeht(152). Von der Immunsuppression sind nahezu alle Komponenten der Immunantwort betroffen. So hat TCDD einen starken Einfluss auf die Reifung von hematopoetischen Stammzellen(84;107), beeinflusst die humorale Immunantwort(104), die Reifung von B-Zellen im Knochenmark(142) und verändert die Antigenpräsentation von dendritischen Zellen(150) sowie die Zytokinexpression(62;81). Außerdem kommt es zu einer Atrophie des Thymus. Die Effekte von TCDD auf das Immunsystem werden umfassend in den Übersichtsartikeln von Holsapple(52) und

Kerkvliet(69) diskutiert. Im Folgenden werden einige für diese Arbeit wichtige TCDD-Effekte auf das Immunsystem, insbesondere auf den Thymus, vorgestellt.

TCDD-Belastung führt in der Maus neben der allgemeinen Immunsuppression zu einer Atrophie des Thymus. Der Thymus bildet das Hauptorgan der T-Zellentwicklung. Er besteht aus zwei miteinander verbundenen Lappen, die jeweils von einer Kapsel aus Bindegewebe umschlossen sind. Zahlreiche Septen, die in den Innenraum hereinragen, bilden ein Netzwerk von miteinander verbundenen Kompartimenten, die jeweils in einen äußeren, den (Kortex) und einen inneren Bereich (Medulla) unterschieden werden. Die Epithelzellen von Medulla und Kortex unterscheiden sich voneinander und geben jeweils wichtige Signale für die Reifung von Thymozyten ab. Das Milieu für die Thymozytenreifung wird darüber hinaus von einer geringen Anzahl von Makrophagen und dendritischen Zellen, welche beide zu den Antigenpräsentierenden Zellen (APZ) zählen, komplettiert. Pluripotente hematopoetische Vorläuferzellen wandern aus dem Knochenmark in den Thymus und reifen hier in einem komplexen Zusammenspiel aus intrinsischen und extrinsischen Signalen des Thymusstromas und den auf den Thymozytenvorläufern exprimierten Rezeptoren. Diese Entwicklung verläuft in mehreren, gut durch die Expression von Oberflächenproteinen nachvollziehbaren Schritten ab. Die Vorläufer können im Thymus anhand der Expression der Oberflächenmarker CD25, CD44, CD4 und CD8 in mindestens sieben Entwicklungsstadien unterteilt werden. Während der ersten vier Reifungsschritte sind die Zellen $CD4^-CD8^-$ und werden daher im Folgenden als doppelt negativ (DN) bezeichnet. Diese aus vier Reifungsstadien bestehende Phase kann anhand der CD25- und CD44-Expression in die Stadien DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$) DN3 ($CD44^-CD25^+$) sowie DN4 ($CD44^-CD25^-$) unterschieden werden, wobei diese noch in weitere Zellentwicklungsstadien unterteilt werden können. In diesen Phasen findet die Rekombination der T-Zellrezeptor (TZR)-Gensegmente statt. Nach erfolgreicher Rekombination der αβ-Ketten des TZRs wird dieser erstmalig in geringer Dichte auf der Oberfläche ausgeprägt. Parallel werden nun die beiden kostimmulatorischen Moleküle CD4 und CD8 ausgeprägt. Daher wird dieses Stadium als doppelt positiv (DP) bezeichnet. Durch 2-Photonen mikroskopische Analysen konnte gezeigt werden, dass unreife Thymozyten mit vielen epithelialen Thymuszellen (ETZ) interagieren(120). Thymusepithelzellen prägen in großer Menge MHC (engl.: Major Histocompatibility Complex) I- bzw. MHCII- Moleküle aus, über die sie Selbstantigene präsentieren. Bei ausbleibender Stimulation durch die Bindung zwischen MHC und TZR sterben die T-Zellen apoptotisch. Dieser Vorgang wird auch als positive Selektion bezeichnet und gewährleistet, dass die T-Zellen MHC-restringiert sind. Resultiert die Interaktion von TZR mit MHC in einem starken Signal, kommt es

ebenfalls zu Apoptose der T-Zellen, der so genannten negativen Selektion, wodurch T-Zellreaktionen gegen Selbstpeptide und somit Autoimmunerkrankungen verhindert werden. Durch beide Prozesse werden ca. 99% aller reifenden T-Zellen deletiert. Findet die funktionelle Interaktion der TZR mit MHCI oder MHCII statt, werden die entsprechend nicht benötigten kostimulatorischen Moleküle nicht mehr exprimiert, so dass die Zellen mit MHCI-Spezifität CD8 positiv und solche mit MHCII-Spezifität CD4 positiv sind. Reife Thymozyten migrieren anschließend in die peripheren lymphatischen Organe, wie Lymphknoten und Milz und zirkulieren im Blut und der Lymphe(16). Die gesamte Reifung untersteht einer stringenten Kontrolle durch eine Reihe von Transkriptionsfaktoren, wie Beispielsweise c-myb, Ikaros, GATA-3 oder Notch-1. Diese Kontrolle wird umfassend von Anderson diskutiert(1).

Bei einer exogenen Aktivierung des AhR durch TCDD wird die T-Zellentwicklung durch eine Vielzahl adverser Effekte beeinflusst. Dabei sind sowohl Effekte auf Thymusepithelzellen (TEZ), als auch ein direkter Effekt auf die Thymozyten beschrieben worden. So sind Thymozytenvorläufer aus dem Knochenmark nach Exposition mit TCDD in ihrer Funktion und Fähigkeit zur Reifung eingeschränkt(33;128). Im fötalen und adulten Thymus induziert TCDD eine nicht vollständige Blockade während der DN Reifungsstadien(93;138). TCB, ebenfalls ein AhR-Ligand, inhibiert die Proliferation unreifer Thymozyten und verändert die T-Zellfrequenzen zugunsten von $CD8^+$-T-Zellen(29;82). Darüber hinaus induziert TCDD eine gesteigerte, CD44v10-abhängige Emigration von unreifen Thymozyten(30), die sowohl im fötalen als auch im adulten Thymus potentiell regulatorisch wirkende Thymusemigranten generiert(93;138)[63]. Durch den Einsatz von Chimären, in denen nur das Thymusepithel, nicht jedoch die hematopoetischen Zellen, den AhR ausprägen, konnte gezeigt werden, das die TCDD induzierte Thymusatrophie primär die hematopoetischen Stammzellen betrifft.(130). Getrennte Behandlung der Thymozyten bzw. Thymusepithelzellen aus Thymusorgankulturen, mit TCDD zeigte, dass eine alleinige Behandlung des Thymusepithel für eine veränderte Differenzierung der Thymozyten ausreicht(76). Die Daten deuten darauf hin, dass eine AhR-Expression in Thymozyten zwar für die verminderte Proliferation, nicht jedoch für die Veränderung der Differenzierung der T-Zellen notwendig ist. Daher ist ein genaues Verständnis der adversen Effekte von TCDD auf hematopoetische und epitheliale Thymuszellen wichtig. Aus diesem Grund sollte in dieser Arbeit sohl die TCDD-induzierten Expressionsänderungen in Thymozyten, als auch in TEZ untersucht werden.

Es ist zurzeit unklar, ob die Effekte im Thymus in einem direkten kausalen Zusammenhang zur Immunsuppression stehen und über welchen molekularen Mechanismus sie hervorgerufen werden, also welche Gene als direkte AhR-Effektormechanismen wirken.
Neben den beschriebenen Effekten sind eine Reihe weiterer Effekte auf T-Zellen, bzw. durch T-Zellen vermittelte Immunantworten bekannt. So reduziert TCDD die humorale und

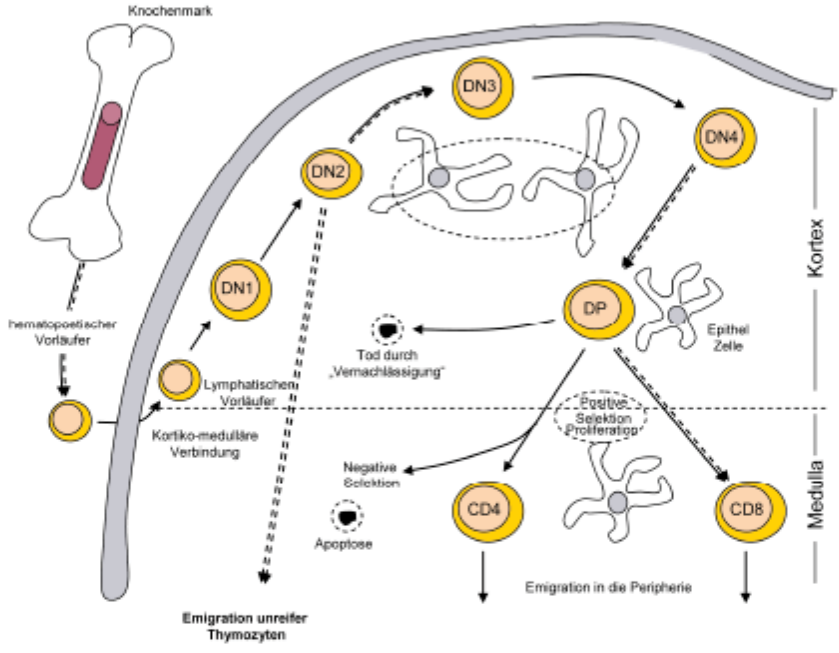


Abbildung 5: Auswirkungen einer AhR-Aktivierung auf die T-Zellentwicklung im Thymus

Hematopoetische Vorläuferzellen wandern aus dem Knochenmark in den Thymus. In diesem frühen Stadium exprimieren diese Zellen weder den TZR, noch CD4 oder CD8 und werden als „doppelt negativ" (DN) bezeichnet. DN-Thymozyten können anhand ihrer Oberflächenmarker in vier Stadien unterteilt werden (DN1, $CD44^+CD25^-$; DN2, $CD44^+CD25^+$; DN3, $CD44^-CD25^+$; DN4, $CD44^-CD25^-$). Nach erfolgreicher Rekombination der TZR-Gensegmente exprimieren die Thymozyten sowohl CD4, als auch CD8 [„doppelt positiv"(DP)] und werden positiv und negativ selektiert. Die überlebenden T-Zellen emigrieren als einfach positive $CD4^+$- oder $CD8^+$-T-Zellen aus

dem Thymus in die Peripherie. Eine Aktivierung des AhR verändert mehrere Stufen dieses Prozesses. Die durch eine AhR-Aktivierung beeinträchtigten Prozesse und Zelltypen sind gestrichelt, oder gestrichelt eingekreist gezeichnet.

zellvermittelte Reaktion gegen pulmonale Influenzaviren(102;159). Dabei zeigte sich jedoch, dass der Effekt sich nur auf das „priming" der $CD8^+$-T-Zellen, nicht jedoch auf die T-Gedächtniszellen auswirkte(85). Kerkvliet et. al. konnten zeigen, dass für die Suppression einer zytotoxischen Lymphozytenantwort durch TCDD die Expression des AhR in T-Zellen notwendig ist(70). Außerdem entstehen nach einer TCDD-Behandlung vermehrt $CD4^+CD25^+$-regulatorische T-Zellen(36). Es ist unklar, ob welche Rolle regulatorische T-Zellen bei der TCDD-vermittelten Immunsuppression spielen, und ob diese Effekte von den beobachteten Veränderungen im Thymus abhängen. Neben den $CD4^+CD25^+$ regulatorischen Zellen entstehen sowohl in adulten Mäusen *in vivo*, als auch *ex vivo* in FTOC DN Thymusemigranten, die *in vitro* bei Kokultivierung mit ConA-stimulierten Milzzellen eine Proliferation von $CD4^+$-T-Zellen inhibieren (93;138).

Gegen eine direkte Rolle des Thymus bei der TCDD-vermittelten Immunsuppression spricht jedoch, dass eine neonatale Entfernung des Thymus nicht die TCDD-induzierte Immunsuppression im Ohrschwellungstest verhindert. Während des Ohrschwellungsstests kommt es zu einer TCDD-abhängigen Akkumulation von dendritischen Zellen (DZs) in den drainierenden Lymphknoten, was darauf hindeutet, dass in dieser Reaktion die DZs das primäre Ziel von TCDD sind (Marc Majora, persönliche Mitteilung). Für keine der oben beschriebenen Reaktionen sind die molekularen Mechanismen auf Expressionsebene bekannt. Die Charakterisierung der TCDD-Zielgene in Zellen des peripheren Immunsystems würde hierfür wertvolle Ansatzpunkte liefern.

Die Unterschiedlichkeit der Zellpopulationen des Immunsystems, sowie die unterschiedlichen Arten der Immunantwort gegen verschiedene Pathogene deuten darauf hin, dass die T-Zellvermittelte Immunsuppression je nach untersuchtem Modell auf verschiedenen Mechanismen beruht. Daher ist es wichtig, die Rolle des AhR in den verschiedenen Kompartimenten des Immunsystems vor allem unter physiologischen Bedingungen zu verstehen.

1.5 Mausmodelle des AhR

Da der AhR für die meisten toxischen Effekte von HAKs essentiell ist, wurden schon früh Experimente mit Zell- und Tiermodellen mit modifizierter Ausprägung von Komponenten des AhR Systems durchgeführt. Neben diversen Zelllinien mit einer funktionellen Inhibierung von Komponenten der AhR-Signalkaskade(129;162) stehen hierzu inzwischen einige Mausmodelle zu Verfügung. Modelle für eine konstitutive Aktivität des AhR, $AhR^{-/-}$-Mäuse, zelltypspezifische Ablationen des AhR, Modelle mit einer Modifikation im Kernsignal des AhR,sowie Modelle für potentielle Interaktionspartner (Arnt, Arnt2, Hsp90 oder Aip) und AhR-Zielgene wurden beschrieben. Verschiedene dieser Modelle zeigen massive physiologische Beeinträchtigungen. Cyp1a1- defiziente-Mäuse sind gegen TCDD induzierte Effekte weitestgehend resistent(148). $Cyp1b1^{-/-}$-Mäuse besitzen eine erhöhte Resistenz gegenüber 7,12-Dimethylbenzo[a]anthrazen (DMBA)-induzierter Tumorbildung. Unter physiologischen Bedingungen weisen sie jedoch eine erhöhte Inzidenz von progressiver Glomerulonephritis, sowie von histozytären Sarcomen auf. Dabei zeigten Makrophagen aus $Cyp1b1^{-/-}$-Mäusen ein erniedrigte Kapazität zur Phagozytose von apoptotischen und nekrotischen Zellen(156). Arnt-, Arnt2- und Aip-defiziente Mäuse sind schon im frühen Embryonalstadium letal und eignen sich daher nicht für eine Untersuchung des AhR-Signalweges(89;124).

Mäuse mit konstitutiv aktiviertem AhR (CA-tgAhR) zeigen einen mit TCDD exponierten Mäusen vergleichbaren Phänotyp. Dazu gehören Veränderungen von Leber, Niere und Herz, geringere Fertilität, sowie eine deutlich geringere Zellularität des Thymus. Darüber hinaus treten in diesen Mäusen Tumoren der Leber und des Magen-Darm-Traktes verstärkt auf(2;9;103). In Mäusen, in denen der AhR nur in Keratinozyten konstitutiv exprimiert ist, treten vermehrtes Hautjucken und Hautläsionen auf. Diese werden von Entzündungsreaktionen begleitet, die einer atopischen Dermatitis ähneln. Eine T-Zell spezifische CA-tgAhR-Mauslinie zeigt die nach TCDD-Exposition beobachtete Thymusatrophie und eine Unterdrückung der durch eine Immunisierung induzierten Vergrößerung der Milz(108;111;137). Zurzeit ist keines dieser Modelle umfassend immunologisch charakterisiert.

Neben diesen Modellen gibt es drei unabhängig voneinander generierte $AhR^{-/-}$-Stämme. Diese Modelle unterscheiden sich zum Teil sehr deutlich voneinander. Die von F. Gonzales und P.M. Fernandez-Salguero hergestellte Maus hat eine Deletion und Rekombination mit einer Neomycin-Sequenz in Exon 1(31). Diese Maus wird im Folgenden als $AhR^{\Delta 1/\Delta 1}$ bezeichnet.

In den beiden anderen Modellen aus den Laboren von C. Bradfield und Y. Fujii-Kuriyama ist Exon 2 deletiert. Diese Mäuse werden respektiv im Folgenden als $AhR^{\Delta 2/\Delta 2B}$ und $AhR^{\Delta 2/\Delta 2F}$ bezeichnet(101).

Alle drei Mausstämme zeigen eine weitestgehende Resistenz gegenüber TCDD und zeigen damit, dass die TCDD-induzierten adversen Effekte kausal AhR-abhängig sind. Dabei sind sowohl die hepatotoxische Wirkung, als auch die Immunsuppression und die Thymusatrophie betroffen. Durch den gezielten Einsatz dieser Mausmodelle kann darüber hinaus die AhR-Abhängigkeit einzelner Effekte auf spezielle Zelltypen untersucht werden. Knochenmarks-Chimären, in denen der AhR nur in T-Zellen deletiert ist demonstrieren, dass eine Deletion im T-Zellkompartiment für die Blockade der TCDD-induzierten, generellen Thymusatrophie ausreicht(130).

Durch die Anwendung der $AhR^{-/-}$-Maus bei der Untersuchung der Toxizität einzelner HAK kann die direkte AhR-abhängigkeit aufgeklärt werden. So wird die B[a]P-vermittelte chemische Kanzerogenese, die von einer AhR-induzierten metabolischen Aktivierung von B[a]P durch Cyp1a1/Cyp1b1 abhängig ist unterbunden(127). Im Fall der DMBA induzierten Pre-B-Zellapoptose, die ebenfalls von einer metabolischen Aktivierung der Substanz über Cyp1b1 abhängig ist, bewirkt eine AhR-Ablation jedoch nur eine geringe Verminderung der Toxizität(50).

Studien in allen drei Mausstämme weisen darüber hinaus pathologische Veränderungen auf, was auf eine wichtige physiologische Rolle des AhR hindeutet. Diese sind in Abhängigkeit der verwendeten Mauslinie und der angewendeten Verpaarungsstrategie etwas unterschiedlich.

$AhR^{\Delta 1/\Delta 1}$ weisen eine erhöhte neonatale Letalität, vermindertes Wachstum während der ersten vier Wochen, eine Reduzierung der Fertiliät, verringertes Lebergewicht, eine Blockade der Induzierbarkeit von *Cyp1a1* und *Cyp1b1*, sowie einige Veränderungen der peripheren lymphatischen Organe, wie der Milz, auf. Eine initiale Charakterisierung der $AhR^{\Delta 2/\Delta 2B}$ Maus zeigte unabhängig vom Untersuchungszeitpunkt keine Veränderung der Letalität. Auch dieser Mausstamm weist eine geringere Fertilität, eine Blockade der Induzierbarkeit von Cyp1a1 und Cyp1b1, sowie eine deutliche morphologische Veränderung der Leber auf. Die Leberphänotypen beider Mausstämme unterscheiden sich dabei jedoch. So treten in der $AhR^{\Delta 1/\Delta 1}$–Maus verstärkt Fibrosen des Portaltraktes, Entzündungen des Gallengangs, verringerte Glykogenkonzentrationen, zentroloboläre Hyperzellularität, sowie eine Eosinophilie auf. In $AhR^{\Delta 2/\Delta 2B}$ kommt es zu einer verspäteten Schließung des Duktus Venosus, und damit zu einer veränderten Blutzirkulation. Diese wird von einer extensiven

mikrovesikulären Metamorphose der Hepatozyten, milder Fibrose der Portalregion, sowie einer allgemeinen persistenten Verkleinerung der Leber begleitet. Im adulten Stadium weisen diese Mäuse darüber hinaus eine Vergrößerung der Milz auf(80).

Im Gegensatz zu einer Überaktivierung des AhR durch Xenobiotika weisen alle hier besprochenen AhR-defizienten Mäuse keine Frequenzverschiebung der Lymphozyten-Subpopulationen in Milz und Thymus auf. Untersuchungen der durch zytotoxische T-Zellen vermittelten Transplantatabstoßung oder der humoral vermittelten Reaktion gegen rote Blutkörper des Schafs zeigen jedoch in beiden Mausstämmen, dass diese beiden primären Immunantworten weder im $AhR^{\Delta 1/\Delta 1}$ – noch im $AhR^{\Delta 2/\Delta 2B}$ – Mäusen inhibiert sind(151). Eine Analyse der Immunglobulinproduktion in $AhR^{\Delta 1/\Delta 1}$ – Mäusen zeigt ebenfalls keine Unterschiede. Obwohl die Immunantwort gegen rote Blutkörper des Schafs sowie in der Transplantatabstoßung, keine veränderte Immunantwort aufweisen, zeigt sich eine erhöhte Produktion von IFN-γ und IL-12 in der Milz dieser Mäuse(121). Das zeigt, dass der Effekt einer AhR-Deletion auf das Immunsystem noch wenig verstanden ist.

Bisher untersuchen wenige Studien die Auswirkung einer AhR-Deletion auf den Fötus, und die AhR-induzierten teratogenen Effekte. Für ein Gesamtverständnis der Rolle des AhR, insbesondere die Wechselwirkung des fötalen Fremdstoffmetabolismus, des endokrinen Systems und des Immunsystems und die daraus resultierenden Wirkmechanismen einer AhR-Aktivierung im Fötus, sind solche Studien jedoch äußerst wichtig. Föten, die aus einer Verpaarung von $AhR^{+/-}$-Männchen und $AhR^{-/-}$-Weibchen ($AhR^{\Delta 2/\Delta 2B}$) stammen und *in utero* mit TCDD oder Dexamethasone (DEX) behandelt wurden, zeigen eine deutlich erhöhte Embryotoxizität. Eine Messung der Verteilung dieser Teratogene zeigt dabei, dass es zu einem Anfluten, also einer erhöhten Konzentration beider Stoffe im Embryo kommt. Der zugrunde liegende Mechanismus hierfür ist zurzeit nicht bekannt, kann jedoch mit der verspäteten Ausbildung des Duktus Venosus und der daraus Veränderung des enterohepatischen Kreislaufs zu tun haben. Dadurch würde eine initiale Metabolisierung von xenobiotischen Substanzen reduziert und sie würden in erhöhter Konzentration in der Peripherie wirken können(141).

Darüber hinaus zeigen Nachkommen einer Verpaarung von $AhR^{+/-}$-Männchen und $AhR^{-/-}$-Weibchen ($AhR^{\Delta 2/\Delta 2B}$) eine veränderte Durchblutung und Ausbildung des Herzmuskels (Hyperplasie), was zu kardiovaskulären Veränderungen und einer erhöhten Anfälligkeit gegenüber Ischämien führt ($AhR^{\Delta 1/\Delta 1}$)(54). Außerdem zeigen diese Mäuse in der pränatalen, frühen postnatalen Phase und im Alter (7 Monate) eine Veränderung des Insulinstoffwechsels,

die phänotypisch Ähnlichkeiten mit einer Typ II Diabetes aufweisen, jedoch funktionell nicht zu Hyperglukämie führt(139;140).

Bei der Untersuchung von $AhR^{-/-}$-Mäusen ist also die Auswahl des Verpaarungsschemas und die Berücksichtigung der geringeren Fertiltität, gemessen an der Anzahl der überlebenden Nachkommen, äußerst wichtig. In der hier vorgelegten Arbeit $AhR^{\Delta 2/\Delta 2B}$-Mäuse in einer $AhR^{-/-}$-Männchen und $AhR^{+/-}$-Weibchen verwendet.

Die oben diskutierten Erkenntnisse über AhR-defiziente Mäuse, zeigen deutlich, dass der AhR neben seiner Funktion als Transkriptionsfaktor physiologisch noch weitere wichtige Funktionen besitzt, die im Folgenden diskutiert werden sollen.

1.6 Das duale Modell des AhR der AhR-Aktivität

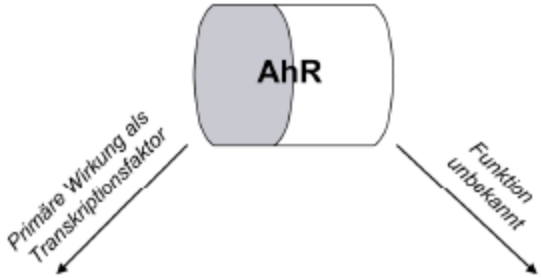
Zum Verständnis der AhR-Aktivität und der TCDD-vermittelten Transkriptionsänderungen ist es wichtig, sowohl die Funktion des AhR als Transkriptionsfaktor, als auch seine Proteininteraktionen zu verstehen.

Der AhR besitzt eine Reihe physiologischer Funktionen. Die am umfassendsten untersuchte ist seine Funktion als Transkriptionsfaktor. Untersuchungen an AhR-defizienten Mäusen, beziehungsweise an Modellen, die eine Fehlen bzw. eine Mutation von Arnt aufweisen, zeigen, dass viele adverse Effekte, die nach einer Belastung mit TCDD oder einem anderen AhR-Liganden auftreten, direkt von der Funktion des AhR als Transkriptionsfaktor abhängen. Darüber hinaus interagiert der AhR mit einer Reihe von Proteinen, wie z.B. dem Retinoblastomaprotein oder seinen zytosolischen Bindungspartnern(38). Desweiteren konnte gezeigt werden, dass der AhR ein Bestandteil eines Cullin-Ligase-Komplexes ($CUL^{AhR)}$ ist. Im CUL^{AhR} wirkt der AhR als Adapterkomplex, durch den Sexsteroidrezeptoren für ihre Degradation markiert werden(113). Inwieweit solche Mechanismen bei einer AhR-Überaktivierung, aber vor allem im Modell der $AhR^{-/-}$-Maus eine Rolle spielen ist erst wenig verstanden.

Bei der Suche nach den Wirkmechanismen und Funktionen des AhR muss also ein duales Modell des AhR zugrunde gelegt werden. Zum einen, seine Funktion als Transkriptionsfaktor und zum anderen seine Interaktion mit verschiedenen anderen Proteinen und Signalwegen. In der hier vorgelegten Arbeit wird, durch die Anwendung globaler Transkriptionsanalysen, die zelltypspezischische Auswirkung einer AhR-Aktivierung sowie einer AhR-Defizienz auf die Transkription analysiert. Durch diesen Ansatz sollen die Gene charakterisiert werden, deren Expression direkt durch den AhR reguliert sind.

Insbesondere AhR-defiziente Mäuse bieten hierbei die Möglichkeit die Konsequenz einer fehlenden Potein-Protein Wechselwirkung zu untersuchen. Für das Modell der AhR-defizienten Maus wurden bisher nur wenige Transkriptionsprofile aufgenommen, nämlich von glatten Muskelzellen der Aorta(66) und der Leber(143;161). In beiden Modellen ist der AhR transkriptionell sehr aktiv, wie die parallel untersuchte Genexpressionsänderung durch AhR-Liganden demonstriert. Daher ist eine Trennung der Funktion als Transkriptionsfaktor und der Regulation über direkte AhR-Proteinwechselwirkungen in diesen Modellen nicht möglich. Für die Untersuchung der, von der Rolle als Transkriptionsfaktor unabhängigen Funktionen, wäre die Bestimmung der Genexpression in Zellen wünschenswert, in denen der AhR transkriptionell nicht, oder nur wenig aktiv ist.

Der Dualismus des AhR-Systems ist schematisch in Abbildung 6 dargestellt.



Liganden induzierte Toxizität
- Hepatotoxizität
- Kanzerogenität
- „Wasting Syndrom“
- Teratogenität
- Allgemeine Immunsuppression
- Thymusatrophie

Phänotyp des AhR$^{-/-}$ -Modells
- Abhängig vom verwendeten Modell und Verpaarungsschema
  - veränderte Fertilität
  - veränderte Morphologie der Leber
  - kardiovaskuläre Defekte
  - erhöhte Anfälligkeit gegenüber teratogenen Noxen
  - Splenomegalie
  - veränderte Zytokinausschüttung

Abbildung 6: Duales Modell des AhR

Während die meisten toxischen Effekte von AhR-Liganden ihre Wirkung durch die Funktion des AhR als Transkriptionsfaktor entfalten, ist der molekulare Mechanismus, der dem Phänotyp des AhR$^{-/-}$-Modells zugrunde liegt, bisher weitgehend unbekannt.

1.7 Toxicogenomic des AhR-Systems

Transkriptionsanalysen von AhR-defizienten Mäusen können zwar dabei helfen, die physiologische, von der Funktion als Transkriptionsfaktor unabhängige, Funktion des AhR aufzuklären, liefern jedoch nur begrenzt Informationen, über potentielle Effektormechanismen, die den toxischen Effekten einer AhR-Aktivierung durch exogene Liganden zugrunde liegen. Führt eine AhR-Aktivierung in jedem Zelltyp zu vergleichbaren Expressionsänderungen? Liegt also den toxischen Effekten ein gemeinsamer Wirkmechanismus zu Grunde?

Um solche Fragen zu beantworten, sind Transkriptionsanalysen in mehreren Zelltypen notwendig. Aus diesem Grund sollte im Rahmen dieser Arbeit die TCDD-induzierte Expressionsänderung in einer Reihe primärer hochaufgereinigter Immunzellen untersucht werden.

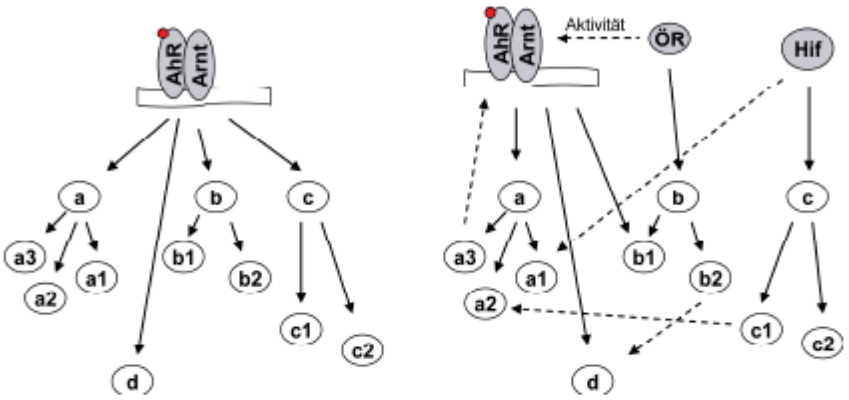
Für die durch AhR-Aktivierung veränderte Expressionssignatur kann man dabei grundsätzlich zwei Modelle diskutieren. Im Ersteren gehen alle Expressionsänderungen direkt vom AhR als Transkriptionsfaktor aus und setzen sich über die Regulation weiterer Signalwege fort. Man kann in diesem Modell also zwischen primären AhR-Zielgenen, und sekundären durch die primären Zielgene beeinflussten Genen unterscheiden. Dieses Modell wird im Weiteren als „primär AhR“ gesteuert bezeichnet. Im zweiten Modell erfolgt die Regulation auf mehreren Ebenen und beruht auf der Wechselwirkung des AhR-Signalweges mit anderen Signalwegen. Dieses Modell wird im Folgenden als „Multi-Faktor-Modell“ bezeichnet. Die beiden Ansätze sind schematisch in Abbildung 7 dargestellt.

Beide Modelle eröffnen eine Reihe von Fragen, wie:

Welche Gene werden reguliert und sind diese Änderungen direkt durch den AhR vermittelt (primäre Zielgene) oder reguliert der AhR andere Transkriptionsfaktoren, deren Aktivität wiederum zur Veränderung der Transkription führt (sekundäre Zielgene)? Gibt es so etwas wie eine allgemeine Batterie von AhR-Zielgenen, die in jedem Zelltyp oder Gewebe reguliert wird? Und wenn ja, besitzen diese Gene prädiktives Potential um andere Substanzen oder pathophysiologische Beobachtungen auf eine Regulation des AhR zu untersuchen? Mit welchen anderen Signalwegen interagiert der AhR um die beobachteten Expressionsänderungen hervorzurufen? Also, was ist das passende Modell, um diese Transkriptionsänderungen darzustellen?

Abbildung 7: Modelle der AhR-induzierten Transkriptionsänderungen

a) Primär AhR-gesteuertes Modell **b) Multi-Faktor Modell**



Der zeitliche Ablauf der TCDD-vermittelten Transkriptionsänderungen (dargestellt durch die Länge der Pfeile) kann auf einer alleinigen transkriptionellen Aktivität des AhR beruhen, die Zielgene reguliert (primäre Zielgene), die dann wiederum weitere Gene regulieren (sekundäre Zielgene). Ein weiteres Modell beruht auf der Interaktion des AhR mit anderen Transkriptionsfaktoren und Signalwegen. In diesem Modell hat der AhR eine Wirkung auf die Aktivität dieser Faktoren oder reguliert deren Zielgene. Diese Regulation kann dabei in beide Richtungen stattfinden (gestrichelte Linien), so dass ein regulatorisches Netzwerk entsteht.

Viele dieser Fragen lassen sich durch eine Kombination von Genexpressionprofilen mit entsprechender Unterstützung durch bioinformatische Analysemethoden bearbeiten. Microarrays („Chips“) ermöglichen die parallele Analyse der Expression von Tausenden von Genen und stellen somit ein probates Mittel zur Identifikation von Zielgenen dar(123). Durch die Standardisierung der experimentellen und analytischen Verfahren, sind die Ergebnisse innerhalb eines bestimmten Chiptypen und im Bereich der gut detektierbaren Gene über verschiedene Chiptypen gut vergleichbar. Insbesondere Microarrays der Firmen Agilent und Affymetrix liefern gut reproduzierbare Ergebnisse(115;126). Die Verwendung eines Chiptyps, auf dem nur jeweils eine Probe hybridisiert wird (Einfarbenarray, z.B. PE-Färbung in der Affymetrix-Technologie), ermöglicht einen Vergleich von Expressionsdaten mit Daten, die unabhängig von verschiedenen Arbeitsgruppen oder zu verschiedenen Zeitpunkten

aufgenommen wurden. Durch den Aufbau von Expressionsdatenbanken und der Identifikation von gemeinsamen Zielgenen eines bestimmten Signalweges könnten sich Markergene charakterisieren lassen, die zur Identifizierung einer Beteiligung des untersuchten Signalweges oder zur Unterscheidung verschiedener Krankheitsgrade, wie Krebs verwendet werden können(8;157;158). Ein Beispiel mit Bedeutung für den AhR-Signalweg stellt die, von der Arbeitsgruppe von C. Bradfield betriebene Datenbank EDGE dar(47). In dieser Arbeit wurden eine Reihe von AhR-, CAR und PPAR-Antagonisten, Hemmern des Hämstoffwechsels sowie weiteren Faktoren analysiert, um mit Hilfe von Clustering-Algorithmen den Eintrag der durch sie repräsentierten Signalwege in die von unbekannten Substanzen verursachte Genexpression zu ermitteln. Ein weiteres Beispiel von allgemeiner toxikologischer Relevanz ist ein Datenbankprojekt von ILSI HESI unter der Leitung von C. Afshari und H. Hamadeh. In diesem Projekt werden Genexprofile der Kontrolltiere von toxikogenomischen Studien an Rattenleber, insgesamt 536 Affymetrix-Microarrays aus 18 Laboren, verwendet, um die basale Varianz der Genexpression zu charakterisieren. Zu diesem Zweck werden alle Arrays gemeinsam normalisiert und mit den gleichen Algorithmen ausgewertet (Persönliche Mitteilung C. Afshari).

Im Rahmen der hier vorgelegten Arbeit sollen die gesammelten Expressionsdaten verschiedener Immunzellen zur Charakterisierung von Markergenen dienen. Die Markergene sollen anschließend zur Identifizierung AhR-abhängiger Prozesse in einer zu diesem Zweck aufzubauenden Genexpressionsdatenbank dienen.

Werden Expressionsdaten über mehrere Zeitpunkte aufgenommen, so lassen sich aus der zeitlichen Aufeinanderfolge der Expressionsänderungen Rückschlüsse auf gemeinsame Mechanismen der Regulation ziehen. Dabei wird vorausgesetzt, dass Gene, die ein vergleichbares Expressionsmuster aufweisen, z.B. durch die Aktivität eines gemeinsamen Transkriptionsfaktors reguliert werden. Kombiniert man diese Technik mit der Analyse der regulatorischen Regionen der gemeinsam regulierten Gene, ermöglicht dieses im Optimalfall die Identifikation von überrepräsentierten, regulatorischen Elementen(72;74;78;135). Werden die *in silico* Daten experimentell für den Eintrag dieser Elemente in die beobachteten Expressionsänderungen validiert, lassen sich so Aussagen über die Beteiligung dieser Elemente treffen. Dieser Ansatz führte in verschiedenen Modellen, wie z.B. der Hefe, zur Entdeckung von primären und sekundären Zielgenen, bis hin zu globalen regulatorischen Netzwerken(55-57). Dieser Ansatz bietet also potentiell die Möglichkeit primäre Zielgene von sekundären, durch die Wirkung anderer Transkriptionsfaktoren regulierte Gene zu unterscheiden.

Dabei ist die Auswahl des zu untersuchenden Systems von essentieller Bedeutung. Idealerweise sollten alle beobachteten Änderungen auf der direkten transkriptionellen Regulation durch die untersuchte Substanz beruhen. Bei gemischten Zellpopulationen, wie sie z.B. in Organen vorliegen, verursacht häufig z.B. eine Veränderung der Anzahl einzelner Zelltypen eine massive, mehrere hundert Gene betreffende Änderung der Expression. Gleiches gilt für die Veränderung von Reifungszuständen. Ein für diese Arbeit wichtiges Beispiel stellt die differentielle Genexpression zwischen den einzelnen Schritten der T-Zellentwicklung dar. Hoffmann et.al. konnten zeigen, dass während der T-Zellentwicklung mehr als 1000 Transkripte differentiell exprimiert werden, was ein Diskriminierung von TCDD-Zielgenen in diesem Zusammenhang deutlich erschwert(51).

Um genügend regulierte Gene für eine statistisch gesicherte Aussage zu analysieren, muss das verwendete Modell möglichst stark auf die Exposition reagieren, dabei jedoch keine allgemeine Stressantwort oder toxikologische Effekte aufweisen, da der Eintrag dieser Reaktionen die Identifikation von spezifischen Signalkaskaden erschwert. Daher müssen massiver Stress und die Induktion umfangreicher toxischer Effekte, charakterisiert durch die differentielle Expression von Hunderten bis Tausenden Genen verhindert werden. Für die Anwendung auf den AhR Signalweg bietet sich besonders der Thymus als Modell an, da eine AhR-Aktivierung im Thymus zu massiven Effekten führt, die sowohl das Thymusstroma, als auch die hematopoetischen Zellen betreffen. Aus diesem Grund sollte in dieser Arbeit die TCDD-induzierte Genexpressionsänderung in Thymozyten und TEZ untersucht werden.

Bei der Identifikation der regulatorischen Elemente ist die Wahl der verwendeten Methodik von entscheidender Bedeutung. Die meisten regulatorischen Elemente besitzen nur eine kurze, oft nicht sequenzkonservierte Bindestelle (5-15 Nukleotide). Die Wahrscheinlichkeit eines zufälligen Vorkommens einer kurzen, sechs Nukleotid-langen Sequenz in einer zufällig generierten DNA-Sequenz liegt mit $0{,}25^6$ (4 Basen, gleiche Wahrscheinlichkeit für jedes der 6 Nukleotide), bei einem alle 4096 bp. Berücksichtigt man die Tatsache, dass diese Sequenz auf beiden DNA-Strängen und in beide Richtungen vorkommen kann, sinkt diese Zahl auf $0{,}25^5$, also 1 alle 1024 bp. Bei der computergestützten ab initio Analyse regulatorischer Regionen besteht also eine hohe Wahrscheinlichkeit falsch positiver Treffer. Um deren Anteil möglichst klein zu halten, gibt es verschiedene Ansätze. Zum einen die Anwendung möglichst stringenter Algorithmen und ein Vergleich mit Kontrollgruppen aus zufällig gewählten regulatorischen Regionen(26;86). Ein weiterer Ansatz ist die Analyse der Konserviertheit der Sequenzen über verschiedene Spezies. Dieser Ansatz geht davon aus, dass funktionelle Elemente über selektive Prozesse besonders konserviert werden, wie dies beispielsweise für

die Promotoren von Referenzgenen oder Transkriptionsfaktoren demonstriert werden konnte(60;88). Dieser Ansatz ist stark von der Qualität der zugrunde liegenden Sequenzen abhängig. Bedenkt man dabei, dass beispielsweise die letzte Veröffentlichung des Rattengenoms im Jahr 2004 stattfand, werden die Limitationen dieses Ansatzes deutlich (39). Der phylogenetische Ansatz ist jedoch hochgradig stringent, so dass die Rate an falsch positiven Entdeckungen niedriger liegt, als mit vergleichbaren Algorithmen. Durch die Restriktion auf konservierte Sequenzen ist jedoch die Rate der falsch negativen Ergebnisse deutlich höher. Der interessierte Leser sei auf den exzellenten Review von Maston et.al. 2006 hingewiesen, der dieses Thema umfassend bearbeitet(94). In dieser Arbeit wurde ein stringenter ab initio Algorithmus verwendet. Durch den Einsatz einer aus zufällig ausgewählten Promotoren bestehenden Kontrollgruppe, wurde dabei der Anteil an falsch positiven Ergebnissen möglichst gering gehalten.

In jedem Fall müssen die identifizierten, regulatorischen Elemente oder Transkriptionsfaktor-bindestellen und ihr Einfluss auf die beobachteten Expressionsänderungen experimentell validiert werden. Hierzu steht eine Reihe von Techniken zu Verfügung, die in unterschiedlichem Maße zur Validierung verwendet werden können(72). Klassischerweise erfolgen solche Validierungen durch den Einsatz von Reportergenanalysen. Diese sind jedoch aufgrund der umfangreichen Klonierungsarbeiten auf wenige Gene limitiert und liefern nur eine Aussage über ein artifizielles System.

Eine weitere Möglichkeit stellt die Analyse der Bindung des analysierten Transkriptionsfaktors durch Chromatinimmunopräzipitation (CHIP) dar. Für eine Reihe von Transkriptionsfaktoren ist dieser Ansatz inzwischen im Hochdurchsatzverfahren als Tilingarrays oder durch CHIP-Assays an cDNA-Bibliotheken, gekoppelt mit Sequenzierungstechniken, möglich. Es ist jedoch zu bedenken, dass die Bindung des Transkriptionsfaktors noch keinen endgültigen Nachweis für seine Aktivität darstellt.

Eine weitere Möglichkeit, die *in silico* gewonnenen Ergebnisse zu validieren ist der Einsatz der parallelen Aktivierung eines identifizierten Transkriptionsfaktors, und der Aktivierung des AhR. Ein Bespiel hierfür wäre die parallele Induktion des Östrogenrezeptors und des AhR. Verändert sich die Expression der Zielgene eines der Faktoren, ist dies ein deutlicher Hinweis auf eine Wechselwirkung beider Faktoren. Da diese Methode direkt die Funktionalität einer Wechselwirkung angibt, wurde sie im Rahmen dieser Arbeit verwendet.

Optimal wäre eine Kombination aller Methoden, was jedoch oft aus logistischen Gründen nicht möglich ist (72).

1.8 Fragestellung

Die hier vorgelegte Dissertation besteht aus drei Teilen, die zusammen der Charakterisierung der primären und sekundären Signalkaskaden des AhR und ihrer Beteiligung an immunologischen Prozessen dienen.

Der erste Teil dieser Arbeit dient durch globale Genexpressionsanalysen der Identifizierung der Zielgene einer TCDD-induzierten transkriptionellen Veränderung in einer Reihe von primären Zellen des Immunsystems nach *in vivo* oder *in vitro* Behandlung mit TCDD. In ausgewählten Zelltypen, die keine/wenige TCDD-vermittelten Änderungen aufwiesen, sollte die Wirkung einer AhR-Deletion auf die Transkription charakterisiert werden. Ziel dieses Teils der Arbeit war die Identifikation von Markergenen und eines potentiellen gemeinsamen AhR-Effektormechanismus. Desweiteren ermöglicht er die Identifizierung eines geeigneten Modellsystems, welches sich für die kinetische Analyse der Expressionsänderung besonders eignent.

Im zweiten Teil sollte aus veröffentlichten Transkriptionsprofilen eine direkt vergleichbare Genexpressionsdatenbank aufgebaut werden. Unter Verwendung der im ersten Teil identifizierten Markergene dient diese der Analyse der zell-/gewebsspezifischen Ausprägung von Komponenten des AhR-Systems und der Identifikation von physiologischen Prozessen, insbesondere des Immunsystems, mit einer potentiellen Beteiligung des AhR-Signalweges dienen. Diese Analyse unterstützt die Auswahl eines möglichst relevanten Zellmodells für die im dritten Teil der Arbeit durchgeführte, detaillierte Analyse der zeitabhängigen Transkriptionsänderung.

Im dritten Teil sollten die primären und sekundären durch TCDD-induzierten Genexpressionsänderungen in ihrem Zeitverlauf untersucht werden. Durch Kombination mit Promoteranalysen ermöglicht dies die Unterscheidung, welche der veränderten Transkriptsignaturen, sich der Wirkung bestimmter Transkriptionsfaktoren zuordnen lassen. Damit sollte bestimmt werden, ob die beobachteten Transkriptionsänderungen am besten durch ein primär AhR gesteuertes Modell erklärbar sind, oder ob sie auf einer Wechselwirkung des AhR-Signalweges mit andern Signalwegen beruhen, und so durch ein Multifaktormodell erklärbar sind. Die Anwendung dieser Ergebnisse auf die im zweiten Teil aufgebaute Datenbank kann deren Möglichkeiten zur Identifikation einer AhR-Beteiligung und potentieller Effekte einer AhR-Aktivierung verstärken.

2 Ergebnisse

2.1 Veröffentlichungen als Erstautor

Im Folgenden werden die Ergebnisse der im Anhang A beigefügten Veröffentlichungen als Erstautor noch einmal kurz im Zusammenhang dargestellt. Die Aufteilung in die Veröffentlichungen spiegelt dabei die Zielsetzungen der drei Arbeitsteile wieder. Diese waren:

I Untersuchung der zelltypspezifischen AhR-abhängigen Transkriptionsveränderung in Zellen des Immunsystems und Identifikation von Markergenen für eine AhR-Aktivierung.

II Charakterisierung der Transkription des AhR und AhR-assoziierter Gene in einer direkt vergleichbaren Expressionsdatenbank und Identifizierung von physiologischen Bedingungen in denen AhR-Zielgene differentiell exprimiert werden.

III Analyse der Transkriptionsveränderung nach AhR-Aktivierung in einem kontrollierten Zellmodell und Identifikation von Interaktionen des AhR-Signalweges mit anderen Signalwegen (eingereicht).

2.1.1 Transcriptional signatures of immune cells in aryl hydrocarbon receptor (AhR)- proficient and AhR-deficient mice

Frericks M, Temchura VV, Majora M, Stutte S, Esser C
Biol Chem. 2006 Sep;387(9):1219-26

Zellen des Immunsystems reagieren in Abhängigkeit ihres Reifungsgrades, ihrer Lokalisation und dem Zeitpunkt der Behandlung sehr unterschiedlich auf eine TCDD-Belastung. Die zu Grunde liegenden Genexpressionsänderungen sind bisher weitgehend ungeklärt. Daher wurden Genexpressionsprofile von verschiedenen aufgereinigten primären Zellpopulationen des Immunsystems aufgenommen, um eine Reihe von allgemeinen Zielgenen und somit potentiell gemeinsame Effektormechanismen zu charakterisieren. Um die Effekte von TCDD auf die Genexpression während der Reifung von T-Zellen zu untersuchen, wurden fötale $CD4^-CD8^-$ (DN) Thymusemigranten und adulte $CD4^-CD8^-$ (DN) Thymozyten bzw. Thymusemigranten verwendet. Die Ausprägung des AhR war dabei in allen drei Zelltypen vergleichbar. Daher ist es überraschend, dass fötale DN Thymusemigranten nach TCDD-Exposition, in fötaler Thymusorgankultur mit ca. 300 mehr als zweifach unterschiedlich ausgeprägten Transkripten stark reagierten, während adulte DN Thymozyten und Thymusemigranten, also Zellen mit vergleichbaren Reifungsgrad, mit 35 bzw. 45 Transkripten deutlich schwächer reagtierten. Zwischen den Zelltypen zeigten nur die Gene *Cyp1b1*, *Lgals3* und *Scin* eine Regulation in allen drei Zelltypen. Deren Reifungsgrad war durch TCDD zu einem unreiferen Phänotyp verschoben, so dass nicht geklärt werden konnte, ob die Änderungen der Expression primär auf die transkriptionelle Wirkung des AhR oder sekundär auf dem veränderten Reifegrad beruhten.

Zur Analyse der Wirkung TCDD-Belastung auf das periphere Immunsystem, wurden dendritische Zellen als Vertreter der antigenpräsentierenden Zellen, sowie $CD4^+$ und $CD8^+$-T-Zellen aus der Milz analysiert. 24h nach einer i.p. Injektion von 10µg/kg Körpergewicht TCDD zeigten DZ mit 192 veränderten Transkripten eine deutlich stärkere Reaktion als die $CD4^+$ und $CD8^+$ T-Zellen (12 bzw. 6 veränderte Transkripte). $CD4^+$-Zellen aus $AhR^{-/-}$-Mäusen wiesen keinerlei Expressionsänderungen auf, was demonstriert, dass die in Wildtyp-Mäusen beobachteten Änderungen AhR abhängig waren. Weder *Cyp1a1* noch *Cyp1b1*, ansonsten als typische AhR-Zielgene bekannt, zeigten eine Veränderung.

Zwischen allen in dieser Arbeit verglichenen Zeltypen gab es kein gemeinsames TCDD-induziertes Gen. Das zeigt deutlich, dass das Konzept einer für alle Zelltypen gültigen

Batterie von AhR-Zielgenen, und damit einem einheitlichen Effektormechanismus, nicht haltbar ist.

Eine Ausweitung der Suche nach Zielgenen auf veröffentlichte Genexpressionsänderung nach AhR-Aktivierung/-Deletion zeigte, dass einige Gene wie *Cyp1a1*, *Cyp1b1*, *Scin* oder *Tiparp* vermehrt differentiell exprimiert werden. Es gab jedoch kein Gen, welches immer reguliert wurde und somit als Indikator einer AhR-Aktivität prädiktiv sein könnte. Das beweist, das der Einsatz eines einzelnen AhR-Markergens für die Bestimmung einer AhR-Aktivität nicht ausreicht.

Bei den TCDD-induzierten Expressionsänderungen weisen viele Gene eine verminderte Expression auf, was nicht durch das klassische Modell des AhR als induzierender Transkriptionsfaktor zu erklären ist.

Neben der Analyse TCDD-induzierter Expressionsänderungen in primären aufgereinigten Zellpopulationen wurde in dieser Arbeit erstmals die Auswirkung einer AhR-Deletion auf die Transkription eines Zelltyp untersucht, der nur eine geringe AhR-Expression sowie wenige TCDD-induzierte Expressionsänderungen aufweist. Im Vergleich von $CD4^+$-Zellen aus $AhR^{+/+}$- und $AhR^{-/-}$-Mäusen waren mehr als 300 Transkripte unterschiedlich ausgeprägt. Der molekulare Mechanismus dieser Expressionsänderung ist zurzeit unklar. Weder im Thymus noch in der Milz der $AhR^{-/-}$-Mäuse war eine Änderung des Reifegrads (gemessen an den Verschiebungen der Populationsfrequenzen), was diesen Befund erklären könnte, nachweisbar. Auch die Möglichkeit der Aktivität eines endogenen Liganden in diesem Zelltyp ist aufgrund der geringen Transkriptionsänderung durch TCDD nicht wahrscheinlich, kann jedoch für andere Zellen der Milz nicht ausgeschlossen werden, die ihrerseits durch veränderte Signalausschüttung den beobachteten Phänotyp beeinflussen können. Die in dieser Arbeit gezeigten Ergebnisse deuten darauf hin, dass die Expressionsänderungen auf einer physiologischen Rolle des AhR beruhen, die von der Funktion als Transkriptionsfaktor unabhängig ist.

2.1.2 Microarray analysis of the AhR system: tissue-specific flexibility in signal and target genes

Frericks M, Meissner M, Esser C

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Um die Ausprägung des AhR und seine Aktivität unter Bedingungen zu untersuchen, in denen keine Aktivierung, Inhibierung oder Deletion des AhR stattfand, wurde eine direkt vergleichbare Genexpressionsdatenbank aufgebaut. In typischen Expressionsstudien werden meist nur die differentiell exprimierten Gene untersucht (meist weniger als 1%), während die parallel gemessene Expressionsstärke aller Transkripte meist nicht betrachtet werden. Daher bieten diese Daten eine umfangreiche, über das jeweilige Ziel der Arbeit hinausgehende, Informationsquelle. Die Reproduzierbarkeit von Expressionsdaten über einen einzelnen Chiptyp und bei gleicher Normalisierung ist dabei sehr hoch. Unter Verwendung von 1967 murinen Affymetrix U74aV2 GeneChips®, die alle mit dem MAS5.0-Algorithmus analysiert wurden, konnte daher eine Expressionsdatenbank aufgebaut werden. Die Datensätze stammen von Mäusen unterschiedlichen Alters, Geschlechts, Genotyps, verschiedenen Behandlungen, sowie einer Reihe von Zellkultursystemen. Nach Median-Skalierung waren diese Datensätze zwischen verschiedenen Laboratorien vergleichbar, spiegelten aus der Literatur bekannte Expressionsmuster wieder und ließen sich mit quantitativer RT-PCR reproduzieren.

Die Datenbank erlaubt also für jedes enthaltene Transkript eine umfassende Analyse der Transkriptionsstärke, seiner Verteilung, seiner Koexpressionen mit anderen Transkripten, sowie seiner differentiellen Expression zwischen Behandlungen (Reifegrad, Genotyp und andere Parameter). Angewendet auf den AhR ergaben sich folgende Ergebnisse:

i) der AhR zeigt keine Koexpression zu anderen Transkripten, was als Hinweis auf gemeinsame Regulation gewertet werden könnte, ii) die Expressionsstärke des AhR korreliert vielfach mit dem Differenzierungszustand von Zellen. So ist er beispielsweise in unreifen ($CD4^-CD8^-$- und $CD4^+CD8^+$- Thymozyten) sowie in ausgereiften $CD4^+CD25^+$ regulatorischen T-Zellen des Pankreas gut detektierbar, während die Ausprägung des AhR in reifen Thymozyten und T-Zelllymphomen am Rande der Nachweisgrenze liegt. Ähnlich verhält es sich in hematopoetischen Stammzellen und dendritischen Zellen, während Makrophagen nur eine schwache AhR-Ausprägung aufweisen.

Da die Datenbank viele Bedingungen differenzieller Expression (z.B. Behandlungen, Vergleich von Wildtyp zu gendefizienten-Tieren, Tieren unterschiedlichen Alters oder

Geschlechts u.v.m.) beinhaltet, eignet sie sich besonders zur Untersuchung der differentiellen Expression von Transkripten. Dabei zeigte sich, dass der AhR unter 53 von 308 analysierten Bedingungen mehr als 2-fach differentiell exprimiert war, was auf eine Funktion des AhR in diesen Bedingungen hindeutet. Eine Ausweitung auf bekannte AhR-Zielgene, und Gene, die in mindestens drei unabhängigen Expressionsstudien im AhR-Kontext (Aktivierung durch Ligand oder $AhR^{-/-}$-Modell) reguliert waren, zeigte, dass die meisten in mehr als 20% aller Bedingungen reguliert waren. Um eine funktionell relevante Expressionsänderung von falsch positiven Ergebnissen zu trennen, wurde eine Regulation von mindestens vier dieser Markergene als Indikator einer AhR-Beteiligung gewertet. Durch diesen Ansatz konnten viele Prozesse identifiziert werden, unter denen der AhR eine bekannte Funktion besitzt (z.B. Reifung von B-Zellen) aber auch viele weitere Kandidaten identifiziert werden. Diese Daten deuten darauf hin, dass der AhR gerade in vielen Entwicklungsprozessen (Muskeln, Hoden, Ovarien, frühe Entwicklung von der Oozyte zur Blastula), und unter pathophysiologischen Bedingungen in Herz und Gehirn, aber auch in medullären Thymusepithelzellen bei der Expression von Selbstpeptiden und damit bei der Reifung- und Ausbildung des T-Zellrepertoires eine Rolle spielt. Erstaunlicherweise scheint der AhR dabei auch in einigen Zelltypen, in denen die Arnt-Expression unter der Nachweisgrenze des Chipsystems lag, transkriptionell aktiv zu sein. Dieser Befund deutet darauf hin, dass der AhR in Abwesenheit von Arnt eine alternative physiologische Funktion besitzt.
Die aus der erstellten Datenbank gewonnenen Daten zur Expression des AhR und seiner potentiellen Beteiligung veröffentlichten Expressionsänderungen, stellen eine in diesem Umfang bisher noch nicht veröffentlichte Charakterisierung der Expression des AhR, insbesondere im Immunsystem, dar. Sie zeigen eindeutig die stark zelltypspezifische Ausprägung und Aktivität des AhR und seiner Regulation.

Die vollständigen Ergebnisse inklusive aller in dieser Veröffentlichung enthaltenen Supplementary Figures/Tables sind der Arbeit als Anhang auf DVD beigelegt. Die DVD enthält darüber hinaus die Datenbank, sowie eine umfangreichen Dokumentation davon.

2.1.3 Transcription factor crosstalk controls transcriptional response to AhR-over-activation by TCDD in thymic epithelial cells

Frericks M, Burgoon L.D. , Zacharewski T., Esser C.
[Eingereicht]

Die in 2.1.1 diskutierten Daten demonstrieren deutlich die Wirkung einer AhR-Überaktivierung auf Thymozyten. Aufgrund der TCDD-induzierten Veränderung des Reifungsgrades der Thymusemigranten/Thymozyten, die eine Identifizierung primärer AhR-Zielgene verhindert, eignen sie sich nicht für eine Differenzierung zwischen primären und sekundären Zielgenen des AhR. Neben Thymozyten sind auch Thymusepithelzellen Ziel der TCDD-induzierten adversen Effekte im Thymus. So führt eine selektive Exposition von Thymusepithelzellen zu einer Verschiebung in Richtung der $CD8^+$ T-Zellen. Bisher wurden für Thymusepithelzellen keine direkten toxischen Effekte von TCDD wie Apoptose oder eine veränderte Reifung beschrieben. Daher sind TEZ für die Identifizierung primärer und sekundärer TCDD-Zielgene gut geeignet. Um eine Vermischung der TCDD-Effekte auf medulläre und kortikale Tymusepithelzellen zu vermeiden, wurden ET-Zellen, eine kortikale Thymusepithelzelllinie, für die Identifikation der TCDD-induzierten Expressionsänderungen und somit der primären und sekundären Zielgene verwendet. Bei Belastung mit 5nM TCDD war in ET-Zellen schon nach 30 Minuten eine Translokation des AhR in den Kern zu beobachten. Parallel kam es zu einer verstärkten Transkription von *Cyp1a1* und *Cyp1b1*. Beide Gene sind auch in unbehandeltem Zustand schwach exprimiert. 24 h nach Kontakt mit TCDD fiel die Expression beider Gene unter das Basalniveau. Eine Genexpressionsanalyse über 2, 4 und 6h mit Affymetrix MOE430A Genchips zeigte, dass über diese Zeit 201 Transkripte differentiell exprimiert waren. Dabei waren die TCDD-Behandlungen klar von der Lösungsmittelkontrolle zu trennen. Von den 201 Transkripten waren 158 stärker und 43 schwächer exprimiert. K-means-Clustering unterteilte diese Gene anhand der zeitlichen Verläufe ihrer Änderung in 8 klar voneinander unterscheidbare Gruppen. Um zu erkennen, ob innerhalb dieser Gruppen Gene mit ähnlichen biologischen Funktionen vorlagen, wurde eine Genontologieanalyse durchgeführt. Über alle Transkripte war dabei die negative Regulation der Apoptose als einzige statistisch überrepräsentierte, hochauflösende Kategorie nachweisbar. Diese Gruppe war dabei hauptsächlich der Gruppe mit initial stärkerer Ausprägung und anschließendem Rückgang auf das basale Niveau zuzuordnen. Eine Analyse

der Funktion der differentiell exprimierten Gene zeigte darüber hinaus eine statistisch signifikante Überrepräsentation der Funktionen: Apoptose, intrazellulärer Proteintransport, Regulation der Transkription, Migration/Lokalisation sowie der Zell-Zell-Kommunikation.
Um die der Transkriptionsänderung zu Grunde liegenden Mechanismen aufzuklären, wurde eine detaillierte Analyse der regulatorischen Regionen im Bereich -10.000 bis +5.000 bp um den annotierten Transkriptionsstart auf die statistisch signifikante Überrepräsentation von Sequenzmotiven durchgeführt. Im Vergleich zu einer Kontrollgruppe von zufällig ausgewählten Genen, waren dabei insgesamt 6.754 kurze Nukleotidsequenzen (5-10 Nukleotide) überrepräsentiert. Um diese bereits bekannten Transkriptionsfaktoren zuzuweisen, wurden sie mit den Bindestellen bekannter in Transkriptionsfaktordatenbanken TRANSFAC und JASPAR hinterlegten Motive verglichen. Dabei ergab das Clustering der Motive 439 einzelne Konsensussequenzen, deren Positionsgewichtungsmatrizen (PWM) 52 Transkriptions-faktorbindestellen (TFBS) aus der JASPAR- und 114 aus der TRANSFAC-Datenbank entsprachen, während 12 Bindestellen in beiden Datenbanken keine Entsprechung hatten. Zur Ermittlung der Sequenzspezifität der Bindestellen wurden für alle 52 JASPAR-Bindestellen die Verteilung im Bereich von -5.000 Bp bis zum Transkriptionsstart in allen murinen und humanen Promotoren identifiziert. Als sequenzspezifisch wurden dabei solche Bindestellen angesehen, die durchschnittlich pro 1.000 Nukleotide nicht mehr als zweifach detektiert werden konnten. Diese waren neben den Bindestellen für das AhR/Arnt-Hetreodimer, NfkB-, Pax4-, RoR-alpha-, MADS-, Hypoxia responsive und RREB- Elemente. Die Anwendung der dabei identifizierten Eigenschaften der Bindestellen auf die aus TRANSFAC erhaltenen TFBS identifizierte 25 weitere Bindestellen, unter anderem Glucokortikoid responsive Elemente (GRE), Östrogen responsive Elemente (ÖRE), PPAR, sowie die für Mitglieder der bHLH-Familie typische E-Box-Motive.
Eine Bestätigung der *in silico* gewonnenen Ergebnisse würde vermuten lassen, dass auch die identifizierten, regulatorischen Elemente in der TCDD-vermittelten Toxizität unter physiologischen Bedingungen eine Rolle spielen. Um die biologische Relevanz der *in silico* Ergebnisse zu überprüfen, wurden parallele Behandlungen mit $CoCl_2$, welches wie hypoxische Bedingungen Hif1a stabilisiert und so eine Konkurrenz um Arnt auslösen kann, und 17-β-Estradiol, welches beide Östrogenrezeptoren aktiviert, durchgeführt. Beide Substanzen veränderten die TCDD-induzierte Expressionsänderung und können so potentiell die AhR-Zielgene und damit die AhR-vermittelten toxischen Effekte beeinflussen. Da viele Gewebe grundsätzlich eher hypoxisch vorliegen, bzw. eine Östrogenabhängigkeit aufweisen, ist ein Einfluss beider Signalwege auf den AhR-Signalweg wahrscheinlich. Da für die meisten

der anderen identifizierten Transkriptionsfaktoren bisher keine spezifischen Inhibitoren oder Aktivatoren vorliegen, wäre ihre Rolle in entsprechenden gendefizienten Tieren oder Zelllinien zu untersuchen.
Diese Daten deuten klar darauf hin, dass ein einfaches Modell, in dem alle Expressionsänderungen nur über den AhR vermittelt werden, nicht haltbar ist. Eine genaue Trennung der involvierten Signalkaskaden war jedoch nicht möglich.

Neben der eingereichten Veröffentlichung wurden sämtliche zusätzlichen Tabellen auf der DVD beigefügt.

2.2 Für diese Arbeit wichtige Veröffentlichungen als Koautor

Im Folgenden werden die Ergebnisse der im Anhang B beigefügten Veröffentlichungen als Koautor noch einmal kurz im Zusammenhang der hier bearbeiteten Fragestellung dargestellt. Alle Auswertungen der in diesen Veröffentlichungen dargestellten Microarraydaten wurden vom Autor dieser Dissertation durchgeführt und im Rahmen der unter 2.1.1 diskutierten Arbeit verwendet. Die ersten beiden Veröffentlichungen behandeln die veränderte Reifung und Migration von unreifen Thymozyten nach TCDD-Behandlung. Sowohl im fötalen als auch im adulten Thymus führte eine TCDD-Exposition zu einer Veränderung der T-Zellreifung und zu einer präferentiellen Emigration von unreifen Thymozyten. Bei der Analyse der Expressionsprofile der DN Thymozyten und Thymusemigranten war eine Diskriminierung zwischen direkten AhR-Zielgenen und Genen, deren Expression durch den veränderten Reifungsgrad unterschiedlich ausgeprägt war, nicht möglich.
Neben der Wirkung auf den Thymus inhibierte TCDD die Immunantwort bei der Kontaktsensibilisierung. In dieser Reaktion spielen Langerhans Zellen eine wichtige Rolle als primäre dermale antigenpräsentierende Zellen. Dementsprechend sollten auch hier potentielle Effektormechanismen aufgeklärt werden. Die Analyse der TCDD-induzierten Expressionsänderungen steht also in direktem Bezug zu den unter 2.1.1. beschriebenen Daten.

2.2.1 Role of the aryl hydrocarbon receptor in thymocyte emigration in vivo

Temchura VV, **Frericks M**, Nacken W, Esser C
Eur J Immunol. 2005 Sep;35(9):2738-47

Eine Aktivierung der AhR-Signalkaskade durch TCDD führt zu einer massiven Atrophie des Thymus. Diese wird durch eine Reihe von unterschiedlichen Prozessen ausgelöst, die besonders $CD4^-CD8^-$ Thymozyten (DN) betreffen. In der vorgelegten Arbeit wurde gezeigt, dass es nach AhR-Überaktivierung zu einer vermehrten Emigration von DN Zellen aus dem Thymus kommt, die sich in der Milz nachweisen lassen. Einige dieser unreifen Thymusemigranten hatten einen bisher nicht beschriebenen Phänotyp, der durch die Ausprägung Oberflächenmarkern, die typisch für reife und unreife T-Zellen sind, gekennzeichnet ist ($CD3^-TCRbeta^-CD25^{+/int}CD44^-CD45RB^{+/int}CD62L^+CD69^-$ Zellen). Kultivierte man diese Emigranten mit T-Zellen der Milz, inhibierten sie deren ConA-induzierte Proliferation. Der Vergleich der Expressionsprofile von TCDD-Emigranten mit

veröffentlichen Expressionsprofilen von 8 verschiedenen Entwicklungsstufen der Thymozytenentwicklung zeigte, dass sich die Emigranten vermehrt in den frühen Phasen der T-Zellentwicklung befanden. Das ließ sich durch intrazelluläre Färbung der vorläufigen α-Kette des T-Zellrezeptors bestätigen. TCDD induziert eine Änderung der Transkription in DN Thymozyten und Thymusepithelzellen. Durch den unterschiedlichen Reifungsgrad der analysierten Zellpopulationen war es nicht möglich primäre TCDD-induzierte Expressionsänderungen von zelltypspezifischen zu unterscheiden. Zu den regulierten Genen gehörte unter anderem *S100A9*, ein Molekül, welches in Migrationsprozessen involviert ist. Eine Exposition von S100A9 defizienten Mäusen mit TCDD zeigte darüber hinaus, dass S100A9 aktiv an der TCDD induzierten Thymusemigration beteiligt ist.

Der Verfasser der Dissertation führte sämtliche Genexpressionsanalysen und deren Vergleich mit bereits veröffentlichten Expressionsdaten von Thymozytenpopulationen durch. Die Expressionsdaten gingen direkt in die unter 2.1.1. beschriebene Analyse mit ein.

2.2.2 Detection of a novel population of fetal thymocytes characterized by preferential emigration and a TCR gammadelta$^+$ T cell fate after dioxin exposure

Majora M, **Frericks M**, Temchura VV, Reichmann G, Esser C
Int Immunopharmacol. 2005 Nov;5(12):1659-74.

Die fötale T-Zellentwicklung in T-Zellrezeptor αβ$^+$ TZR und γδ$^+$ TZR Zellen aus CD4$^-$CD8$^-$ (DN) Vorläuferzellen im Thymus wird durch die geordnete Regulation einer Vielzahl von Genen reguliert. Der überaktivierte AhR beeinflusst diese Entwicklung auf verschiedenen Ebenen, insbesondere im DN Stadium. In dieser Studie wurde die Rolle des AhR auf die Reifung und Migration von DN fötalen Zellen im Modell der fötalen Thymusorgankultur (FTOC) untersucht. Nach AhR-Aktivierung durch TCDD zeigte sich ein vermehrtes Auftreten von γδ$^+$ TZR DN Zellen. Es konnte eine neue Population von CD25$^{(int/low)}$CD44$^{(hi)}$, was einem sehr unreifen T-Zellstadium entspricht, identifiziert werden, die präferentiell aus dem Thymus emigrierten und γδ$^+$ TZR waren. Sortierte γδ$^+$ TZR Emigranten proliferierten in Gegenwart von IL-2 und waren darüber hinaus bei Ko-Kultivierung in der Lage, die Proliferation von aktivierten CD4$^+$-T-Zellen zu unterdrücken. Genexpressionsanalysen von

DN Thymusemigranten zeigten mehr als 200 differentiell exprimierte Gene, von denen ca. 10% mit dem Immunsystem und viele mit der T-Zellentwicklung assoziiert waren. Unter anderem waren die Gene Rag-1 und TdT, beide normalerweise nur in den DN3 und DN4 Phasen der Thymusentwicklung exprimiert, nach TCDD-Exposition nicht mehr detektierbar. Diese und weitere Gene sind im Einklang mit der phänotypisch detektierbaren Barriere im DN2 Stadium. Darüber hinaus wurde jedoch auch eine Reihe von Genen stärker exprimiert, die sonst primär in reifen T-Zellen ausgeprägt werden, was auf einen unreifen, aktivierten Phänotyp hindeutet. Insgesamt zeigen diese Ergebnisse, dass TCDD-Exposition zu einer Veränderung des Differenzierungsprogramms während der Thymusentwicklung führt.

Im Zusammenhang mit der unter 2.2.1 beschriebenen Arbeit wird deutlich, dass TCDD sowohl im fötalen als auch im adulten Thymus direkt in die Entwicklung und das Migrationsverhalten von unreifen Thymozyten eingreift. Zwischen den TCDD-induzierten Expressionsänderungen in fötalen DN Thymusemigranten und den in adulten Thymozyten und Thymusemigranten beobachteten Änderungen gab es so gut wie keine Übereinstimmungen. Das deutet darauf hin, dass die den jeweils beobachteten phänotypischen Änderungen zugrunde liegenden Effektormechanismen verschieden sind.

Der Verfasser der Dissertation führte alle Expressionsanalysen und die damit verbundenen experimentellen Schritte, wie RNA-Isolation, RNA-Amplifikation und Hybridisierungen durch. Die Expressionsdaten flossen direkt in die unter 2.1.1. dargestellte Arbeit ein. Da in den untersuchten Zelltypen aufgrund der starken Differenzierungsunterschiede eine Identifikation der direkten AhR-Zielgene nicht möglich war, führten die hier dargestellten Ergebnisse dazu, die Technik der fötalen Thymusorgankultur für die Analyse der primären und sekundären Zielgene zu verwerfen.

2.2.3 Impairment of maturational competence of cultured Langerhans cells from aryl hydrocarbon receptor (AhR) null mice and suppression of AhR signalling points to its role in LC tolerogenic strategy

Jux B, **Frericks M**, Esser C
[Eingereicht]

TCDD induziert eine Suppression des Immunsystems der Haut, was sich zum Beispiel in einer verminderten Reaktion im Ohrschwellungstest zeigt. Die zu Grunde liegenden Mechanismen dieser Inhibition sind bisher nicht vollständig aufgeklärt. Bei Immunantworten der Haut, sowie bei der Kontrolle durch Fremdstoffe induzierter allergischer Reaktionen, spielen Langerhans Zellen eine wichtige Rolle.
In LZ aus C57BL/6 Mäusen ist sowohl das AhR-Transkript, als auch das AhR-Protein gut detektierbar. Erstaunlicherweise führte eine 24h *in vivo* TCDD-Exposition zu keiner Transkriptionsänderung in den LZ. Eine mögliche Erklärung hierfür ist die in dieser Arbeit erstmals nachgewiesene starke konstitutive Expression des AhR-Repressors, welcher als ein negativer Regulator des AhR-Signalwegs gilt. Durch eine Unterdrückung der AhR-vermittelten Verstoffwechselung von Fremdstoffen, kann eine Bildung von allergene Zwischenprodukten verhindert, und somit eine allergische Reaktion gegen viele Fremdstoffe vermieden werden. Die Unterdrückung der AhR-Aktivität stellt somit einen potentiellen Mechanismus für Tolerogenität dar. In LZ von AhR defizienten Mäusen war die AhRR-Ausprägung nur schwach nachweisbar. Darüber hinaus zeigten die LZ aus AhR-defizienten Mäusen eine eingeschränkte Reifung, was sich in ihrer geringeren Größe und Granularität äußerte. In *in vitro* Differenzierungsstudien zeigten sie keine Aufregulation der kostimulatorischen Moleküle CD40, CD80 und CD24. Auch der Verlust der phagozytischen Aktivität, wie es für reife Langerhanszellen zu erwarten wäre, blieb aus. Ein möglicher Mechanismus hierfür wäre die nachweislich geringere Produktion von GMCSF in AhR-null-Mäusen. Diese Daten deuten darauf hin, dass der AhR für die tolerogene Aktivität und Reifung von LZ eine wichtige Rolle spielt.

Der Autor der Dissertation führte die primäre Analyse der Expressionsprofile durch. Die für diese Veröffentlichung durchgeführten Expressionsanalysen nach TCDD-Belastung in Langerhans Zellen erweitern die in 2.2.1 beschriebenen Untersuchungen der TCDD-vermittelten Transkriptionsänderung um einen weiteren Zelltyp des peripheren

Immunsystems. Im Gegensatz zu allen anderen untersuchten Zelltypen, besitzen LZ eine hohe AhR-Expression, weisen aber keine TCDD-induzierten Expressionsänderungen auf. Damit wird die These unterstützt, dass es keine einheitliche AhR-Zielgenbatterie gibt. Das zeigt deutlich, das die physiologischen Funktionen des AhR in vielen Bereichen noch weiter untersucht werden müssen(63).

3 Diskussion

3.1 TCDD-induzierte Expressionsänderungen

Der AhR ist ein Modell für einen ligandeninduzierten Transkriptionsfaktor. Da er zusammen mit PPAR (Peroxisome proliferator-activated receptor) und CAR (Constitutive androstane receptor) die Verstoffwechselung der meisten Pharmazeutika kontrolliert, wurden die AhR-vermittelten Transkriptionsänderungen in einer Reihe von verschiedenen Zell- und Organtypen umfangreich untersucht. Die meisten toxikogenomischen Analysen beschränken sich dabei jedoch entweder auf einzelne Zelllinien, oder komplette Organe. Beide Ansätze haben einige Vor- und Nachteile. Während die Analyse von Zelllinien nie die komplexe Reaktion eines Organs abbilden kann, bieten sie ein kontrolliertes System, welches nach Belieben manipuliert werden kann. Organe setzen sich dahingegen aus einer Vielzahl von Zelltypen zusammen, die untereinander interagieren. Expressionuntersuchungen in Organen bilden immer nur die Summe der Veränderungen ab. Diese kann jedoch z.B. allein dadurch beeinflusst werden, dass eine der Zellpopulationen in erhöhter Frequenz oder mit verändertem Reifungsgrad vorkommt. So unterscheiden sich beispielsweise allein die 8 verschiedenen Entwicklungsstadien der Thymozyten während ihrer Entwicklung im Thymus in mehr als 1000 Transkripten (51).

Um die Nachteile beider Systeme zu umgehen wurde in der hier vorgelegten Arbeit daher erstmalig eine Reihe von hochgradig aufgereinigten (alle Reinheiten >90%) primären Zellpopulationen verwendet, um die TCDD-vermittelten Effekte auf die Transkription zu erfassen. Die Zelltypen wurden so ausgewählt, dass sie verschiedene Stadien der T-Zellentwicklung bzw. verschiedene Arten von Antigenpräsentierenden Zellen abbilden. Dadurch war es möglich, den Einfluss des Alters, durch den Vergleich fötaler und adulter DN Thymusemigranten, der Lokalisation, durch den Vergleich von DN Thymozyten gegenüber Thymusemigranten, bzw. DZ aus der Milz und der Haut und des Reifungsgrads, durch einen Vergleich unreifer DN-Thymozyten zu $CD4^+$ - und $CD8^+$- T-Zellen der Milz, auf die Expression des AhR und die durch TCDD induzierte differentielle Expression zu untersuchen.

Es zeigte sich, dass zwischen den TCDD-induzierten Expressionsänderungen der einzelnen Zelltypen in dieser Arbeit verwendeten Zelltypen nahezu keine Übereinstimmung bestand. Selbst nah verwandte Zelltypen, wie die verschiedenen in dieser Arbeit untersuchten T-Zellpopulationen, weisen unterschiedliche Transkriptionssignaturen auf. Gleiches gilt auch

für die beiden DZ-Populationen, von denen DZ aus der Milz ca. 200 differentiell ausgeprägte Gene aufwiesen, während LZ aus der Haut jedoch, im Rahmen der verwendeten Schwellenwertes einer 2-fachen Regulation, nicht ein einziges verändertes Gen zeigten.

Es gibt mehrere Ansätze, die Unterschiede der beobachteten Transkriptionsänderungen zu erklären.

Die Zelltypen, in denen nach TCDD-Belastung eine größere Anzahl von Genen differentiell reguliert waren, wiesen eine gut detektierbare AhR-Expression auf. In den Effektor-T-Zellen der Peripherie, die nur wenige TCDD-vermittelte Expressionsänderungen aufweisen, war der AhR im Vergleich dazu nur in sehr geringen Mengen ausgeprägt. Dies weist darauf hin, dass eine hohe AhR-Expression nach TCDD-Belastung eine größere Menge differentiell transkribierter Gene impliziert. Diese Korrelation deckt sich mit den Beobachtungen für andere Organe, die stark auf eine AhR-Aktivierung reagieren. Beispiele hierfür sind Leber oder Uterus, die eine hohe AhR-Expression aufweisen und in denen nach TCDD-Exposition mehrere hundert Gene eine veränderte Transkription zeigen (7;24;119;143).

Fötale DN Thymusemigranten, sowie adulte DN Thymozyten und Thymusemigranten exprimierten den AhR in vergleichbarer Stärke, wiesen jedoch eine sehr unterschiedliche Menge von unterschiedlich ausgeprägten Transkripten nach TCDD-Belastung auf. Die Höhe der AhR-Expression allein ist also nicht ausreichend, um die unterschiedliche Wirkung auf die unreifen DN-Thymozytenstadien zu erklären. In allen drei Zelltypen verändert TCDD die Reifung, wodurch eine Identifikation von primären AhR-Zielgenen stark erschwert wird. So kommt es z.B in fötalen DN Thymusemigranten zu einem Block der T-Zellreifung im DN2-Stadium. Daher unterscheiden sich die Reifungsstadien von TCDD- und Lösungsmittel-behandelten Zellpopulationen. Hoffmann et. al. konnten demonstrieren, dass sich unterschiedliche Reifungsstadien von Thymozyten in mehr als 1000 Transkripten voneinander unterschieden (51). Es könnte also sein, dass viele der hier beobachteten Expressionsänderungen auf der Veränderung in der Häufigkeit von Zellen unterschiedlichen Reifungsgrads in der Population beruhen und eine Identifikation von direkten AhR-Zielgenen daher nicht möglich ist.

Um die unterschiedliche Anzahl differentiell ausgeprägter Gene zu erklären, muss auch die Wirkung anderer regulatorischer Mechanismen berücksichtigt werden. Genau wie in den drei unreifen T-Zellstadien ist die Stärke der AhR-Expression in den beiden DZ-Populationen aus Milz und Haut vergleichbar. Während Milz-DZ nach TCDD-Belastung mehr als 200 differentiell exprimierte Gene ausweisen, zeigen LZ keinerlei TCDD-Einfluss auf ihre

Transkriptionssignatur. Daher ist hier erneut die Stärke der AhR-Expression nicht ausreichend, um die unterschiedliche TCDD-induzierte Expressionssignatur zu erklären.
Ein mögliches Erklärungsmodell ist die Expression des AhRR, der in LZ basal eine hohe Expression zeigt. Der AhRR, der keine transaktivierende Domäne hat, kann mit dem AhR sowohl um Arnt, als auch um die DREs konkurrieren und die Transkription von Zielgenen unterbinden. Primäre Fibroblasten, die ebenso wie die LZ eine hohe basale AhRR-Ausprägung aufweisen, reagieren auf TCDD-Belastung genau wie LZ nicht mit einer Transkriptionsänderung. Dabei ist zu bedenken, dass in Fibroblasten bisher nur wenige Gene untersucht wurden, so dass die genaue (41;42).
Es gibt also eine Reihe von Faktoren, die den Umfang und die Art der TCDD-induzierten Transkriptionsänderung beeinflussen.

3.2 Verminderte Genexpression nach AhR-Aktivierung

Das klassische Modell des AhR-Signalwegs beschreibt nur die Geninduktion nach AhR-Aktivierung. In den hier vorgelegten Expressionsstudien wiesen viele Gene jedoch neben der Geninduktion auch eine verminderte Expression auf. Dieser Befund deckt sich mit den Ergebnissen anderer Arbeitsgruppen, die nach Belastung mit einem AhR-Liganden in verschiedenen Organ-/ Zellmodellen ebenfalls eine verminderte Expression vieler Transkripte beobachteten(24;47;48;66;119). In den im Rahmen dieser Arbeit erstellten Transkriptionsprofilen sowie in veröffentlichten Expressionssignaturen waren jeweils unterschiedliche Prozentzahlen von Genen induziert oder geringer ausgeprägt. Wie lässt sich die verminderte Transkription nach AhR-Aktivierung erklären?
Ein möglicher Mechanismus wäre die TCDD-induzierte Aktiviät von regulatorischen RNAs. Moffat et. al. konnten jedoch zeigen, dass TCDD in Leberzellen nur einen geringen Einfluss auf die Konzentration verschiedener miRNA-Spezies hat(104). In wiefern miRNAs die verminderte Expression in anderen Zelltypen beeinflussen, ist jedoch unklar und bedarf weiterer Forschung. Die Entwicklung von Microarrays, die nach regulatorischen RNAs suchen, bietet eine gute Möglichkeit, deren Regulation zu untersuchen. Da sich die miRNA-Ausstattung verschiedener Zelltypen unterscheidet, wäre hier eine zelltypspezifische Analyse, kombiniert mit der parallelen Messung der Transkriptionsänderung, ein probates Mittel, einen besseren Einblick in diesen Regulationsmechanismus zu bekommen.
Eine Veränderung des Reifungsgrads, wie in den DN Thymusemigranten, oder der Zellzusammensetzung des betrachteten Organs, wie z.B. die Infiltration von Leukozyten im

Uterus nach TCDD-Exposition (Cora Fong, persönliche Mittteilung), ist eine weitere Möglichkeit, die verminderte Genexpression zu erklären. Es ist jedoch nicht ausreichend, um alle Effekte in Zellkultursystemen zu erklären. So weisen beispielsweise auch Hepa1c1c7 – Zellen nach TCDD-Exposition einen hohen Anteil an vermindert ausgeprägten Genen auf(24).

Um die verminderte Expression von Genen nach TCDD-Belastung zu erklären, wird eine Konkurrenz um Transkriptionsfaktorbindestellen diskutiert. Mehrere Arbeitsgruppen versuchen derzeit diese Frage durch CHIP-Chip (Magdalena Adamska, persönliche Mitteilung), bzw. CHIP-Sequenziermethoden zu beantworten (Jeremy Burt, persönliche Mitteilung). Die Arbeiten des ENCODE-Konsortiums zeigen, dass es parallel bei mehreren hundert bis tausend Genen im Promoterbereich zu einer gehäuften Bindung von mehreren Transkriptionsfaktoren kommt(5). Des Weiteren konnten die im Rahmen der initialen Phase des ENCODE-Projekts erhobenen Daten keine direkte Korrelation zwischen der Bindung einer Transkriptionsfaktors an den Promoter und der Genexpression zeigen. Die DNA-Protein-Wechselwirkungen wurden dabei in einer Auflösung von einigen hundert Basenpaaren gemessen.

Bei der *in silico* Analyse der Abundanz putativer Transkriptionsfaktorbindestellen zeigte sich, dass diese präferentiell im Bereich von -1500 bis +1500 bp um den Transkriptionsstart auftraten(5). Eine solche Verteilung konnte auch für DRE und HRE gezeigt werden(86;134). Dies konnte im Rahmen in dieser Arbeit durchgeführten Promoteranalysen bestätigt und für alle untersuchten TFBS mit konservieten Bindungmotiven erweitert werden (Daten nicht gezeigt). Dabei zeigte sich, dass viele Bindestellen der verschiedenen Transkriptionsfaktoren oft überlappen (Daten nicht gezeigt). Die geringe Auflösung der erwähnten Hochdurchsatzverfahren gepaart mit der Häufung putativer Bindestellen um den Transkriptionsstart zeigen verhindern zurzeit noch eine Demonstration der direkte Kompetition um TFBS(5). Darüber hinaus sind die Experimente mit Tiling-Arrays bisher noch auf wenige Transkriptionsfaktoren beschränkt, für die es gute, für diese Technik nutzbare Antikörper gibt. Mit Verbesserung der Technik, der zugrunde liegenden Bioinformatik und einer breiten Palette von geeigneten Antikörpern, ermöglichen Tiling-Arrays wichtige neue Erkenntnisse für die Kontrolle des AhR-Signalwegs und der AhR-vermittelten Geninduktion gewonnen.

Für die Erklärung der verminderten Genexpression nach TCDD-Belastung werden alle hier besprochenen Mechanismen eine Rolle spielen. Die Erkenntnis, dass die AhR-vermittelten Expressionsänderung in hohem Maß vom untersuchten Zelltyp abhängig ist, deutet darauf

hin, dass auch die jeweiligen molekularen Mechanismen die diese Änderungen bewirken zelltypspezifisch sind.

3.3 Identifikation einer AhR-Zielgenbatterie

Ein Ziel dieser Arbeit war die Identifikationen von allgemeinen Zielgenen, also von Markergenen, für eine AhR-Aktivierung. Zu diesem Zweck wurden verschiedene primäre Zellen des Immunsystems auf TCDD-vermittelte Expressionsänderungen untersucht. Zwischen den TCDD-induzierten Genexpressionsänderungen der in dieser Arbeit untersuchten T- und DZ-Populationen gab es so gut wie keine Überlappungen. Auffällig ist, dass keines der klassischen „Markergene", wie *Cyp1a1*, *Cyp1a2*, *AhRR*, *Aldh3a1*, *Gstm* oder *Nqo1* in einer Vielzahl der untersuchten Zelltypen gehäuft differentiell exprimiert waren. Die meisten der Gene dieser klassischen AhR-Batterie haben eine wichtige Funktion im Fremdstoffmetabolismus und werden hauptsächlich in der Leber ausgeprägt. Die Ausstattung verschiedener Organe und Zelltypen mit fremdstoffmetabolisierenden Enzymen ist selbst innerhalb eines Organs stark unterschiedlich(90;114). Zellen des Immunsystems haben allgemein eine geringe fremdstoffmetabolisierende Kapazität, weshalb hier auch eine geringe Expression und Induzierbarkeit der entsprechenden Enzyme zu erwarten ist. Im Gegensatz dazu konnten in dieser Arbeit erstmals gezeigt werden, dass in ET-Zellen eine basale Expression und Induzierbarkeit großer Teile der klassischen Markergene nachweisbar ist. Diese Ergebnisse konnten durch die Analyse von veröffentlichten Expressionsprofilen primärer kortikalen TEZ, die ebenfalls eine gut nachweisbare basale Ausprägung von *Cyp1b1*, *Nqo1* oder *Gstm* aufweisen, bestätigt werden(23). Das eine Aktivität des Fremdstoffwechsels im Zusammenhang mit den Ahr-abhängigen adversen Effekte auf den Thymus steht konnte für die Cyp1b1 gezeigt werden(147), bedarf jedoch für andere Gene weiterer Forschung.

Cyp1b1 war als einziges Gen in vier der untersuchten Zelltypen, nämlich fötale Thymusemigranten, adulten Thymozyten und Thymusemigranten sowie ET-Zellen, nach TCDD-Exposition differentiell reguliert und kann damit am ehesten als Markergen bezeichnet werden. Um eine allgemeine AhR-Zielgenbatterie zu identifizieren, wurde daher die Analyse auf veröffentlichte Expressionsprofile ausgeweitet, in denen der AhR aktiviert, inhiebiert oder deletiert wurde. Dabei bestätigten sich die in Immunzellen gemachten Beobachtungen, nämlich, dass es keine Gene gibt, die immer eine AhR-abhängige Regulation zeigen.

Kombiniert man alle untersuchten Expressionsprofile, die der primären Immunzellen und veröffentlichte im AhR-Kontext, gibt es jedoch eine Reihe von Genen, die vermehrt differentiell ausgeprägt werden. Von diesen zeigt *Cyp1a1*, gefolgt von *Cyp1b1*, am häufigsten eine differentielle Transkription. Durch den Vergleich der Gene, die im AhR-Zusammenhang (Aktivierung oder Deletion) gehäuft (mehr als drei Experimente) differentiell exprimiert werden, gelang es so eine allgemeine Markergenbatterie zu identifizieren. Beim hier verwendeten Vergleich von Listen differentiell exprimierter Gene, die mit unterschiedlichen Microarraytypen erstellt wurden, muss jedoch berücksichtigt werden, dass Microarrays verschiedener Hersteller sich oft erheblich in der Zusammensetzung und der Nachweisgrenze der einzelnen Gene unterscheiden. Daher kann es sein, dass manche Transkripte, insbesondere wenig charakterisierte, wie *Tiparp* oder *Xist*, mit vielen Chiptypen nicht mit gemessen werden. Dadurch kommt es zu einer bevorzugten Entdeckung von gut charakterisierten Genen. Idealerweise müsste man also die Suche nach Markergenen auf einen Expressionsanalysen eines Chiptyps beschränken. Zum Aufbau der in dieser Arbeit verwendeten Expressionsdatenbank wurde ein solcher Ansatz gewählt. Die zunehmende Standardisierung von Expressionsanalysen, die steigende Anzahl veröffentlichter Expressionsdaten sowie die Tatsache, dass die meisten Verlage eine gleichzeitige Veröffentlichung der ursprünglich gemessenen Microarraydaten verlangen, wird eine solch umfassende spezies- und chiptypspezifische Analyse bald zulassen. Die daraus gewonnenen Erkenntnisse werden zu einer deutlich genaueren Identifikation von Markergenbatterien führen.

Die definierte allgemeine Markergenbatterie wurde nun dazu verwendet, die Expression des AhR, sowie eine Beteiligung des AhR an pathophysiologischen Prozessen, wie Erkrankungen oder verschiedenen Entwicklungsvorgängen, zu untersuchen. Dazu wurde eine Datenbank aus veröffentlichten Expressionsprofilen aufgebaut, bei deren Erstellung der AhR weder aktiviert, inhibiert noch deletiert war.

Dabei zeigte sich, dass die meisten Gene in mehr als einem Drittel aller in der Datenbank enthaltenen Experimente differentiell ausgeprägt wurden. Das galt auch für die AhR-Markergene. Diese Erkenntnis deutet darauf hin, dass die meisten Gene durch multiple Prozesse reguliert werden, und die Betrachtung einzelner Gene als Marker für eine Beteiligung eines Signalwegs an einem Prozess nicht ausreicht.

Beim Aufbau der Datenbank standen für viele Behandlungen jedoch nicht mehr als zwei Replikate zur Verfügung, was die statistische Aussagekraft einer solchen Analyse schwächt. Erschwerend kommt hinzu, dass Microarrays, wie die meisten anderen Hoch-

durchsatzmethoden, eine gewisse „Hintergrundproblematik“ aufweisen. Das heißt, dass schwach transkribierte Gene, deren Detektierbarkeit nur wenig über dem Hintergrundsignal liegt, falsch positive oder negative Ergebnisse liefern können(126). Um diese Problematik zu umgehen, ist es notwendig, mehr als ein Transkript als Marker zu verwenden, um zufällige Befunde möglichst gering zu halten.

Zusammengefasst lässt sich sagen, dass die TCDD-induzierten Expressionsänderungen vom untersuchten Zelltyp, dessen Reifungsgrad bzw. Alter sowie der basalen Expression von Genen mit Einfluss auf den AhR-Signalweg, wie die AhRR-Expression, abhängen. Außerdem können AhR-Zielgene zelltypabhängig eine verminderte oder erhöhte Expression aufweisen. Darüber hinaus bedingt die Nachweisgrenze verschiedener Techniken eine unterschiedliche Detektierbarkeit der Zielgene. Das alles zeigt klar, dass für die Identifikation einer AhR-Beteiligung mehrere potentielle Zielgene verwendet werden müssen.

3.4 Anwendung von Zielgenbatterien

Ein Problem der Analyse von differentiellen Expressionssignaturen stellt die Menge der veränderten Transkripte dar. Für viele Gene ist meist nur wenig über ihre Funktion, insbesondere in dem gerade untersuchten Modell, bekannt. Ein mögliches und inzwischen häufig angewendetes Hilfsmittel, ist die Verwendung von Genen, so genannten „Gen-Sets“, die z.B. nach AhR-Aktivierung eine veränderte Ausprägung aufweisen. Bei „Gene Set Enrichment Analysen“ (GSEA) werden aus veröffentlichten Genexpressionssignaturen, die nach Aktivierung oder Inhibition einer Signalkaskade eine veränderte Expression aufweisen, so genannte „Gene-Sets“ entwickelt(132). Ist dieses Gene-Set in einem Datensatz statistisch signifikant überrepräsentiert, wird dies als Beteiligung des Signalweges an der veränderten Genexpression gewertet. Diese Methode ist sehr stark von der Qualität der verwendeten Gene-Sets abhängig. Zudem sind, wie in dieser Arbeit gezeigt, induzierte Transkriptionssignaturen in hohem Maß zelltypspezifisch(35). So ist es für den AhR nicht möglich, Gene zu definieren, die immer in allen Zelltypen differentiell reguliert werden. Unter Idealbedingungen sollten daher nur Gen-Sets verwendet werden, die im gleichen Zelltyp entwickelt wurden, in dem auch die Transkriptionsanalyse durchgeführt wird. Darüber hinaus geht eine GSEA von der gleichen Richtung der Regulation aus, was, wie in dieser Arbeit gezeigt, oft nicht der Fall ist. Daher sind viele GSEA eine Vereinfachung und sollten nur als Hinweis gewertet werden. Bei hochqualitativen Gene-Sets stellt GSEA jedoch ein leistungsfähiges Werkzeug zur Interpretation von Microarrayergebnissen dar.

Die im Rahmen dieser Arbeit identifizierten Markergene konnten aus Expressionsstudien primärer Immunzellen (2.1.1) und einer Reihe veröffentlichter Expressionsstudien(2.1.2), in denen der AhR experimentell manipuliert, also aktiviert, inhibiert oder deletiert wurde, Gene identifiziert werden, die gehäuft differentiell exprimiert wurden. Diese AhR-Markergene stellen ebenfalls ein Gene-Set dar. Dieses Gene-Set ist jedoch nicht zelltypspezifisch, sondern wurde aus einer Vielzahl von Veröffentlichungen entwickelt. Es ist auch nicht auf eine Richtung der Expressionsänderung beschränkt. Daher eignet es sich gut für die Identifizierung von physiologischen Prozessen mit einer potentiellen Rolle des AhR an den beobachteten Genexpressionsänderungen.

In dieser Dissertation wurde ein solches, allgemeines AhR-Gene-Set, erstmalig in diesem Umfang, für die Identifikation einer AhR-Beteiligung an pathophysiologischen Prozessen verwendet(35). Dazu wurden öffentlich zugängliche Expressionsprofile verwendet, in denen der AhR a priori nicht manipuliert, also deletiert oder durch einen Liganden aktiviert, wurde. Das ermöglicht es, mehr über die physiologische Rolle des AhR zu erfahren.

In vielen physiologischen Entwicklungsprozessen, wie der Reifung der Hoden, des Uterus, von T-Zellen oder dendritischen Zellen, aber auch bei Ischämien am Herz oder Gehirn, sowie nach Gendeletionen, wie der von Nrf2, waren der AhR und mehrere Zielgene reguliert. Das deutet darauf hin, dass der AhR unter diesen Bedingungen eine physiologische Aktivität aufweist. Es stellt sich die Frage, ob eine intrinsische Aktivität des AhR während dieser Prozesse gleichzeitig eine erhöhte Anfälligkeit gegenüber den adversen Effekten von AhR-Liganden auslöst. Ein Beispiel dafür wäre, ob z.B. unter ischämischen Bedingungen am Herzen oder Gehirn die adversen Effekte durch eine parallele Belastung mit TCDD oder anderen AhR-Liganden verstärkt oder abgeschwächt werden. Alternativ wäre die Untersuchung eines solchen Prozesses und seiner translationalen Umsetzung in der $AhR^{-/-}$-Maus für die Bestätigung der AhR-Beteiligung sinnvoll.

Solche Analysen können im Rahmen der Präventivmedizin äußerst wichtig sein. Dadurch könnten schädliche Einflüsse und Prädispositionen vermindert werden, die zu einer Verstärkung von Krankheitsymptomen oder einem erhöhten Erkrankungsrisiko führen. So wäre z.B eine Vermeidung von PCB-belasteter Nahrung, insbesondere Fisch, unter solchen Bedingungen empfehlenswert, da viele PCBs AhR-Liganden sind, bzw. zu ihnen verstoffwechselt werden können.

3.5 Limitationen und Möglichkeiten der Datenbank NC-GED

Um den AhR und seine Beteiligung an physiologischen Prozessen zu untersuchen, wurde im Rahmen dieser Datenbank eine umfangreiche Datenbank (NC-GED) aus veröffentlichten Expressionsprofilen aufgebaut. Dabei wurden nur Microarrays desselben Chiptyps verwendet, die außerdem alle mit dem gleichen Auswertealgorithmus erstellt wurden(59). Diese Restriktionen sowie ein zusätzlicher Normalisierungsschritt garantiert ein maximales Maß der Vergleichbarkeit(58;115). Wie unter 2.1.2 gezeigt, liefert NC-GED valide Ergebnisse. Das äußert sich durch eine hohe Vergleichbarkeit der Genexpression von Organen, die in verschiedenen Laboratorien erstellt wurden, dadurch, dass sich die Gewebsverteilungen einzelner Gene durch qRT-PCR bestätigen ließen, und dadurch, dass bekannte Verteilungsmuster verschiedener Gene auch in der Datenbank vergleichbar waren. Der zum Aufbau der Datenbank verwendete Ansatz stellt jedoch keine Optimallösung dar, da normalisierte Daten und nicht deren Rohdaten verwendet werden mussten. Die Qualität der Rohdaten ist nicht bekannt. Eine genaue Kontrolle von Microarray-Rohdaten ist zur Vermeidung von Komplikationen, wie einzelne Ausreißer, oder eines zu hohen Hintergrunds, jedoch notwendig(37;58). Daher wäre es wünschenswert, wenn die Originaldaten (.cel-Files) zur Verfügung ständen und gemeinsam mit dem gleichen Algorithmus in der gleichen Version, analysiert werden könnten. Dieser Ansatz wird inzwischen z.B. zum Erstellen von toxikologischen Referenzdatenbanken verwendet (C. Afshari, persönliche Mitteilung). Ein weiteres Beispiel ist die vom Scripps Institut betriebene Datenbank Symatlas(131). Zum Zeitpunkt des Aufbaus der in dieser Arbeit erstellten Datenbank standen neben den tabellarischen, bereits normalisierten Daten nur wenige Rohdaten zur Verfügung. Aus diesem Grund wurde eine genaue Kontrolle der Rohdaten der veröffentlichten Expressionsprofile vorausgesetzt.

Sowohl die gemeinsame Analyse der Rohdaten, als auch die Verwendung bereits normalisierter Datensätze erlauben eine nahezu beliebige Erweiterung der aufgebauten Datenbank. Durch den parallelen Aufbau vergleichbarer Datenbanken für andere Spezies und Chiptypen ist es so z.B. möglich Speziesvergleiche anzustellen. Dies wird es erlauben festzustellen, ob und in welchem Ausmaß z.B. die Induktion von Zielgene des AhR über verschiedene Spezies konserviert ist. Außerdem wäre es möglich, die Relevanz von Expressionsveränderungen in Modellorganismen, wie der Maus, für die Risikobewertung im Menschen abzuschätzen. Der Aufbau solcher Datenbänke ist also in jedem Fall wünschenswert(95;136).

3.6 Nutzung der Datenbank zur Vorhersage von AhR-vermittelten Effekten

Neben der Identifikation AhR-abhängiger Reaktionen ist für ein Verständnis der physiologischen Rolle des AhR und der Vorhersage von Zielen einer AhR-Überaktivierung, auch eine genaue Kenntnis der basalen Expression aller Komponenten des AhR-Signalweges sowie seiner potentiellen Interaktionspartner wichtig. Darüber ist es möglich, das Reaktionsvermögen eines Zelltyps, wie z.B. von Thymozyten, in gewissem Rahmen vorherzusagen, und Experimente zur Bestätigung der so gewonnenen Hypothesen zu erarbeiten. Ein Beispiel hierfür wäre die Prognose, dass unreife Thymozyten aufgrund ihrer gut detektierbaren AhR-Expression und dem Fehlen einer AhRR-Expression ein Ziel einer TCDD-induzierten Veränderung sind. Entsprechend deutet vieles darauf hin, dass T-Zell-Lyphomzelllinien und periphere reife Thymozyten, die eine geringe bis kaum nachweisbare AhR-Expression aufweisen, nur wenig durch eine TCDD-Belastung beeinträchtigt werden. Für die beiden T-Zelllinien Wehi 7.1 und EL-4 (beides $CD4^+$-Effektorzellen) ließ sich dieses Verhalten, gemessen an der Induktion von Genen wie *Cyp1a1* und *Cyp1b1*, auch bestätigen (Daten nicht gezeigt). Auch periphere $CD4^+$ und $CD8^+$-Zellen reagieren nur mit wenigen TCDD-induzierten Expressionsänderungen. Eine weitere Möglichkeit wäre die Vorhersage der Expression von differentiell transkribierten Zielgenen. So ist es wahrscheinlich, dass in Zellen, in denen z.B. *Cyp1a1* und *Cyp1b1* eine Expression aufweisen, in denen das Chromatin offen und der Promoter also für Transkriptionsfaktoren zugänglich ist, TCDD die Expression dieser Gene steigert. Diese Annahme trifft beispielsweise für kortikale Thymusepithelzellen zu.

Mit der Datenbank NC-GED konnten auch einige Zelltypen, insbesondere einige hematopoetische Stammzellen, identifiziert werden, die zwar eine hohe AhR-Expression, aber, im Rahmen der Nachweisgrenze des verwendeten Microarrays, keine Arnt- oder Arnt2-Expression aufweisen. Der AhR benötigt Arnt als Heterodimerisierungspartner, um als Transkriptionsfaktor zu wirken. Welche Funktion besitzt der AhR also in diesen Zelltypen? Mit welchen Proteinen ist er assoziiert? Was passiert in einer solchen Zelle, bei einer Ligandenbindung des AhR? Nach dem bisherigen Modell sollte er nach Bindung eines Liganden in den Kern translozieren und dort seine zytosolischen Bindungspartner freisetzen. Aber was passiert dann? Um solche Fragen zu beantworten sind in jedem Fall weitere Experimente notwendig, beispielsweise die Transkriptionsanalyse aber vor allem Western-Blotanalysen eines solchen Zelltyps.

Mit wachsendem Umfang der Datenbank und wachsender Qualität der hinterlegten Daten wird die Fähigkeit, aus einer solchen Datenbank Vorhersagen zu treffen, verbessert. Diese Fähigkeit wird entscheidend durch die Kenntnis von Parametern gesteigert, welche die TCDD-induzierte Expressionssignatur beeinflussen. Daher wäre eine Erweiterung, bzw. Neuaufbau der Datenbank mit Microarray-Rohdaten ein sehr viel versprechendes Projekt. Allerdings wäre hierfür eine Implementierung in ein professionelles Datenbank-Format, beispielsweise Oracle mit entsprechendem Web-Interface notwendig.

Durch die Kombination der TCDD-induzierten Expressionsänderungen mit hoch auflösenden Transkriptionsfaktoranalysen konnten einige potentielle Interaktionspartner des AhR-Signalwegs identifiziert werden. Neben den erwarteten DREs kommen auch die Bindestellen einer Reihe weiterer Transkriptionsfaktoren, wie NFκB-, Pax4, ÖR oder HIF statistisch signifikant gehäuft in den regulatorischen Regionen der TCDD-regulierten Gene von ET-Zellen vor. Das legt nahe, dass die Aktivität dieser Signalwege einen Einfluß auf die TCDD-induzierte Transkriptions-änderung besitzt, und umgekehrt. Für die beiden hier getesteten Faktoren, HIF und ÖR, ist eine Wechselwirkung bekannt(6;61;86;110;122;160). Für beide Faktoren konnte im Rahmen dieser Arbeit experimentell ein Einfluss auf die TCDD-vermittelte Expressionsänderung in ET-Zellen bestätigt werden (2.1.3). Diese Bestätigung der Ergebnisse der *in silico* Promoteranalysen deutet darauf hin, dass auch die anderen Transkriptions-faktorbindestellen, bzw. ihre Nutzung durch die Aktivität anderer Signalwege, die AhR-Zielgene beeinflussen. Um eine Wechselwirkung des AhR-Signalwegs mit diesen Transkriptionsfaktoren, z.B. durch eine Kompetition um DNA-Bindestellen, zu bestätigen, sind jedoch noch weitere Experimente notwendig. Eine Bestätigung der *in silico* Analysen für die anderen identifizierten TFBS, wie Pax4 oder ROR-alpha, wäre beispielsweise durch die Untersuchung der TCDD-induzierten Genexpression in Mäusen, die eine Deletion des entsprechenden Transkriptionsfaktors besitzen.

Vielfach führen Gendeletionen zu einer deutlichen Beeinträchtigung des Phänotyps. Selbst in Zelltypen, für die keine phänotypischen Änderungen ersichtlich sind, kann es zu einer Veränderung der basalen Genexpression führen. Ein Beispiel hierfür ist die veränderte Transkriptionssignatur von $CD4^+$-T-Zellen aus Wildtyp im Vergleich zu $AhR^{-/-}$-Mäusen. Die Gründe der veränderten Expressionssignatur sind dabei nicht klar definiert. $CD4^+$-Zellen besitzen nur eine geringe AhR-Expression und reagieren mit wenigen (12) Genen auf eine Aktivierung des AhR (2.1.1). Die differentielle Transkription kann also beispielsweise auch auf einer veränderten Aktivität anderer Signalkaskaden beruhen. Zurzeit kann diese Frage nicht endgültig geklärt werden.

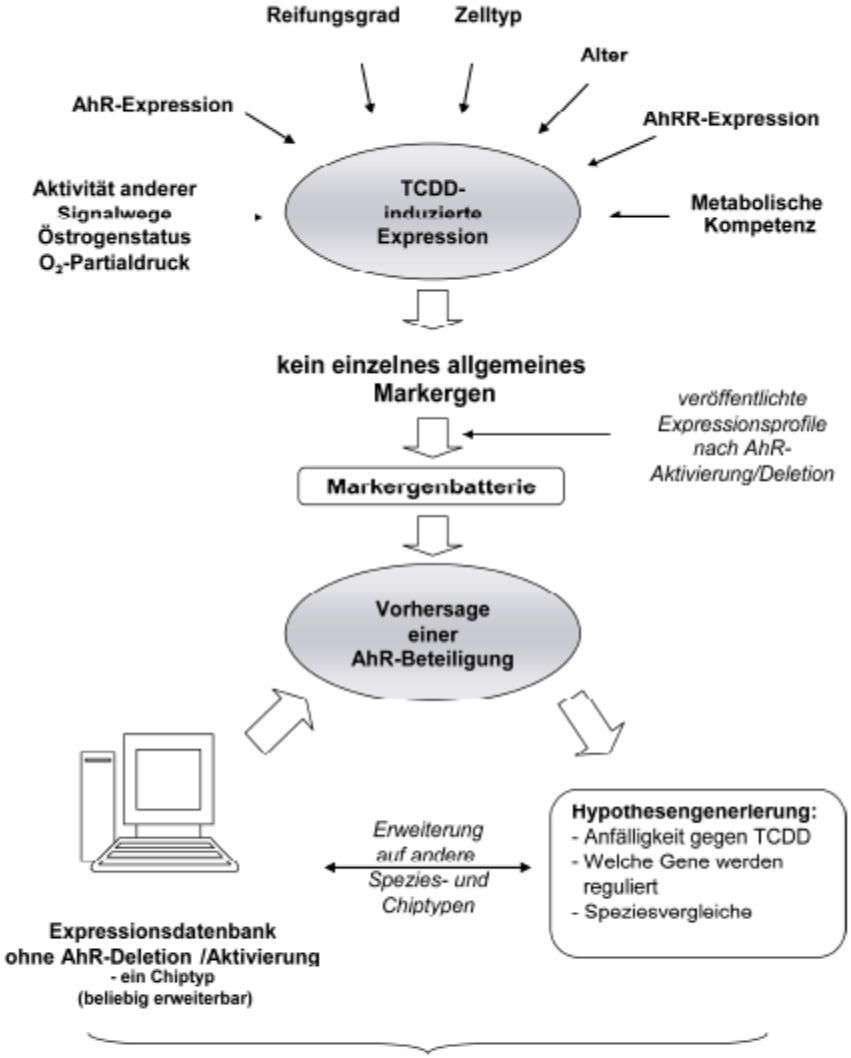

Abbildung 8: Schematische Darstellung der Ergebnisse und ihrer Anwendungsmöglichkeiten.

Durch die Analyse der zelltypspezifischen differentiellen Expressions primärer Zellen des Immunsystems nach TCDD-Belastung konnte gezeigt werden, dass die TCDD-

abhängigen Transkriptionsänderungen von vielen Faktoren beeinflusst werden. Dabei war kein Gen in allen Zelltypen AhR-abhängig reguliert. Durch die Verwendung zusätzlicher, veröffentlichter Expressionsprofile konnte eine Batterie von Markergenen definiert werden, die in mehreren AhR-abhängigen Expressions-untersuchungen reguliert waren. Mit dieser Markergenbatterie ist die Vorhersage einer potentiellen AhR-Beteiligung an physiologischen Prozessen möglich. Zu diesem Zweck wurde eine Datenbank aus veröffentlichten Expressionsprofilen aufgebaut und mit der Markergenbatterie untersucht. Die Vorhersagemöglichkeiten eines solchen Modells sind stark von der Kenntnis aller es beeinflussenden Faktoren abhängig. Weitere beeinflussende Faktoren die einen Einfluss auf die AhR-abhängige Transkriptionsänderung haben wurden durch eine Kombination von kinetischen Expressionsanalysen mit Promoteranalysen definiert. Durch eine Erweiterung der Kenntnis beeinträchtigender Faktoren und der parallelen Erweiterung der Datenbank werden so immer genauere Vorhersagen über die physiologische Funktion des AhR und damit auch über die Folgen einer AhR-Überaktivierung möglich.

Betrachtet man den Einfluss einer Gendeletion auf die TCDD-induzierte Transkription, so muss man bei der Interpretation der Ergebnisse auch auf die veränderte Aktivität anderer Signalwege achten. Für das Beispiel einer Wechselwirkung zwischen dem AhR-Signalweg und dem ÖR-Signalweg ist es also nicht klar, ob z.B. die Deletion des ÖR direkt die TCDD-induzierte Expression beeinträchtigen würde, oder ob eine solche Beeinträchtigung auf einer veränderten Aktivität anderer Signalwege beruht.

Eine andere Möglichkeit eine Wechselwirkung des AhR-Signalweges mit anderen Signalwegen zu untersuchen, wäre die Verwendung eines chemischen Inhibitors oder Aktivators des zu untersuchenden Signalwegs. Für die Wechselwirkung des AhR- mit dem HIF-Signalweg wurde ein solches Experiment bereits veröffentlicht(86).

Für chemische Inhibitoren und Aktivatoren ergibt sich jedoch dieselbe Problematik wie in defizienten Mausmodellen. Man spricht dabei davon, dass ein chemischer Inhibitor nicht „sauber" arbeitet, dass er also neben der spezifischen Inhibition des untersuchten Signalweges auch andere Signalwege beeinflusst. So ist zum Beispiel für das in dieser Arbeit verwendet $CoCl_2$ bekannt, dass es zwar primär eine stabilisierenden Wirkung auf Hif1α auslöst, daneben aber, wenn auch in geringerem Umfang, mit dem NFκB-Signalweg interagieren kann. Ähnlich verhält es sich für E2. E2 bindet mit vergleichbaren Bindekonstanten an ÖRα und

ÖRβ(67;68;77). Es ist also unklar, ob die im Rahmen dieser Arbeit beobachteten Einflüsse von E2 auf einer Bindung an ÖRα oder ÖRβ beruhen. Umfangreiche toxikogenomische und pharmakogenomische Untersuchungen haben gezeigt, dass nahezu keine Substanz nur einen Signalweg beeinflusst (11;45;47;99;105;125). Über die Stöchiometrie und den Umfang einer solchen unspezifischen Wechselwirkung ist wenig bekannt.

Das Vorgehen und die Ergebnisse dieser Arbeit noch einmal schematisch in Abbildung 8 dargestellt. Die Untersuchung der AhR-abhängigen Transkription in primären Zellen des Immunsystems zeigen deutlich, dass die Wirkungen einer AhR-Aktivierung hochgradig komplex sind, und sich nicht komplett durch das bisherige Modell des AhR-Signalwegs erklären lassen. So zeigen die TCDD-induzierten Transkriptionssignaturen verschiedener Zelltypen nahezu keine Gemeinsamkeiten. Die Expressionsänderung wird von vielen Faktoren, wie der AhR-Expressionsstärke, dem Reifungsgrad der Zellen, der Expression des AhRR, der Expression von anderen AhR-assoziierten Genen, sowie der Aktivität anderer Signalwege, die mit dem AhR interagieren können, beeinflusst. Das macht deutlich, dass man von einem multifaktoriellen System der AhR-induzierten Geninduktion ausgehen muss.

Die Erweiterung der Kenntnis dieser Prozesse sowie die Identifizierung der Aktivität der beteiligten Signalwege werden bei ihrer Anwendung auf die steigende Anzahl von öffentlich zugänglichen Expressionsprofilen immer genauere Vorhersagen der AhR-Beteiligung an physiologischen Prozessen zulassen.

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Anhang A

Veröffentlichungen

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Short Communication

Transcriptional signatures of immune cells in aryl hydrocarbon receptor (AHR)-proficient and AHR-deficient mice

Markus Frericks, Vladimir V. Temchura[a], Marc Majora, Susanne Stutte and Charlotte Esser*

Institut für Umweltmedizinische Forschung (IUF) at the Heinrich-Heine University of Düsseldorf, Auf'm Hennekamp 50, D-40225 Düsseldorf, Germany

* Corresponding author
e-mail: chesser@uni-duesseldorf.de

Abstract

The ligand-activated aryl hydrocarbon receptor (AHR) is known to modulate many genes in a highly cell-specific manner, either directly or indirectly via secondary effects. In contrast, little is known about the effects of AHR deficiency on gene expression balance. We compared the transcriptome of CD4 T cells from AHR$^{-/-}$ mice and wild-type mice; 390 genes, many of them immunotypic, were deregulated in AHR-deficient CD4 cells. TCDD-induced transcriptome changes correlated with the AHR expression level in immune cells. However, there was little overlap in AHR-dependent transcripts found in T lineage cells or dendritic cells. Our results demonstrate flexible gene accessibility for the AHR in immune cells. The idea of a universal battery of AHR-responsive genes is not tenable.

Keywords: Affymetrix; aryl hydrocarbon receptor; CD4 T cells; dendritic cells; Lgals3; scinderin; thymocytes.

Early toxicological studies of the aryl hydrocarbon receptor (AHR) analyzed primarily over-activation by stable chemical compounds, such as the highly toxic dioxins and furans. More recently, the role of the AHR in cell physiology and cellular differentiation programs has been appreciated and experimentally addressed, in particular by the analysis of several AHR-deficient, constitutively active AHR-transgenic, or AHR-dimerization partner aryl hydrocarbon receptor nuclear transporter (ARNT)-hypomorphic mouse strains (Schmidt et al., 1996; Ito et al., 2004; Walisser et al., 2004). Thus the central role of the AHR in dioxin toxicity has been unequivocally established. Over the years more and more genes have been identified as regulated by dioxin, although for only a few genes has targeting of their promoters by the AHR been formally shown. As we also found in the present study, not all genes modulated after exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have putative dioxin-responsive elements (DREs) in their promoters (Lai et al., 1996; Sun et al., 2004; Majora et al., 2005). Thus, it has been suggested that many of the genes regulated by the AHR/ligand complex are due to secondary signaling cascades. This notion is supported by the striking cell specificity of AHR-action. While AHR-deficient (–/–) mice are refractory to dioxin-induced toxicity (Fernandez-Salguero et al., 1996), at the same time their phenotype indicates involvement of the AHR in normal growth, development, differentiation and organ functions (Fernandez-Salguero et al., 1995; Schmidt et al., 1996). However, little is known on changes in gene expression by the absence of the AHR in relation to cells or organs known to be sensitive targets of AHR activity. Until now, two such studies have been done with global gene expression profiling, one a comparison of cultivated aortic cells from AHR$^{-/-}$ mice with AHR$^{+/+}$, and the other a study of liver cells of AHR$^{-/-}$ cells (Karyala et al., 2004; Tijet et al., 2006). Those data asserted that constitutive expression of genes can also be dependent on the AHR; i.e., the absence of the AHR influences normal gene expression as well.

Overactivation of the AHR results in a variety of toxic effects, including hepatotoxicity, teratogenicity, cancerogenicity, or cachexia. The sensitivity towards effects seems to be species-specific. The immune system is a sensitive target of the AHR in all species analyzed so far, and we and other groups have previously reported extensive effects of the dioxin-activated AHR on murine thymocyte differentiation programs, and on CD4 T cell activity and dendritic cell function in immune responses (Staples et al., 1998; Kronenberg et al., 2000; Vorderstrasse et al., 2001; Laupeze et al., 2002; Choi et al., 2003; Funatake et al., 2005; Temchura et al., 2005). Here we again study the mouse, but it is important to note that species might differ. For the present study, we isolated fetal immature thymocytes, fetal thymic emigrants, mature adult CD4 and CD8 cells, and splenic dendritic cells (DC) from C57BL6 AHR$^{+/+}$ and determined their global gene expression with Affymetrix™ chips (Affymetrix, Santa Clara, CA, USA). Cells were either from mice treated short-term with 10 μg/kg TCDD for 24 h *in vivo*, or were isolated from fetal thymus organ cultures treated long-term with 10 nM TCDD for 6 days *in vitro*.

In a first series of experiments, we isolated immature CD4$^-$CD8$^-$ thymocytes and thymic emigrants from adult mice treated *in vivo* for 7 days with a moderate single dose of TCDD of 10 μg/kg. The LD_{50} for mice is ~100 μg/kg. Thus, the dose used is not systemically toxic but affects the thymus nonetheless. We chose a

[a]Present address: Department of Molecular and Medical Virology, University of Bochum, Universitätsstrasse 150, D-44801 Bochum, Germany

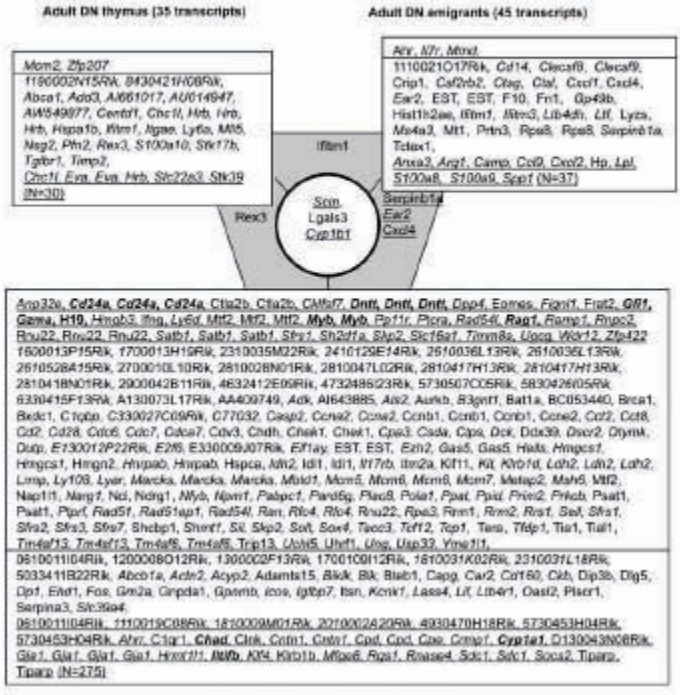

Figure 1 Venn diagram of adult CD4$^-$CD8$^-$ (DN) thymocytes and emigrants.
Mice were injected i.p. with 10 μg/kg TCDD (Promochem, Wesel, Germany) dissolved in olive oil or with solvent alone. Seven days later, mice were sacrificed, thymocytes isolated and CD4$^-$CD8$^-$CD19$^-$ thymocytes separated by magnetic cell sorting (Miltenyi Biotec, Bergisch-Gladbach, Germany) to >95% purity. Adult DN emigrants were isolated after injection of fluorescein isothiocyanate into the thymus of anesthetized mice 5 days after TCDD exposure, and FACS-assisted cell sorting of emigrated, i.e., RTE$^+$ cells, CD4$^-$CD8$^-$ cells 2 days later from the spleen. For fetal emigrants, fetal thymic lobes were taken from embryos at gestation day 15 and cultivated for 6 days in the presence of 10 nM TCDD or solvent only. Emigrants were collected and magnetically depleted of CD4- and CD8-bearing cells. Total RNA was isolated from all cell types, linear amplified (MessageAmp™ Kit of Ambion, Woodward St. Austin, TX, USA) and hybridized to Affymetrix® MOE430A chips after ascertaining integrity. Data were analyzed with the bioconductor affy package provided by the manufacturer. As cut-off point a two-fold change is presented here. Two independent experiments were performed. Only transcripts (i.e., differentially transcribed genes) found in both experiments are shown. Affymetrix arrays contain multiple ProbeSets that map to the same gene. Thus, the same gene name can appear several times. We counted and depicted all ProbeSets as transcripts here. Genes with putative DREs according to the analysis by Sun et al. (2004) are written in italics. EST, expressed sequence tag; xxxxxxRIK, cDNA sequences coding for unknown proteins, as identified in the Japanese RIKEN database. Gene names are sorted alphabetically. Transcripts validated by quantitative RT-PCR are indicated in bold letters. Genes modulated >3-fold are underlined. Upper group in box, downregulation; lower group in box, upregulation after TCDD treatment.

threshold of two-fold differential expression to include as many potential biological meaningful target genes as possible and validated several by PCR in an exemplary fashion. We found that the immature thymocytes had changes in 35 probe sets on the chip, i.e., different transcripts (see legend of Figure 1), the emigrants had changes in 44 probe sets, while the fetal emigrants, treated for 6 days with 10 nM TCDD, had a striking 282 changes. However, only four genes (*interferon induced transmembrane protein 1*, *scinderin* and *galectin-3*, *Cyp1b1*) were found regulated in both adult subsets, and only the latter three were also upregulated in TCDD-exposed fetal immature emigrants (see Figure 1). Overlaps between adult thymus/fetal emigrants (four genes) and adult emigrants/fetal emigrants (six genes) were equally small. Only three genes overlapped between all three subsets, *scinderin*, *galectin 3* and *Cyp1b1*. Surprisingly, *Cyp1a1*, considered a universal marker gene for TCDD exposure, was only upregulated in fetal CD4$^-$CD8$^-$ cells, but not in the other immature immune cells, although the AHR is abundant in these cells, as measured by RT-PCR (Figures 1 and 3). The TCDD-mediated induction of *scinderin* in thymocytes has been found before (Svensson et al., 2002). *Lgals3*, a galactose bind-

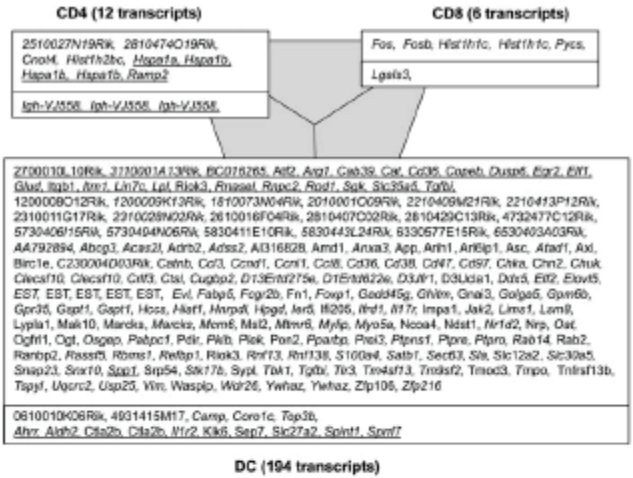

Figure 2 Venn diagram of gene expression changes by TCDD in CD4, CD8 and dendritic cells.
Mice were injected i.p. with 10 μ-g/kg TCDD (Promochem) dissolved in olive oil, or with solvent alone. Twenty-four hours later, mice were sacrificed, and T cells from spleen sorted flow-cytometrically to >99% purity. Dendritic cells were isolated after separation of spleen cells on a BSA gradient, followed by sorting with anti-CD11c coupled magnetic beads. The purity of the sorts was ascertained flow cytometrically. Total RNA was isolated from all cell types and processed and analyzed as described for Figure 1. As cut-off point a two-fold change is presented here. Results are from two independent experiments. Genes modulated >3-fold are underlined. Upper group in box, downregulation; lower group in box, upregulation after TCDD treatment.

ing lectin, is involved in adhesion of thymocytes to the thymic environment (Villa-Verde et al., 2002), and its upregulation is congruent with the disturbance of thymocyte emigration by TCDD (Temchura et al., 2005). Our results suggest that emigration of a cell subset out of the thymus is a cellular event that is accompanied by completely reprogramming the sensitivity to AHR-mediated transcription. For the calcium binding protein *S100A9*, which is upregulated by TCDD in adult emigrants, we previously demonstrated a functional role in emigration (Temchura et al., 2005). Our data also show that similar/equal cell differentiation stages (here: CD4-CD8- cells) can have very different susceptibility to AHR-mediated gene regulation in the fetus and the adult, arguing for special care in the extrapolation of toxic data in risk assessment.

In the experiments described, we analyzed the transcriptome at the end of a long-lasting AHR overactivation (6 or 7 days). Thus, it is possible that changes in gene expression reflect not only first-hand AHR-mediated transcription but are the result of the earlier reprogramming of cells. We therefore generated global gene expression profiles of mature immune cells in the periphery, after a relatively short (24 h) *in vivo* TCDD-exposure. CD4 and CD8 T cells are the carriers of the cellular immune response, and DCs are the most powerful antigen-presenting cells of the immune system. These cell subsets have been discussed and analyzed in the context of dioxin-induced immunosuppression, a hallmark of AHR-mediated adverse effects (Kerkvliet et al., 1996; Kronenberg et al., 2000; Choi et al., 2003; Funatake et al., 2005).

As shown in Figure 2, CD4, CD8 and DC cells displayed completely different effects for dioxin-dependent gene regulation. Firstly, the absolute number of affected transcripts is 194 for DCs but only 12 or six for CD4 and CD8 cells, respectively. More striking, there is no overlap whatsoever, i.e., all cell types regulate a distinct set of genes after AHR over-activation by TCDD. The majority of genes of the DCs have a putative DRE in their promoter regions (Sun et al., 2004). We found congruence between the number of genes expressed after TCDD-exposure and the expression level of the AHR in the cells (see Figure 3), which is in agreement with the pivotal role of the AHR in TCDD-mediated transcriptional changes. It is noteworthy that this appears to be the case independent of the duration of the treatment (24 h *in vivo*, or 6 days *in vitro*). Although only a few genes were changed by TCDD exposure in CD4 cells, these were AHR-dependent, as the gene expression profiles of AHR$^{-/-}$ CD4 cells demonstrate (see below).

Our data point to a degree of cell specificity in response to the AHR, which is even higher than previously estimated. Much of the literature on AHR-mediated gene expression changes has relied heavily on the analysis of liver cells and a few cancer cell lines, such as MCF-7. Surprisingly, transcription of several of the genes, which are designated as belonging to the 'AHR-battery' (Nebert et al., 1993) and were considered robust marker genes for TCDD, was not increased in various immune cells. Thus, *Cyp1a1*, *Cyp1b1*, *Cyp1a2*, *Gst*, *Nqo*, or *Ald3* were not detectable as upregulated in all cells reported here, even though transcripts of the AHR were

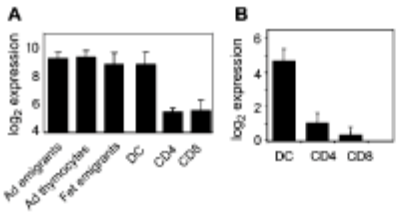

Figure 3 Expression level of the AHR in immune cell subsets. Cells were isolated as described in Figures 1 and 2. RNA was either hybridized to Affymetrix chips (A) or reverse transcribed into cDNA for semi-quantitative RT-PCR (B) with the Light-Cycler™ (Roche Diagnostics, Mannheim, Germany). (A) Data from chips were normalized with the affy package, and log2 expression values determined, making hybridization intensity signals comparable between chips. (B) Expression of AHR determined with semi-quantitative RT-PCR. Note that the 4 log difference, i.e., the ~32-fold higher expression of the AHR in DC versus T cells is found by RT-PCR and by chip hybridization. Ad: adult; Fet: fetal.

abundant in these cells (see Figure 3). *Cyp1b1* was found upregulated in CD4-CD8$^-$ emigrants and thymocytes (fetal or adult alike), but not on CD4$^+$ or CD8$^+$ mature peripheral T cells, or DCs. *Cyp1a1* was only detected in fetal CD4-CD8$^-$ emigrant thymocytes. In DCs, we could detect *Cyp1a1* by RT-PCR, but not on the arrays; in CD4 and CD8 T cells, *Cyp1a1* was virtually absent after TCDD treatment, and could hardly be detected by RT-PCR in CD4 cells, and not at all in CD8 cells. Thus, for low abundance of *Cyp1a1* transcripts in a given cell type, the Affymetrix chip appears to be too insensitive.

We chose CD4 T cells for an analysis of the effects of the absence of the AHR on global gene expression (Figure 4). CD4 T cells were sorted to high purity (99%) from spleens of AHR$^{-/-}$ and wild-type mice injected with 10 μg/kg bodyweight 24 h before. CD4 cells express the AHR (Figure 3) and are responsive to its activation by TCDD. This subset of immune cells plays a functional role in TCDD-induced immunosuppression (Kerkvliet et al., 2002). While no changes of expression were detectable following TCDD treatment, strong transcriptional differences were observed comparing CD4 cells of wild-type to AHR$^{-/-}$ mice. The expression of a total of 390 genes (i.e., probe sets) was changed at least two-fold in CD4 cells of AHR$^{-/-}$ mice (Figure 5). As shown in Figure 4A (upper graph) and Figure 5, the direction of changes was not uniform, i.e., 157 genes were upregulated and 233 were downregulated compared to the expression in wild-type mice. Incidentally, none of these genes in AHR$^{-/-}$ was responsive to TCDD alone, i.e., treatment of AHR$^{-/-}$ mice for 24 h with 10 μg/kg bodyweight TCDD did not change gene expression at all (Figure 4A, lower graph). This finding is in good agreement with a recent report by Tijet et al. (2006), who found that virtually all TCDD-inducible genes in the liver were refractory to TCDD induction in AHR$^{-/-}$ mice, even at the very high dose of exposure of 1000 μg/kg TCDD. Thus, not only in the liver, but also in the immune system, transcriptome changes of TCDD require the AHR. However, the link of a certain gene modulation to a certain toxic effect is known or proven for only very few genes.

Shown in Figure 4B is a cluster analysis of the results comparing the effects of TCDD-treatment for gene expression changes in AHR$^{-/-}$ and AHR$^{+/+}$ mice, respectively. Within the wild-type mice, hierarchical clustering detects the distinct differences in gene expression with and without TCDD exposure. This difference is not detectable in the AHR$^{-/-}$ mice, i.e., here the largest differences reflect only experimental variation, but not TCDD treatment.

The biggest difference is between AHR$^{-/-}$ mice and their wild-type counterparts, reflecting the large numbers of genes targeted by TCDD plus the large number of genes deregulated by the absence of the AHR. In conclusion, the findings of a large number of genes whose expressions change in the absence of the AHR, i.e., are inverse-

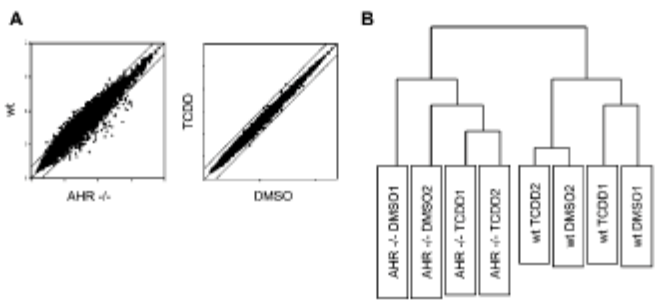

Figure 4 Dot plot analysis of transcriptome changes in AHR$^{-/-}$ mice and C57BL6 wild-type mice.
Mice were injected with 10 μg/kg bodyweight TCDD or solvent alone. Twenty-four hours later, spleens were harvested and depleted of B cells with anti-CD19 magnetic beads (Miltenyi Biotec). CD4 cells were then sorted flow cytometrically. Purity of cells was >99%. RNA was prepared, processed and hybridized to MOE340A chips as described in Figure 1. (A) Each dot represents the expression level of a particular gene on the chip, with normalized fluorescence signal for the mouse or treatment groups as indicated on the axes. Genes expressed at equal levels in both experimental groups fall onto the diagonal line. The two lines to the left and right of the diagonal delimit a two-fold up- or down-regulation threshold. (B) Graph of hierarchical clustering of the eight chip hybridizations performed. The graph was generated with BRB ArrayTools developed by Richard Simon and Amy Peng Lam.

Figure 5 Up- and down-regulation of transcripts in CD4 T cells of AHR-deficient mice.
RNA from CD4 T cells from AHR$^{-/-}$ mice was isolated and hybridized to chips as described in Figure 4. Gene names of ProbeSets (transcripts) identified as expressed higher or lower in AHR$^{-/-}$ versus wild-type mice are shown: light gray shaded area, genes with a higher than four-fold change; dark gray area, genes with a more than three-fold change; white area, genes with a more than two-fold change. Transcript changes verified independently by RT-PCR are written in bold letters. All genes with a putative DRE in their promoters, as identified in Sun et al. (2004) are in italics. EST, expressed sequence tag; xxxxxxRIK, cDNA sequences coding for unknown proteins, as identified in the Japanese RIKEN database. Gene names are sorted alphabetically.

ly AHR-dependent, emphasizes the physiological role of the AHR. The reason for the vast changes remains unclear. One potential explanation is the direct interaction of the AHR with cellular proteins, such as the retinoblastoma protein. Deregulation of this protein-protein interaction might be one cause for differentially expressed genes. At the same time the data suggest the presence of unknown physiological AHR agonists and antagonists in normal CD4 T cell development. Putative agonistic ligands are indole derivates, bilirubin metabolic breakdown products, or photoproducts of tryptophan (Adachi et al., 2001; Denison and Nagy, 2003). We could identify an AHR activating activity in supernatants of cultivated murine thymocytes (unpublished observation).

Surprisingly, also many of the genes, which are down-regulated in CD4 cells of AHR-deficient mice, have DREs in their promoters. We think this points to a suppressive role of an antagonist-activated AHR on DRE-containing promoters. Endogenous antagonists are not known, although AHR-ligands have been described which possess antagonistic qualities (Henry and Gasiewicz, 2003). It is currently not clear whether one or several endogenous ligands exist. Possibly the ligands vary depending on the tissue. The high cell specificity of gene expression changes, which implicates tight control of the AHR signaling pathway, suggests such flexibility.

What are the functions associated with AHR-dependent genes (either directly or inversely)? It is noteworthy that many genes identified as modulated have functions in the immune system. This holds true either after TCDD-induced AHR-activation (Figures 1 and 2) or inversely by the absence of the AHR (Figure 5). We would like to mention in particular the genes of the major histocompatibility complex and chemokines involved in cell migration. *Interferon* γ, whose transcription is upregulated in CD4 T cells of AHR-deficient mice, was recently shown to be

Table 1 Literature survey of transcript expression of 'AHR-battery' genes.

Tissue/cell type	Chip	Treatment	Cyp1a1	Cyb1b1	AHRR	Reference
Fetal heart (m)[a]	A[b]	TCDD[c]	++[e]	+	+	Thackaberry et al., 2005
Liver (m)	C 7,952	TCDD	+	+	-	Boverhof et al., 2005
Liver (m)	SAGE	TCDD	+	-	-	Kurachi et al., 2002
Liver (r)	C 4,608	TCDD	+	-	-	Kondraganti et al., 2005
Heart (z)	C 4,896	TCDD	+	+	+	Handley-Goldstone et al., 2005
Liver (m)	A	Ca-AhR-mice	+	-	-	Moennikes et al., 2004
Brain	C ?	TCDD	+	-	-	Volz et al., 2005
Liver			+	-		
Testis (o)			-	-		
HepG2 (h)	C 4076	TCDD	+	-	-	Puga et al., 2000
Hepatocytes (h,r)	C 302 toxchip	Aroclor 1254	+	+	-	Kel et al., 2004
C3H10T1/2 fibroblasts (h)	A	TCDD	+	+	-	Hanlon et al., 2005
Glandular stomach (m)	M	CA-AHR	+	-	-	Kuznetsov et al., 2005
Liver (r)	A	TCDD,	+	+	-	Vezina et al., 2004
		PeCDF,	+	+	-	
		PCB126,	+	+	-	
		PCB153	-	(+)	-	
Vascular smooth muscle (m)	O 13,664	B[a]P, wt, AHR[-/-]	-	+	-	Karyala et al., 2004
Airway epithelial cells 2 cell lines (h)	ToxChip 1.0	TCDD	+	+	-	Martinez et al., 2002
			-	+	-	
Placenta (r)	C 13, 000	TCDD	+	+	-	Mizutani et al., 2004
Hepa1c1c7 (m)	O 7,600	TCDD	-	-	-	Jin et al., 2004
CD4$^+$KJ$^+$ T cells (m)	O 96 apoptosis	TCDD	-	-	-	Funatake et al., 2004
Uterus (m)	A	TCDD	+	unclear	+	Watanabe et al., 2004
Liver (r)	O Drug Safety	3-MC	+	+	-	Gerhold et al., 2001
Liver (r)	A	A-277249	+	+	-	Waring et al., 2002
		3-MC	+	+	-	
		Aroclor 1254	+	+	-	
Liver (m)	O	6-NF	+	-	-	Bartosiewicz et al., 2000
Hepa1 cells (m)	O 13,433	B[a]P	+	-	-	Wei et al., 2004
Fetal CD4$^-$CD8$^-$ thymus emigrants (m)	A	TCDD	-	+	+	Majora et al., 2005
Adult CD4$^-$CD8$^-$ thymocytes (m)	A	TCDD	-	+	-	Temchura et al., 2005
emigrants			-	+	-	
Splenic CD4$^+$	A	TCDD	-	-	-	this paper
CD8$^+$ (m)			-	-	-	
DC (m)			++	-	+	
CD4$^+$ T cells AHR-/- (m)	A	TCDD	-	-	-	this paper

[a]The tissue of which RNA was prepared is indicated: (m) *Mus musculus*, mouse; (h) *Homo sapiens*, human; (r) *Rattus norvegicus*, rat; (z) *Danio rerio*, zebra fish; (o) *Oryzias latipes*, medaka, a fish species.
[b]A, Affymetrix chip; C, cDNA chip, the number indicates the number of spotted genes; O, other chip, such as oligo-DNA chip, the number indicates the number of spotted genes; M, macroarray.
[c]Treatment. Only the type of ligand, not the dose regimen is given. For details see references. [d]'CA-transgenic', constitutively active mutant of the AHR in transgenic mice. TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3-MC, 3-methylcholantrene; B[a]P, benzo[a]pyrene; PCB, polychlorinated biphenyl.
[e]The '+' symbol indicates a higher transcript abundance of the gene in question under the indicated ligand exposure.
[f]Not detected on the MO430A chip, but detectable by RT-PCR.

also increased at the protein level in antigen-activated AHR$^{-/-}$ splenocytes. It was therefore suggested that the AHR plays a role in downregulating *IFN*γ (Rodriguez-Sosa et al., 2005). We found the deregulation of genes of the immunoglobulin locus (Ig heavy chain, V28 and VJ588 light chains) intriguing. T and B cells are derived from the same stem cell, yet their lineages are separated early, and the genes of the immunoglobulin locus, as well as of the T cell receptor locus, are accessible only in the respective committed cells, i.e., B or T cells. Possibly, the AHR also plays a role in this lineage decision, as it does for several other lymphoid differentiation checkpoints (Staples et al., 1998; Kronenberg et al., 2000; Majora et al., 2005). Such questions must be addressed experimentally in the future.

The biochemistry of the AHR pathway and its involvement in AHR-mediated toxicity has been analyzed in great detail in numerous species. After binding of the ligand and shedding its associated chaperoning proteins p23, hsp90 and AIP, the AHR translocates to the nucleus, dimerizes with ARNT, binds to dioxin responsive elements and induces gene expression after orchestrating the appropriate transcriptional co-factors (Schmidt and Bradfield, 1996; Hankinson, 2005). However, our data reaffirm that AHR-mediated gene induction is not a slippery slope, once started by the ligand, but tightly controlled, leading to a tiered accessibility of genes to AHR signaling.

How extensive is the cell specificity of AHR-mediated transcription? We searched the available publications of

AHR-dependent transcriptome analyses (Affymetrix, oligo-arrays, SAGE analysis). Table 1 contains a list of 24 relevant publications from human, rat, mice and fish species. *Cyp1a1* was the most accessible gene for the AHR, indeed it was upregulated in almost all cases, and consequently has often proved its usefulness as a 'high control' for AHR-mediated dioxin activity under all conditions. Nonetheless, it is not accessible in all cells and under all circumstances. *Cyp1b1* was reported as upregulated only in 19/34 experimental settings (i.e., different tissues, ligands or doses). Other genes of the AHR-battery, such as *Nqo1* (seven array experiments), *Cyp1a2* (six array experiments), or the more recently added *AHR repressor* are found as AHR-accessible in significantly fewer experimental settings (data for *Cyp1a1*, *Cyp1b1* and *AHRR* shown in Table 1). From the array experiments many genes are reported as changed by AHR activation. Depending on the cell type or circumstances of exposure, the number and type of genes affected differ substantially. In the reports cited in Table 1, 20 genes were found regulated in four of the different conditions, 62 in three and 221 in only two (see references in Table 1 for details). In our hands, under the seven conditions reported by us here we found no gene modulated in all circumstances. *Lgals3* transcripts were upregulated in 4/7 experimental settings, five genes (*Cyp1b1*, *Scin*, *Ctsl*, *Ear2*, *D3Jfr1*) in 3/7 conditions, and 61 genes in 2/7 conditions. All other changes in transcription were uniquely specific to one cell type and AHR-modus (TCDD over-activated or deficient). Thus, the idea of a TCDD-specific signature across many cell types or even within closely related cell subsets of a single organ is not tenable. The underlying causes for this highly cell-specific outcome of AHR signaling may involve transcriptional co-factors (Hankinson, 2005), promoter accessibility defined by repressing factors or DNA methylation (Nakajima et al., 2003), or secondary signaling via AHR chaperoning proteins, or the very genes that are primary targets of the AHR, such as the AHR repressor.

Acknowledgments

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Microarray analysis of the AHR system: Tissue-specific flexibility in signal and target genes

Markus Frericks, Marc Meissner, Charlotte Esser*

Institut für Umweltmedizinische Forschung (IUF) at the Heinrich Heine-University of Düsseldorf, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany

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Abstract

Data mining published microarray experiments require that expression profiles are directly comparable. We performed linear global normalization on the data of 1967 Affymetrix U74av2 microarrays, i.e. the transcriptomes of >100 murine tissues or cell types. The mathematical transformation effectively nullifies inter-experimental or inter-laboratory differences between microarrays. The correctness of expression values was validated by quantitative RT-PCR. Using the database we analyze components of the aryl hydrocarbon receptor (AHR) signaling pathway in various tissues. We identified lineage and differentiation specific variant expression of AHR, ARNT, and HIF1α in the T-cell lineage and high expression of CYP1A1 in immature B cells and dendritic cells. Performing co-expression analysis we found unorthodox expression of the AHR in the absence of ARNT, particularly in stem cell populations, and can reject the hypothesis that ARNT2 takes over and is highly expressed when ARNT expression is low or absent. Furthermore the AHR shows no co-expression with any other transcript present on the chip. Analysis of differential gene expression under 308 conditions revealed 53 conditions under which the AHR is regulated, numerous conditions under which an intrinsic AHR action is modified as well as conditions activating the AHR even in the absence of known AHR ligands. Thus meta-analysis of published expression profiles is a powerful tool to gain novel insights into known and unknown systems.

Keywords: Aryl hydrocarbon receptor; PAS-bHLH; CYP1A1; Microarray; T cells; Meta-analysis

Introduction

Microarrays are applied in all fields of molecular biology and have become an important part of toxicological testing. By measuring the expression of thousands of genes in a single experiment, microarrays provide a wealth of information beyond the scope of the original study in question. To make these data available to the scientific community, repository databases like the Gene Expression Omnibus (GEO), ArrayExpress and CIBEX were established (Barrett et al., 2005; Brazma et al., 2003; Parkinson et al., 2005; Ikeo et al., 2003). These databases contain thousands of expression profiles from hundreds of platforms and technologies, such as SAGE, two-color microarrays or the Affymetrix arrays. Data generated by different technologies are not directly comparable, moreover, even results from the same original probes but generated on different versions of the same platform, or on an altogether different platforms can differ strongly. Consequently, it is desirable to make data comparable (Park et al., 2004; Irizarry et al., 2005; Cope et al., 2004; Stoeckert et al., 2002; Kong et al., 2005; Larkin et al., 2005; Severgnini et al., 2006).

One way to address this need was the use of databases like the RIKEN expression array database (READ), which is based on a custom designed array and highly standardized methods (Bono et al., 2002; Cope et al., 2004). However, comparison is restricted to those arrays, whereas other platforms with their wealth of information remain untapped. The wide application and highly standardized protocols, including core laboratory facilities, of

Abbreviations: AHR, aryl hydrocarbon receptor; AIP, AHR interacting protein; ARNT, AHR nuclear translocator; EST, expressed sequence tags; GEO, Gene Expression Omnibus; HPRT, hypoxanthine phosphoribosyl transferase; MAS4.0/5.0, Affymetrix Microarray Suite 4.0/5.0; MG-U74av2, Affymetrix murine genome U74 Chip A Version 2 Gene Chip; RAG1, recombination activating gene; READ, RIKEN expression array database; RPS6, ribosomal protein subunit six; HIF1α, hypoxia inducing factor alpha; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TdT, terminal deoxynucleotidyl transferase.

* Corresponding author. Fax: +49 211 3190910.
E-mail address: chesser@uni-duesseldorf.de (C. Esser).

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the Affymetrix microarray platform render this platform especially suitable for meta-analysis of public expression profiles (Cope et al., 2004; Kong et al., 2005).

We selected 1967 Affymetrix MG-U74av2 gene expression profiles from GEO and generated a directly comparable database by mathematical normalization. We use this database for transcriptome analysis and present here our results for the aryl hydrocarbon (AHR) and its associated proteins. The AHR senses small chemical molecules, and its activation leads to tissue-specific transcriptional changes (Frericks et al., 2006; Hanlon et al., 2005; Fletcher et al., 2005; Puga et al., 2000; Tijet et al., 2006; Karyala et al., 2004). For many environmental ligands with high affinity and low degradability, net toxic effects are the consequence, including tumor promotion, teratogenicity, immune suppression and thymus atrophy (Mimura and Fujii-Kuriyama, 2003; Okey et al., 1994). The AHR is associated with chaperoning proteins AIP and HSP90 in the cytosol (Carver et al., 1994b; Chen and Perdew, 1994; Meyer and Perdew, 1999). Upon activation it shuttles to the nucleus, sheds the HSP90 complex and dimerizes with ARNT, a member of the same bHLH protein family. The AHR/ARNT heterodimer then can act as a transcription factor (Swanson, 2002). In particular, genes of the xenobiotic metabolizing enzymes were found inducible, whose products in turn can metabolize the inducing chemicals. ARNT can partner with other proteins, such as hypoxia inducible factor 1 α (HIF1α), to form yet another transcription factor (Wood et al., 1996; Kinoshita et al., 2004). The curious tissue-specific constitutive abundance of the AHR has been noted early (Carver et al., 1994a; Fitzgerald et al., 1996; Lawrence et al., 1996), but little is known on the distribution of the associated proteins; the collateral endogenous ligand(s), which must be assumed, remain enigmatic. Currently, the mechanisms controlling tissue-specific changes and the role of the AHR in physiology and normal development, in the absence of exogenous ligands, are subjects of intensive investigations (Shimada et al., 2003; Forkert, 1997; Frericks et al., 2006). Using our newly generated database as a tool we here provide a detailed "*in silico*" view of this model system.

Materials and methods

Mice. C57BL/6 mice were bred in our animal facility and kept under standard conditions in accordance with German regulations. Male mice were used at 8–12 weeks of age.

RNA isolation. Liver, spleen kidney, brain, and bone marrow were surgically removed from sacrificed mice and homogenized by gently pressing them through nylon meshes. Total cellular RNA of 1×10^7 cells/organ was isolated with TRIzol (Invitrogen Life Technologies, Karlsruhe, Germany). RNA pellets were washed with ethanol, air-dried for 10 min, and dissolved in water. The RNA quality was assessed by analyzing the A260/A280 ratio and by evaluating the integrity of the 28S and 18S RNA bands on 1% agarose gels.

cDNA synthesis and quantitative real-time PCR. Reverse transcription was performed on 1 μg total RNA for 1 h at 37 °C (Invitrogen, Karlsruhe, Germany). Real-time PCR was performed with a LightCycler (Roche, Mannheim, Germany) in a 20 μl reaction containing 10 μl 2× QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany), 1 μl cDNA and 1 μl of each primer (10 μM). Samples were amplified by repeated cycles of 94 °C 1 min, 55 °C (*Rps6*, *Hprt*, *Arnt*, *Abr*, and *Aip*) 1 min, and 72 °C 1 min with 35 amplification cycles.

List of primers. *Rps6*: forward: 5′-ATTCCTGGACTGACAGACAC-3′, reverse: 5′-GTTCTTCTTAGTGCGTTGCT-3′; *Hprt*: forward: 5′-GTAATGATCAGTCAACGGGGGAC-3′, reverse: 5′-CAGCAAGCTTGCAACCTTAACCA-3′; *Aip*: forward: 5′-GCTCCGTTATAGATGACAGC-3′, reverse: 5′-ATCTCGATGTGGAAGATGAG-3′; *Arnt*: forward: 5′-GGTCTAGTTCCAGTGAGCAG-3′, reverse: 5′-TGGAAAGGGTACATATCAGG-3′; *Abr*: forward: 5′-AGGACCAAACACAAGCTAGA-3′, reverse: 5′-TGGAGATCTCGTACAACACA-3′. Expression was calculated by the ΔΔ-CP method. Briefly, RPS6 and HPRT were used as housekeeping genes and the normalization performed on the mean expression of both. The ΔΔ-CP values for each gene was calculated and the mean expression set to the same level as the mean expression in the microarray database. The resulting distribution was highly similar between database and RT-PCR data.

Retrieval and processing of data. All data sets used for our database were accessed from GEO (http://www.ncbi.nlm.nih.gov/geo/). Only MG-U74aV2 expression profiles, analyzed by MAS4.0, MAS5.0 (Affymetrix), or dChip, were used. The data were downloaded in html format (newer data in SOFT-format) and exported to MS Excel. For each expression profile the MIAME description was stored in a second file (Brazma et al., 2001). In general, the tabular expression data, as available in GEO, are already normalized within the experimental series, thus limiting the available normalization procedures to make them directly comparable. The tabular expression values were sorted alphanumerically. At this step data sets with missing values were discarded. Assuming that the same amount of mRNA results in the same fluorescence intensity, and variations in the distribution between arrays are a result of experimental variance and do not represent biological variability, the equally formatted expression profiles were normalized using a linear scaling procedure based on the median signal intensity. Briefly, the median signal intensity for each array (MSI) was calculated. A scaling median (SM) was set at 400 and for each expression profile a scaling factor (SF) was computed using the following formula:

$$SF = SM/MSI$$

The data were normalized by applying the SF to each expression profile. An SM of 400 was chosen because it was close to the value of the median fluorescence intensity over all arrays. The scaling method is linear, so in principle any SM would be possible, albeit if the SM is chosen very low or far from the average intensity of the chip, low abundance transcripts might be not detectable. In this linear normalization the data are not centered as this would probably result in a skewing of the expression values. More sophisticated non-linear normalization procedures can only be applied to highly similar expression profiles, a prerequisite not met when using data from different cell types.

Normalized data were log2 transformed and stored in a relational database consisting of the data files. As a result, our data are presented in units of fluorescence intensity covering a range of 20 log2 units. Array hybridization does not provide a negative control, i.e. the intensity of a hybridization signal indicates the abundance of a transcript as well as the efficiency and specificity of the hybridization. Therefore, determination of a threshold of absence for any transcript in a tissue is possible before as well as after normalization. Affymetrix present/absent calls may indicate whether a gene is expressed in a given sample. Yet for many included expression profiles these are not available and therefore not included in the analysis.

A list of all experimental files and the annotation table provided by Affymetrix (NetAffx, dated June, 23, 2004) is part of the database. A complete list of the included expression profiles and their MIAME description is given in Supplementary Table 1. As graphical front ends MS Excel and R 2.3 (in basic configuration) were used. We named the database NC-GED (*N*ormalized *C*omparative *G*ene *E*xpression *D*atabase).

On Affymetrix arrays, several ProbeSets may be spotted which detect a single gene. In all of the following analysis each ProbeSet was analyzed individually. Note that for some genes multiple ProbeSets show exactly the same behavior while others differ strongly.

Identification of differentially expressed genes. Where feasible we used Student's *t*-test for the identification. In most studies only two repeat arrays were available between experimental conditions. In these cases and all other

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studies, a cut-off value of two-fold (1 log2) difference was chosen. The heat map was generated using Microsoft Excel. A black bar depicts down-regulation, a gray one up-regulation in the given condition.

Results

NC-GED content

The database contains 1967 expression profiles. We grouped them according to their origin, as given by the MIAME information, into more than one hundred organs, cell types, or tissues. The largest groups in NC-GED are the nervous system (305 samples), muscular tissues (251), and the immune system (210) followed by cardiac tissues (189) and stem cells (180). There are also 134 expression profiles from lung and 126 from liver. It has been estimated and was observed by us, too, that in a typical setting of differential gene expression less than 5% of all transcripts are differentially expressed (Supplementary Tables 2 and 3 and Lockhart and Winzeler, 2000).

Comparison of expression strength of selected genes in NC-GED

We compared the normalized expression of the genes *Aip* (AHR interacting protein), *Arnt*, *Hif*α, and *Hprt* (as a housekeeping gene) in heart expression profiles. Heart was chosen as an example since 80 expression profiles, generated in seven independent laboratories, were available and are included in our database. As shown in Fig. 1, the expression levels of *Aip*, *Arnt*, *Hif*α, and *Hprt* are highly similar for all seven laboratories after normalization. With only one exception (HIF1α in laboratory g, 2.5-fold) no significant deviation from the mean was seen which was >2-fold. Comparable results were observed for hippocampus, uterus, and liver for 20 randomly chosen ProbeSets (see Supplementary Figs. 1–4 and Supplementary Table 2). When we chose a 3-fold cut-off for differential expression only 3% of the ProbeSets showed a deviation from the mean for the given organ (respectively 2.5-fold=5.9%). Likewise, results did not differ between laboratories (Supplementary Fig. 5).

Validation by RT-PCR

We analyzed whether the expression strengths determined for individual genes in the database NC-GED were reproducible by RT-PCR. Five genes, exemplifying typical patterns of differential gene expression identified in our database, i.e. genes expressed in all tissues, in some tissues only, or expressed at high versus low levels, were chosen. RNA from bone marrow, brain, kidney, liver, and spleen of 8–12-week-old male mice was isolated and used in semi-quantitative real-time RT-PCR to measure the expression of *Ahr*, *Aip*, *Arnt*, *Hprt*, and *Rps6* (ribosomal protein subunit 6). For *Aip*, *Hprt*, and *Rps6* the expression values were equal to those determined by microarray analysis (Fig. 2). *Ahr* expression in the liver was underestimated on the microarrays in comparison to RT-PCR. In all other tissues, *Ahr* expression as determined in the database reflected the results from RT-PCR. *Arnt* expression matched for brain, kidney, and spleen, but not for bone marrow and liver. In conclusion, 22 of 25 results were in complete concordance between NC-GED and quantitative RT-PCR, which is an approximate 90% rate of reproducibility.

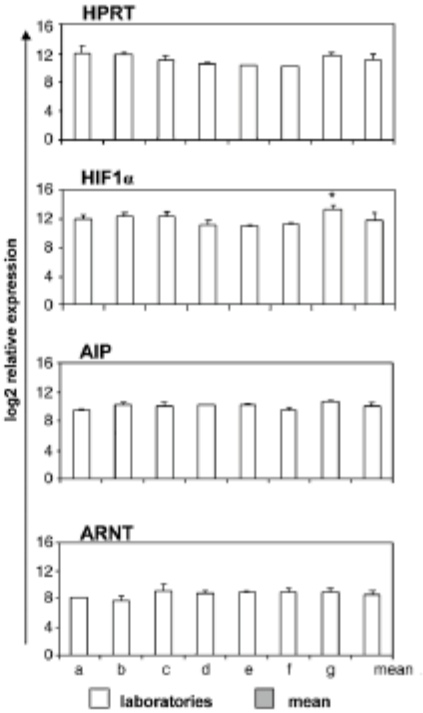

Fig. 1. Log2 relative expression levels in NC-GED of 5 selected genes, from arrays generated in seven independent laboratories (a–g). Data from microarrays performed on cardiac tissue was downloaded and included in NC-GED after normalization.

Tissue distribution

The tissue distribution of transcripts (and by cautious implication of the encoded proteins) is considered physiologically relevant and consequently may correlate to tissue-specific effects of toxins targeting those proteins (Abbott et al., 1999a, b). Typically, tissue-specific transcript abundance is measured by semi-quantitative RT-PCR, i.e. calibrated against housekeeping genes and total RNA content. Drawing on our database, which is normalized to RNA content, we assessed the tissue distribution for >9500 genes in more than 100 tissues/cell types (see Supplementary Table 3). The data can be used to either confirm or extend current knowledge. For instance, the immunoglobulin heavy chain was found only expressed in B cells, which is no surprise. Likewise, the IL7-receptor was found expressed several powers of 2 higher in bone marrow and

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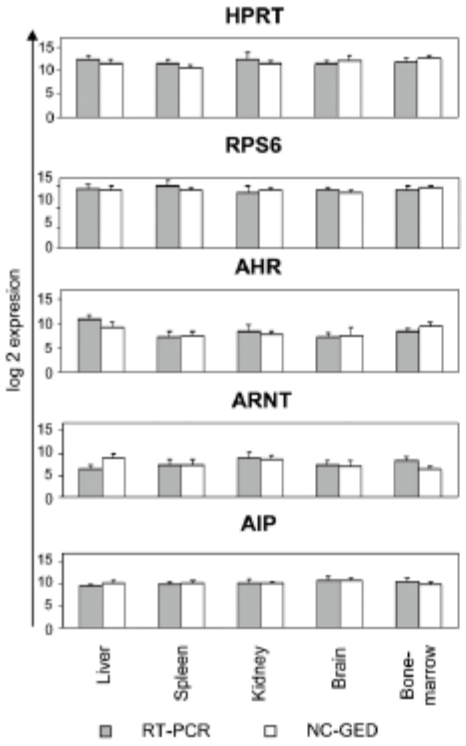

Fig. 2. Comparison of normalized expression levels of 5 genes in NC-GED to the respective experimentally generated expression levels. RNA from five tissues was prepared from C57BL/6 mice (n= 3), and RT-PCR was performed as described in Materials and methods. Shown are the log2 expression levels from NC-GED (white bars) compared to RT-PCR results (gray bars). The experiment was repeated three times.

various T-cell lineages than in other tissues, as expected. However, the IL7-receptor was similarly highly expressed in muscle and the cerebellar cortex, which was previously unknown. Galactose-1-phosphate uridyl transferase, whose lack leads to galactosemia, and may be associated with female infertility, was expressed not only in the liver and heart (as known and expected) but also in the uterus and in immune cells, even several powers of 2 higher than in liver. Other examples of unexpected high or low expression of individual genes in non-typical tissues are easy to find. Again, it should be stressed that values in NC-GED are not absolute, i.e. a very low value for a given gene in the table does not necessarily reflect negligible expression or even absence of the transcript in the respective tissue, but possibly simply the detection sensitivity of that gene on the microarray.

Expression of genes of the AHR system in the T-cell lineage

We used NC-GED to analyze ontogenic and tissue-specific expression of members of the AHR signaling pathway. AHR signaling has been studied in particular with respect to dioxin toxicity. Recently, interest in the AHR has shifted towards its physiological role, for instance in the immune or endocrine system, or with respect to cell differentiation fates.

In recent years we have studied the immunotoxic effects of AHR over-activation by dioxins on the immune system and on lineage decisions of thymocytes and T cells (Lai et al., 1997; Majora et al., 2005; Vogel et al., 1997). In Fig. 3 we show an analysis of the expression levels of AHR system members, of AHR-targeted genes, and of T-cell-specific genes in a variety of T-cell subsets and lineages. High expression of *CD3ε* and *Thy1* was found as expected for T cells (except lack of *Thy1* in one cell line). Other components of the *CD3* complex were highly expressed, too (data not shown). Likewise, *Rag1* which is required for the rearrangement of the T-cell receptor genes in immature thymocytes showed the expected exclusive expression in this cell type.

Surprisingly, we detected a variation of AHR expression in a T-cell lineage dependent fashion. In T-cell subsets, the *Ahr* was differentially expressed, highest in immature T cells, approximately 4 times lower in primary CD8 T cells, and even lower in two typical T-cell lines (EL4 and D10G4), which have been perpetuated in culture for decades. Interestingly, regulatory T cells ($CD4^+CD25^+$) from the pancreas have a comparatively high, while $CD4^+CD25^{low/-}$ showed only weak *Ahr* expression. However, even the highest expression in immature T cells was several log2 orders of magnitude lower than *Ahr* expression in other tissues such as liver or lung (see Supplementary Table 3). This pattern of a decreasing expression of the AHR by T-cell maturation is not followed by the AHR's dimerization partner ARNT. In contrast, where *Ahr* levels decrease, *Arnt* levels appeared to increase. This is particularly noteworthy in the cell lines, where *Arnt* expression was not lost, compared to primary T cells. Transcript expression of neither the AHR chaperone *Aip* nor of *Hif1α* changed much in different T-cell subsets. Upon activation, the AHR/ARNT heterodimer acts as a transcription factor; we therefore looked at two well-known target genes, *Cyp1a1* and *Cyp1b1*. Compared to the median expression level in the database, both are expressed at low levels in the T-cell lineage.

We extended the analysis with NC-GED to lymphoid and myeloid cells, namely early B cells, macrophages, haematopoietic stem cells (HSC), dendritic cells (DCs) and thymic epithelial cells. The results are shown in Fig. 4. Compared to the median value of all arrays, the *Ahr* was expressed several log2 orders higher in immature B cells than the average of all tissues. For other immature lymphoid/myeloid cells derived from bone marrow or fetal liver *Ahr* expression was at least as high, or even higher, than the average. Splenic dendritic cells, but not *in vitro* generated DCs, displayed high *Ahr* expression. Interestingly, some macrophages had very low *Ahr* expression and thus differed from most other immune cells. Note that in more mature stages (i.e. peritoneal macrophages) *Ahr* expression is higher.

Arnt and *Aip* were expressed at average levels almost uniformly across the lymphoid/myeloid cell subsets shown in Fig. 4. However, again the immature B cells differed, i.e. they had

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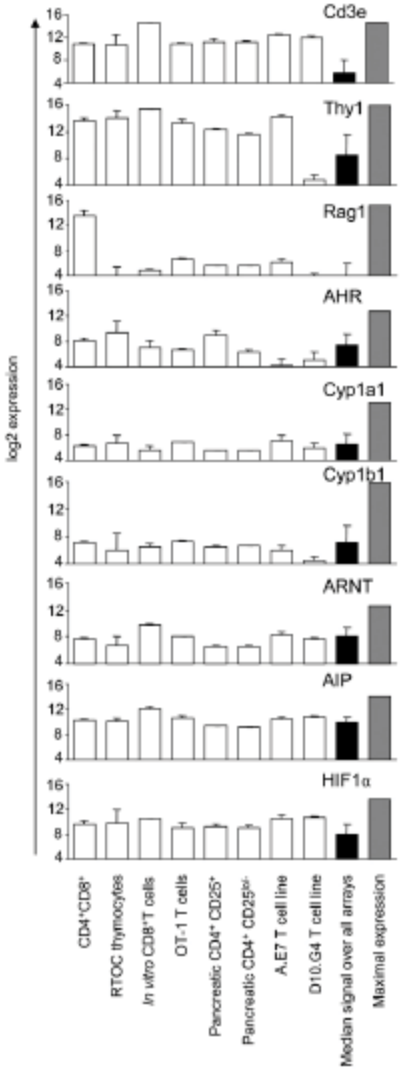

Fig. 3. Lineage and developmental specific expression of the members of the AHR system in T-cell populations. Analysis of lineage specific relative expression levels (log2 values ± SD in NC-GED) of T-cell-specific genes (*Cd3e*, *Thy1* and *Rag1*) as well as genes associated with the AHR system. The median expression over all 1967 included arrays (black bars) and the maximal expression of the respective gene found in any tissue (gray bars) are included for comparison.

above average *Arnt*, below average *Aip*, and, surprisingly, high *Cyp1a1* expression. Another target gene of the transcription factor AHR, *Cyp1b1*, appeared to be independently expressed from *Ahr*, *Arnt*, *Aip*, i.e. there is no obvious pattern of co-expression or non-expression. Note that constitutive *Cyp1b1* expression was generally lower than the average in all tissues of the database, in lymphoid/myeloid cell subsets, whereas for *Cyp1a1* this was not the case.

Typical expression levels of *Ahr*, *Arnt*, *Arnt2*, *Ahrr*, *Cyp1a1* and *Cyp1b1* in non-immunotypic organs and cell types are given in Table 1. Of special notice here is the high *Ahr* and *Ahrr* expression in Schwann cells, accompanied by low levels of *Arnt* and *Arnt2*. This finding of the potential negative feedback loop of the AHR transcriptional activity remains enigmatic.

Co-expression analysis

Expression of two or more genes in the same cell at the same time ("co-expression") can be a first hint at a physiological connection, more so if co-expression is co-regulated by external or internal signals, or when looking at proteins of a signaling pathway. Co-expression can be analyzed by linear regression. For data in NC-GED, we used the graphical approach of scatter plots, which depicts co-expression of two genes. The results of some combinations are shown in Fig. 5. It is important to note that different ProbeSets differ in their binding kinetics and that the same expression value does not necessarily reflect the same amount of RNA bound. For low transcript concentrations, the hybridization follows the shape of a langmuir adsorption isotherm, while higher concentrations result in a deviation from this linear approach (Hekstra et al., 2003; Held et al., 2006).

First, we looked at co-expression of the *Ahr* with its dimerization partner *Arnt*, and with the two well-known AHR-inducible genes, *Cyp1a1* and *Cyp1b1*. Conceivably, *Ahr* and *Arnt* should be expressed together as both molecules are needed to form a functional transcription factor. However, this is not the case. A variety of stem cell populations express the *Ahr* in the absence of *Arnt* (dots in lower right of graph). It is also noteworthy that expression levels do not fall on a line, i.e. there are cells/tissues with $Ahr^{low}Arnt^{high}$ or with $Ahr^{high}Arnt^{low}$.

Similarly, no uniform correlation between *Ahr* and its target genes *Cyp1a1* and *Cyp1b1* was detected. For both combinations a number of tissues were detectable with relatively high expression of *Cyp450*. High expression of *Cyp1b1* was found in combination with a wide range of *Ahr* abundance and vice versa.

Arnt2 has a very high degree of sequence homology to *Arnt* and it has been discussed whether the formation AHR/ARNT2, HIF1α/ARNT2, or ARNT2/ARNT heterodimers is possible (Barrow et al., 2002; Jain et al., 1998). If true, one function of ARNT2 might be to replace ARNT. Co-expression analysis of *Arnt2*/*Arnt* showed, however, that *Arnt2* was expressed not only in the absence of detectable *Arnt*, but also in tissues with high *Arnt* expression levels. Analysis of the expression of *Arnt-like*, a third sequence homolog to *Arnt*, in this regard showed similar results (data not shown).

Functioning as a cytosolic binding partner to the AHR and many other protein complexes, HSP90 is formed as a dimer of HSP90a1 and HSP90b1. The co-expression analysis shows that both genes are always strongly expressed with a high degree of correlation and thus are presumably not a limiting factor in the AHR system.

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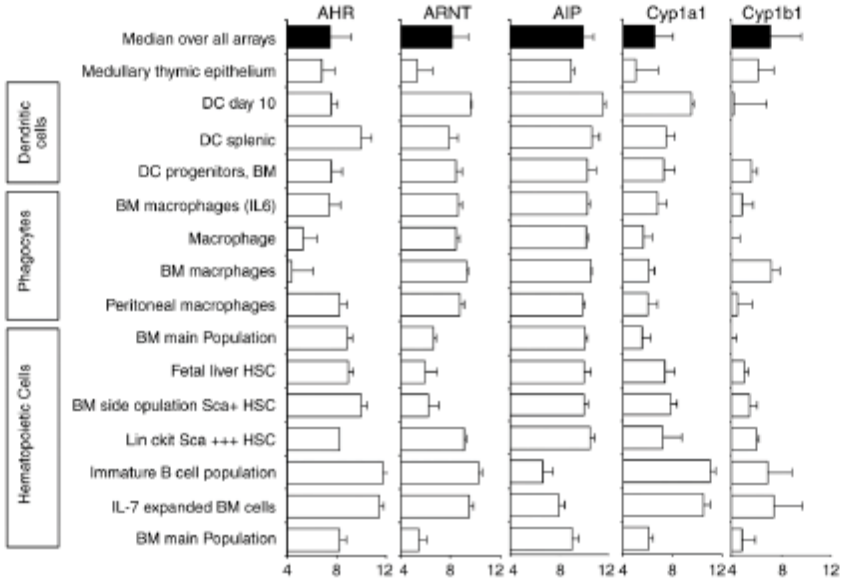

Fig. 4. Relative expression levels (log2 values±SD identified in NC-GED) of AHR associated genes in cells of the immune system. For comparison the median over all arrays is shown (black bar).

Two genes of S100 family, *S100a8* and *S100a9*, which have been reported to be differentially regulated following AHR over-activation, are co-expressed (Temchura et al., 2005). *S100a8* deficient mice, but not *S100a9* deficient mice are embryonically lethal. The co-expression analysis revealed a high degree of correlation between both genes on mRNA level. Deviations from the correlation line were detected only in the lower intensity ranges. This suggests a regulation on protein level to be responsible for the lethal knock-out effect (Passey et al., 1999).

Expression dynamics of the AHR system and associated genes

The constitutive expression levels of the *Ahr* have been determined in several organs and cell types, before and after ligand activation, e.g. by dioxin exposure. The data have revealed a stable and cell-specific expression of the *Ahr*, which is not up-regulated by ligand activation (Fitzgerald et al., 1998; Ma and Baldwin, 2000). However, it is not clear whether cell-specific *Ahr* expression is tightly controlled in all cellular si-

Table 1
Mean expression of AHR, AHR interaction partners, and AHR target genes in non-immunologic tissues and cell types

Gene symbol	*Ahr*		*Arnt*		*Ahrr*		*Arnt2*		*Aip*		*Cyp1a1*		*Cyp1b1*	
	Median	SD	Median	SD	Median	SD	Median	SD	Median	SD	Median	SD	Median	SD
Heart	5.8[a]	1.0	8.7	0.6	5.7	1.8	6.1	1.8	10.3	0.4	6.5	1.2	7.6	2.5
Testis	6.8	1.9	7.8	1.1	5.4	1.6	6.3	1.5	9.8	0.7	6.3	1.4	8.5	2.1
Uterus	8.1	0.5	7.2	0.4	5.7	1.0	6.3	0.7	7.9	0.1	4.6	0.6	8.1	1.6
Kidney	7.8	0.5	8.5	0.8	5.2	0.9	7.2	0.8	10.2	0.4	7.1	0.7	9.6	0.6
Lung	9.3	1.2	8.0	0.7	4.3	1.5	5.0	1.6	9.6	0.5	7.7	1.6	6.6	1.7
Liver	9.2	1.0	7.9	1.3	5.1	1.8	6.5	2.2	9.9	0.8	6.6	1.5	6.9	2.4
Brain	7.1	0.9	7.0	1.5	3.9	2.0	6.7	3.0	10.4	0.6	6.3	1.1	6.7	2.1
Hippocampus	6.7	1.2	6.4	1.3	4.7	1.2	7.0	1.3	9.8	0.5	6.0	1.3	6.3	1.8
Schwann cells	10.6	1.4	7.1	0.2	8.9[b]	0.3	7.4	0.3	7.1	0.5	6.1	1.1	4.0	2.5
Ileum	6.4	0.5	8.0	0.5	4.6	0.2	6.5	0.1	9.6	0.0	6.4	0.5	5.3	0.8
Jejunum	7.7	0.3	8.3	0.4	5.7	0.4	6.4	0.4	9.2	0.1	6.0	0.5	6.0	1.4
Rectum	7.6	0.1	8.1	0.1	5.9	0.1	6.0	0.2	9.3	0.1	5.4	0.1	4.6	2.0
Descending colon	7.9	0.1	7.8	0.2	5.4	0.9	6.3	0.6	9.4	0.2	5.5	0.4	4.1	0.4
Corpus	7.7	0.1	7.7	0.2	3.9	0.3	6.3	0.2	9.2	0.1	5.4	0.7	7.7	0.2

[a] All values given in this table are log2 intensities as given in NC-GED.
[b] Maximal level in NC-GED.

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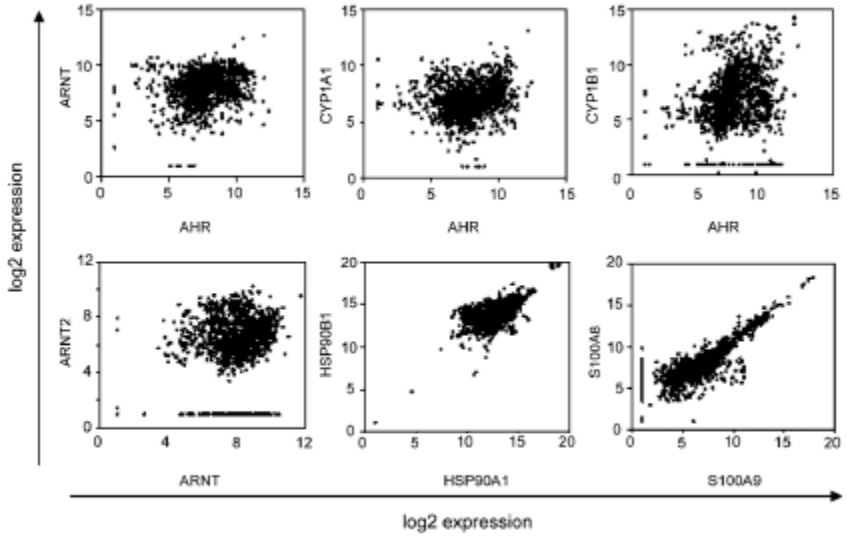

Fig. 5. Co-expression analysis of several gene combinations by scatter plots. Each dot represents the mean log2 values of relative expression intensity of the two respective genes for a single array, i.e. a single cell type or tissue. Note that for each gene the hybridization efficiency might have been different. Array data are relative only, and consequently, the values in NC-GED are relative as well (see Materials and methods).

tuations, or subject to transcriptional regulation, and if so, what might trigger *Ahr* expression changes. To detect such trigger conditions and elucidate the dynamics of AHR-related gene expression, we therefore used the expression levels compiled in NC-GED. The 1967 microarrays in NC-GED are from 158 series of experiments. In GEO these are defined as a set of related samples considered to be part of an experiment (Edgar et al., 2002). For instance, an experimental series can be a cell line which was treated with increasing doses of a growth factor, or a series can be microarrays prepared from brain tissue at different ages of an animal, or a series can be tissues from wild-type versus knock-out mice. Within a series, any different treatment (e.g. a single drug dose, time-point, or gene deficiency) is termed "condition". In previous studies of transcriptomes of dioxin-exposed AHR wild-type versus AHR deficient mice we had identified genes, which were differentially expressed in several cell subsets of the immune system (Frericks et al., 2006) and thus might be connected to the AHR signaling pathway. We here chose 308 conditions for comparison of the expression levels for the *Ahr* and its chaperone *Aip*, 5 other members of the PAS-bHLH family, 6 *Ahr* target genes, and 13 genes found by our own microarray studies. The results are summarily shown in Fig. 6.

This heat map depicts the variability of expression of all investigated genes. Except for *Aip*, all genes are expressed at least 2-fold higher or lower in about 10–30% of conditions. *Aip* showed variability in only 9/308 conditions and was one of the most uniformly expressed genes in the database (data not shown). Unexpectedly, the *Ahr* was differentially expressed by numerous conditions (53/308), including developmental stages in early development, DC activation, Schwann cell development, or in liver and lung after various drug treatment schemes or gene deficiencies, in ovary and testis development, and many more (Table 2a and Supplementary Table 4). These changes were accompanied by expression changes in potential AHR target genes in many, but not in all cases. Conditions which led to an at least 2-fold transcription change of AHR-targetable genes include intestinal tumor development, heart injury, skin components, ischemia and lung and liver treatments, or muscle tissue at different body sites. Table 2a lists some conditions, which we find noteworthy, in particular the increasing *Ahr* expression during hematopoiesis in development of fetal liver cells. A full list of conditions in which the AHR is differentially regulated is given in Supplementary Table 4. For instance, *Ahr, Arnt, Aip* and *HIF1α* all were expressed at least 2-fold higher in the liver of wild-type mice than in $Cry^{-/-}$ gene-deficient mice. The gene *Cry* encodes cryptochrome, which is critical for circadian rhythm and regulation and regulated by CLOCK, yet another gene of the PAS-bHLH family (Oishi et al., 2003).

Potential AHR target genes can be differentially expressed in the absence of ligand

As shown above, *Ahr* expression is affected by numerous conditions. Differential expression of AHR target genes such as *Cyp1a1* and *Cyp1b1* is often considered as an indicator of AHR involvement in processes such as drug treatments, although an AHR-independent induction has been observed in several studies, and they are not exclusively regulated by the AHR. We applied a battery of test genes to query NC-GED to identify

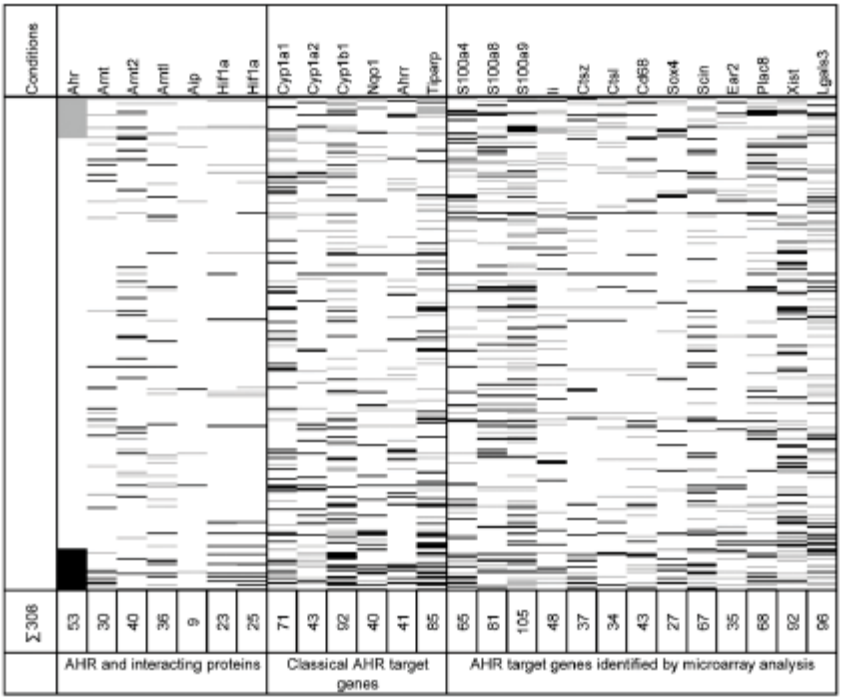

Fig. 6. Heat map graph of differential gene expression for selected genes involved in the AHR signaling pathway (*Ahr*, *Arnt*, *Hif1α* and *Aip*, AHR target genes of the classical AHR gene battery, and putative AHR target genes). The up- or down-regulation for 308 different conditions of various tissues is shown. For a description of "condition" see text. Cut-off threshold is a two-fold or higher difference in expression. Black bar: down regulation, gray bar: up-regulation in the given condition. The number of conditions in which a given gene was differentially expressed is indicated in the lowest row. The table is sorted by differential *Ahr* expression. For exact values and more details refer to Supplementary Tables 2 and 3.

conditions with potential AHR involvement. We used 6 genes from the classical "AHR gene battery", i.e. genes known to be directly inducible by the AHR via AHR-responsive elements in their promoters, and 13 putative AHR-responsive genes, which had previously been identified as TCDD-inducible in immune cells by us (Frericks et al., 2006), albeit it is unclear whether induction is direct or indirect. The results are shown in Table 1. We note that none of the genes was inert to change; *S100a9* was the gene with the highest "flexibility" in the table, i.e. its expression changed in 105 of the 308 conditions. *Ctsz*, *Ctsl*, *Sox4*, and *Ear2* were regulated in less than 40 conditions; others showed a high degree of either up- or down-regulation, depending on the condition tested in the series. This was especially true for *Tiparp*, *S100a8* and *S100a9*, *Xist*, and *Lgals3*, suggesting a higher sensitivity to experimental parameters of these genes. In concordance with this and previous findings from us, there is no direct correlation between the directions of the observed expression changes, i.e. up- versus down-regulation. Yet, linear regression analysis of the observed changes showed a significant correlation between all changes (data not shown).

It is highly probable that single differential gene expression can also be observed by chance or unrelated biological processes. We looked for conditions, in which more than four potential AHR-dependent genes were regulated, and which therefore conceivably are conditions where the AHR might be involved and physiologically active. Table 2b lists several such conditions. A full list of conditions we scored as involving the AHR is given in Supplementary Table 5.

We detected changes of putative AHR-dependent genes in lung, liver, and skin under various conditions. Furthermore, cells of the immune system were prone to such possibly AHR-related transcriptional changes, e.g. in thymocyte developmental stages, medullary thymic epithelium, and during DC differentiation. Muscular tissue, which has been reported previously to have a low abundance of AHR expression (Döhr et al., 1996), showed putative AHR-related gene expression changes in six genes with age. In an asthma model of the lung, 7 putative AHR-dependent genes were affected (Table 2b). Treatment with a PPARγ antagonist, rosaglitazone, led to changes in 6 putative AHR-dependent genes. In heart as well as brain a deregulation of AHR target genes was observed under ischemic conditions and

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Table 2
Differential expression of the AHR and AHR target genes reveals novel physiological processes influenced by the AHR

Condition	Transcriptional change						
	AHR	ARNT	ARNT2	Classical AHR target genes[a]		Microarray identified AHR target genes[b]	
				Up[c]	Down	Up	Down
(a) Regulation of the AHR							
Early differentiation: from oocyte to blastocyst	− − −[d]		− −		3	4	2
Hematopoietic stem cell (HSC) development: fetal liver to day 30 post-partum	++			2		3	4
Testis (day of gestation 11.5–18.5)	(+)			1		4	1
Ovary (day of gestation 11.5–18.5)	+		−	1	1	2	3
Skeletal muscle development	−				2	8	1
Lung development, birth to day 18 wild-type	−			1	1	2	3
Lung development, birth to day 18 T1a knock-out	−			2	2	1	3
DC (CD4 CD8) subset maturation	−	−			3	1	5
Ileum epithelial cell adenoma	+			1	1	2	2
Ileum epithelial cell carcinoma				2	1	5	1
Fibroblast–Myofibroblast	− −	−		1	4	7	3
(b) Regulation of AHR target genes							
Heart — ischemic conditions				2		6	
Cardiac hypertrophy				2		3	
Heart — transplantation (isografts)						1	4
Brain — control — ischemia				2	2	2	5
Ischemia treatment		+	− −		2	3	2
Injection model				2	2	6	3
Fibroblast–Keratinocyte					3	4	3
Medullary thymic epithelial cells AIRE knock-out					3	2	3
Extraocular muscle, age course				2	1	1	2
Aorta rosiglitazone treatment		+		3			3
Lung — asthma model				2		5	
Uterus — ctl2 time course					2	3	
DC b6 CIITA /				2	1	1	1
DC b6 Iab /				2	1	1	

[a] Classical target genes are: CYP1A1, CYP1A2, CYP1B1, NQO1, AHRR, TIPARP.
[b] Genes identified by microarray studies are: S100A4, S100A8, S100A9, Ii, Cs2, Csl, CD68, Sox4, Scin, Ear2, Plac8, Xist, Lgals3.
[c] Number of AHR target genes that are differentially regulated under the given condition.
[d] "+" up-regulation, "−" down-regulation, >2–3-fold change indicated by either + or −, >4-fold=++ or− −, >8-fold by +++ or− − −.

injuries (Table 2b). The high abundance of *Ahr* and *Ahrr* in the development of Schwann cells from neural crest further suggests a role of the AHR in neuronal processes, (Supplementary Table 4). In conclusion, we observed that unexpected conditions exist, where potential AHR target genes can be differentially expressed in tissues in the absence of any exogenous ligand.

Discussion

Beyond tiered "lists of expression", microarray data can be used to data-mine transcriptional interdependence in great detail, albeit this requires direct comparability. Databases like READ, Bodymap (http://bodymap.ims.u-tokyo.ac.jp), or EMAGE are directly comparable, but rely completely on a single set of self spotted array platforms. The leading open source repositories for published array data, like GEO, ArrayExpress, or CIBEX, however, lack direct comparability; they contain huge amounts of microarray profiles (Hishiki et al., 2000; Baldock et al., 2003). Especially the widely used Affymetrix gene chips are included in these open repositories, with a wealth of thousands of expression profiles; currently approximately 40.000 Affymetrix expression profiles are deposited in GEO.

Our approach uses these publicly available expression profiles and renders them comparable by straightforward mathematical normalization. Probe level analysis of the original raw data files would be a more powerful approach, but in most cases, these are not publicly available. However, comparison between data normalized as described here clearly demonstrated that the transcriptomes of distinct tissues derived from different laboratories did not differ with changing "time, space, or person". In addition, we showed experimentally that expression levels in NC-GED corresponded between *in silico* and *in vivo* expression levels; for five sample genes in liver, spleen, kidney, brain, and bone marrow data were reproducible with real-time RT-PCR. Taken together, these criteria demonstrate the validity of our normalization approach in making microarrays comparable.

Only limited information on tissue/cell type distribution is available for most genes on microarrays. This is especially true for all expressed sequence tags (EST) with unknown function. Caused by the absence of published reference data, many po-

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tentially relevant findings concerning differentially regulated ESTs remain unnoticed. Our database NC-GED provides data for the tissue/cell type distribution of more than 9500 genes. In an exemplary fashion, we looked at expression patterns of members of the AHR system, i.e. the AHR itself, its chaperoning proteins, its dimerization partners, token target genes, and its repressor.

The AHR system is the mediator of the toxic effects of several groups of small molecular weight chemicals, in particular of polyhalogenated aromatic hydrocarbons. The high affinity AHR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is hepatotoxic, cancerogenic, teratogenic, and considered an endocrine disruptor. The immune system is a sensitive target of AHR-mediated TCDD toxicity, and immunosuppression and thymus atrophy are hallmarks of dioxin exposure. The molecular mechanisms underlying the pleiotropic and highly tissue-specific effects of AHR activation are a topic of intensive investigation (Puga et al., 2002; Hankinson, 2005; Dalton et al., 2002; Safe et al., 2000). We and others have shown that numerous immunological processes are affected by TCDD, including hematopoietic stem cell development, T- and B-cell differentiation, dendritic cell maturation and function, and an increased abundance of regulatory T cells (Temchura et al., 2005; Funatake et al., 2005; Laiosa et al., 2003; Laupeze et al., 2002).

It was noted early that constitutive expression of the AHR varies strongly between tissues. For instance, the AHR is abundantly expressed in liver, lung, and spleen and was described as almost absent in muscle tissue (Schmidt and Bradfield, 1996; Li et al., 1994). We previously showed that AHR expression, albeit generally high, is stratified in various lymphoid cells (Esser, 2002). Here we confirm and extend these findings and add other genes of the AHR signaling pathway to the analysis. A lineage specific analysis of the AHR in thymocyte subsets revealed medium to high abundance in immature thymocytes, $CD4^+CD25^+$ regulatory T cells, and low abundance in peripheral and mature, cultured cell types, correlating well with the susceptibility to TCDD action in the T-cell lineage (Kerkvliet, 2002). Atrophy of the thymus appears to be caused by several factors, in particular by a block in thymocyte maturation, reduced proliferation, and possibly premature emigration of thymocytes (Silverstone et al., 1994; Kerkvliet, 2002; Funatake et al., 2005; Novosad et al., 2002; Yamaguchi et al., 1997; Laupeze et al., 2002; Temchura et al., 2005). Recently, it was shown that regulatory T cells can be generated by AHR activation (Funatake et al., 2005). Thus, our database demonstrates that, in the T-cell lineage, the very target cells of TCDD action are the cells with the highest transcription of the *Ahr* gene.

Similarly, the systemic immunosuppression observed after TCDD treatment has been associated with phenotypic and functional changes in dendritic cells (Laupeze et al., 2002; Frericks et al., 2006), and the AHR is transcribed in these cells. We note that *Ahr* expression is higher in primary splenic dendritic cells from adults versus young (10 day old) pups, and versus dendritic cell precursors in the bone marrow. Again, as in the T-cell lineage, the developmental stage of a cell type appears to be relevant for *Ahr* expression, suggesting a role for AHR signaling in physiological differentiation events. It is currently unknown what this role could be; we think it reflects primarily the transcription factor function, possibly triggering secondary events after primary gene induction. In this context it is noteworthy that DREs are found as components of many promoters (Sun et al., 2004), and conceivably the AHR might use them to address these promoters. The AHR dimerizes with ARNT to become a functional transcription factor. ARNT in turn can dimerize with other members of the PAS-bHLH family, such as HIF1α. Our co-expression analysis showed, however, that many tissues exist, where *Arnt* is transcribed well, but *Ahr* at low levels or even not at all. The case *vice versa* was much rarer and included in particular hematopoietic stem cells. The stoichiometry of PAS-bHLH partner molecules in a given cell is not known, e.g. to what extent competition for ARNT takes place and may become limiting in AHR function. Our data indicate though that ARNT appears expressed always at sufficiently high levels, probably not regulating AHR activity by competition. It has been suggested that ARNT2, a sequence homolog of ARNT, might also bind to the AHR and thus take over when low levels of ARNT are apparent (Jain et al., 1998). Sekine and co-workers however demonstrated that, although ARNT2 can form heterodimers with HIF1α and with ARNT acting as transcription factor in reporter gene assays, it is very inefficient to form a functional transcription factor with the AHR (Sekine et al., 2006). We find that both isoforms of ARNT can be co-expressed, supporting the possibility of an independent action rather than a mere substitutive role for ARNT2.

However, our data mining of microarrays emphasizes that there is no simple relationship in quantitative terms between *Ahr* expression levels and target gene expression levels, as had been inferred previously from cell culture and molecular data (Esser, 2002). There also does not seem to be a uni-directional co-regulation, i.e. if AHR expression increases, so will target gene expression. Indeed, we identified tissue-specific conditions in NC-GED where *Ahr* transcription is decreased, but target gene transcription such as *Cyp1a1* increased. Although CYP1A1 is considered an important target gene of the AHR, it can also be regulated by other mechanisms. However, our unexpected finding points to either a release of transcriptional repression by the AHR or a role of the AHR system for up- and down-regulation of genes. We are currently investigating this possibility further.

The endogenous ligand of the AHR remains enigmatic. Several small molecular weight chemicals have been identified, which are capable of binding and activating the AHR. However, it is likely that several, tissue-specific ligands exist, which are relevant for different tissues such as skin or liver (Adachi et al., 2001; Sinal and Bend, 1997; Rannug and Fritsche, 2006). We tested NC-GED for conditions, where well-known ("classical") and/or putative AHR target genes, are changed in the absence of any exogenous AHR ligand. Our surprising finding that many such conditions exist might be explained (i) by the action of an endogenous ligand or (ii) by AHR activity without a ligand, or (iii) by coincidental regulation of these genes by other mechanisms, or a combination of them. Any of these possibilities can be tested experimentally; some even have been addressed to some extent. For instance, Elferink et al. have shown that the AHR can act independently of a ligand, by direct interaction

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with the retinoblastoma protein, thereby mediating cell cycle progression in *in vitro* experiments (Ge and Elferink, 1998; Elferink et al., 2001). AHR-responsive genes were differentially regulated in, e.g. lung, the heart, or the brain. What is known about these tissues after exposure to strong AHR ligands, such as TCDD? Immunological responses of the lung after influenza infection have revealed AHR/TCDD-dependent responsiveness of this tissue (Teske et al., 2005). The lung is the point of first contact to all air borne substances, including chemicals needing to be metabolized. Therefore, changes in transcription efficiency of putative AHR-dependent genes during inflammatory reactions like asthma could reflect a natural, endogenous-ligand-driven AHR response.

In treatment of aorta and L3T3-L1 cells with rosiglitazone, a drug used in treatment of airway hyper-responsiveness and type II diabetes, NC-GED identified differential expression of AHR-dependent genes. Rosiglitazone is an antagonist of the peroxisome proliferator activated receptor PPARγ, which in turn is known to exhibit cross-talk to the AHR signaling pathway (Liu and Jefcoate, 2006). It will be interesting to follow up the role of the AHR in asthma, other lung immune responses, and of course, signaling cross-talk.

Likewise, our *in silico* detection of conditions in the heart, which changed expression of putative AHR-dependent genes, is congruent with experimental findings. For instance, the AHR is discussed in the context of heart disease and cardiac development in humans or in the chicken embryo (Walker et al., 1997). Cardiac lesions have been shown in AHR null mice (Thackaberry et al., 2002; Vasquez et al., 2003; Mehrabi et al., 2002). We therefore think it conceivable that changed expression of AHR target genes reflects a changed activity of the AHR, and possibly activity of an endogenous ligand. This, however, will have to be addressed experimentally.

Apart from conditions by which target genes of the AHR are regulated, we report here for the first time a striking number of conditions, where the transcription level of the AHR itself is changed. In many published reports of TCDD action on the AHR system, *Ahr* transcription was not up-regulated upon activation. However, as apparent from our data, the AHR is not only differentially expressed constitutively in different tissues, but also subject to regulation under certain conditions within the same tissue. We find particularly noteworthy the strong down-regulation between the first cell divisions of the fertilized oocyte until the blastocyst (concomitant to HIF1α decrease). Moreover, in uterus and testis development from day 11.5 to day 18.5 post-partum *Ahr* transcription is up-regulated and the expression of target genes is affected. The AHR is known as an endocrine disruptor, and our data support a role for the AHR in reproductive tissues. AHR gene-deficient mice show a marked reduction in fertility and over-activation of the AHR is teratogenic (Gonzalez and Fernandez-Salguero, 1998; Thomae et al., 2004; Mimura et al., 1997).

In summary, we show here that normalization of microarray data can make them directly comparable and accessible for data mining. We established and used the meta-database NC-GED to analyze the AHR signaling pathway and its target genes. Our main findings are (i) a high tissue-specific and cell-stage-specific regulation of *Ahr* expression levels, which is (ii) not co-regulated together with ARNT; (iii) identification of highly uniform constitutive *Aip* expression indicative of its role as chaperone of other proteins in the manner of a housekeeping genes; (iv) no strict co-regulation of *Arnt1* and *Arnt2*; and finally, we found (v) regulation of putative AHR target genes upon changed experimental or physiological conditions not related to exogenous AHR activation. Our data on the tissue-specific flexibility of the AHR system contribute to an increased awareness of the physiological role of AHR signaling system. Long thought to be primarily responsible for xenobiotic metabolism, toxicological, biochemical, and functional studies indicate a breadth of involvement in other bodily functions as well.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2007.01.014.

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Transcription factor crosstalk in the transcriptional response to AhR activation by TCDD in thymic epithelial cells

Frericks M[1]., Burgoon L. D[2]., Zacharewski T.R.[2], Esser C[1].

[1] Institute for Environmental Medical Research (IUF), Düsseldorf, Germany

[2] Department of Biochemistry & Molecular Biology, National Food Safety & Toxicology Center, Center for Integrative Toxicology, Michigan State University, U.S.A.

Running head:

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Address correspondence to:

Dr. Charlotte Esser

Institut für Umweltmedizinische Forschung (IUF) at the Heinrich Heine-University of Düsseldorf

Auf'm Hennekamp 50

40225 Düsseldorf

Germany

Tel.: 0049-211-3389253

Fax: 0049-211-3190910

E-Mail: chesser@uni-duesseldorf.de

Abstract:

Activation of the aryl hydrocarbon receptor (AhR[1]) by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) elicits severe immunosuppression accompanied by thymic atrophy. Previous studies indicate that TCDD targets several steps in T cell development within the thymus; however, the underlying mechanisms remain poorly understood. Evidence suggests that AhR-mediated changes in thymic epithelial cells are relevant for thymocyte development. Global gene expression in the cortical thymic epithelial cell line, ET, at 2, 4, and 6 h following 5nM TCDD exposure resulted in differential regulation of 201 genes. JASPAR and TRANSFAC mapped the statistically over-represented promoter elements in the regulated genes to specific transcription factor binding sites, suggesting a regulatory role in AhR signaling. Over-represented elements included the xenobiotic response element XRE, NFκB-Rel, HRE, PPARγ, GR, PAX-4 and estrogen receptor binding sites. Co-treatment experiments with TCDD and $CoCl_2$, to induce hypoxia, or TCDD and 17-β-estradiol (E2) indicated crosstalk between these transcription factors, in agreement with other experimental models. The computational identification of TFBS and the demonstration of interaction confirm their interactions with AhR signaling and suggests that the other over-represented elements may also be important in the immunosuppressive effects elicited by TCDD.

Abreviations used:

[1]AhR – aryl hydrocarbon receptor, TCDD – 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, TF – transcription factor, TFBS – transcription factor binding site, $CoCl_2$ – Cobaldchloride, E2 - 17β-Estradiol, ER – estrogen receptor, NFκB – nuclear factor of kappa light polypeptide gene enhancer in B-cells, HIF – hypoxia inducible factor, PPARγ - peroxisome proliferative activated receptor, gamma, GR – glucocorticoid receptor, PAX-4 – paired box gene 4, DRE – dioxin responsive element, HRE – hypoxia response elements, PWM – position weight matrix, RR – regulatory regions

Introduction:

Polychlorinated biphenyls (PCBs), polychlorinated dibenzo-dioxins (PCDDs) and polychlorinated furans (PCDFs) are a group of important environmental pollutants. Although the contamination with these substances has decreased in recent years, they are still present in the human food chain[12]. The most toxic congener of this group, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) elicits a range of adverse effects, especially at high doses, including hepatotoxicity, tumor promotion, teratogenicity, and wasting syndrome. In laboratory animals immunosuppression accompanied by severe thymic atrophy is a hallmark of low dose TCDD exposure [57]. TCDD induced immunosuppression affects antibody responses as well as T cell mediated graft versus host responses or anti-viral responses[26, 51]. Especially T cell responses and thymus biology are affected by TCDD. The thymus is the organ of T cell development and maturation. Currently it is unclear, how the TCDD effects on the thymus relate to peripheral immunosuppression. Therefore, it is important to understand the mechanisms underlying the TCDD-elicited thymic phenotype.

The cytosolic ligand-activated transcription factor aryl hydrocarbon receptor (AhR) mediates many of TCDD's toxic responses. AhR exists in the cytosol as an inactive complex associated with HSP90 and AIP[9, 33]. Upon ligand binding, the AhR dissociates from its chaperone proteins and shuttles to the nucleus where it heterodimerizes with the AhR nuclear translocator, ARNT. This heterodimeric complex binds to dioxin response elements (DREs) in the DNA to initiate transcription[30].

Studies in constitutively active AhR T-cell lineage transgenic and AhR-chimeric mice demonstrated the necessity of AhR expression to mediate these effects in thymocytes and thymic epithelial cells[40, 46]. However, the underlying mechanism of thymic phenotype remains obscure as multiple modes of action exist, including reduced thymocyte proliferation and development, aberrant emigration of immature thymocytes and a skewing of the thymic output towards $CD8^+$ T cells [29, 31, 35]. Gene expression analysis in immature $CD3^-CD4^-CD8^-$ triple negative (TN) thymocytes and studies of $CD4^-CD8^-$ double negative (DN) thymocytes and thymic emigrants identified an array of differentially expressed genes, suggesting the role of diverse transcription factors[15, 39]. Changes in thymocyte development and stage- specific differential gene expression confound the differentiation of AhR-mediated responses and those from other transcription factors, and different cell stages. Expression of self

antigens from the periphery is primarily controlled through a tight regulation of transcription factors in thymic epithelial cells. Thymus epithelium provides important signals for thymocyte maturation and differentiation and is targeted by TCDD. As intrinsic transcription factor activity controls thymocyte development at each stage[3], crosstalk between factors conceivably plays a role. In contrast to the TCDD elicited expression changes in thymocytes, no data on thymus epithelial cells is available.

Proposed crosstalk mechanisms include competition for cofactors, direct protein interactions, and competition for DNA binding sites. For instance, AhR crosstalk has been demonstrated with hypoxia inducible factor 1 alpha (Hif1a), estrogen receptor (ER), nuclear factor 2, Ap1, Sp1 and NF-κB in various cell types[32, 33, 48, 10, 28, 37, 47]. For example, over-activation, or inhibition of degradation, of Hif1a results in thymic athrophy[5]. In normoxia, Hif1a is rapidly degraded; however, under hypoxic conditions, or activation by other stimuli, Hif1a is stabilized and heterodimerizes with ARNT. This complex binds to hypoxia responsive elements (HREs), and initiates gene expression[56]. As the thymus is normally hypoxic, AhR will likely crosstalk with Hif1a[21]. Treatment with E2 also induces a severe thymic atrophy, due to effects on thymocytes as well as thymic epithelial cells[58].

Identification of the potential interactions in the regulatory region of target genes is pivotal in elucidating the molecular mechanisms behind AhR mediated thymic atrophy and skewed thymocyte differentiation. To address this, gene expression profiling combined with comprehensive response element identification and analysis identified transcription factors that may interact with AhR signaling in a thymic cortical epithelial cell line. This analysis identified among others DREs, HREs, ERE and NF-κB binding sites as being over-represented. Results from co-treatment experiments of cobalt chloride or 17β-estradiol with TCDD to probe interactions of Hif1a and ER with the AhR, suggest that the HRE and ERE may play a role in AhR-signaling within thymic epithelial cells.

Material and Methods:

Cell culture

ET cells, derived from C57BL/6 mouse thymic cortical epithelial cells ([20, 41]), were cultured in DMEM supplemented with 10% FCS, pen/strep, and non-essential amino acids. Cells were treated at 80-90% confluency with either 5 nM TCDD (Ökometric, Bayreuth, Germany), 100µM $CoCl_2$ (Sigma, Taufkirchen, Germany), or 10nM 17-ß-estradiol (Merck, Darmstadt, Germany) for activation of the AhR, HIF1α, or ER pathway, respectively. Controls received the solvent 0.05% 1,4-dioxane (Sigma, Taufkirchen, Germany), only. For co-treatments, TCDD and either CoCl2 or E2 were added simultaneously at the concentrations stated above.

Immunofluorescence staining

$3x10^3$/ml ET cells were cultivated for 24h on 10 well, 6mm diagnostic slides (Menzel GmbH & Co KG, Braunschweig, Germany). Cells were washed in PBS, fixed for 30 min in 4% paraformaldehyde and then washed in PBS. Staining with anti-AhR (clone RPT9, Alexis Deutschland GmbH, Grünberg, Germany) in a 1:40 dilution in PBS, supplemented with 0.1% Triton, for 1h at 37°C, was followed by labelling with Alexa Fluor® 488 goat anti-mouse IgG (H+L) (MoBiTec, Göttingen, Germany) in 1:500 dilution in PBS/Triton X-100, for 1h at 37°C and washed with PBS. Nuclear counter staining was done with DAPI [10mg/ml, 1:50.000 dilution] (Sigma, Taufkirchen, Germany), for 15 min at room temperature, washed three times with PBS, and slides were mounted with Vector Shield mounting medium (Vektor Laboratories, Hilden, Germany). All images were taken using an Olympus fluorescence microscope equipped with a ColorViewXS camera system at 400X magnification and analyzed using the AnalySIS 3.2 Imaging System (all Olympus, Hamburg, Germany)

Viability testing

$1x10^4$ ET cells were transferred to 96 well-flat bottom plates, allowed to settle for 3 h, treated with 0.05%v/v dioxane, 5nM TCDD, 100µM $CoCl_2$, 10nM E2, or combinations of TCDD with the latter two for 24h. Cells were washed, incubated for 3h with 1/10 Volume MTT [10mg/ml] and lysed in DMSO supplemented with 1% w/v SDS and 0.6 % v/v acetic acid. Optical density was quantified at 570 nm using 630nm as reference in a MRX microplate reader (DYNEX Technologies, Berlin, Germany). One way

ANOVA followed by Tukey's post hoc analysis tested for significance.

RNA-Isolation and Real Time PCR

ET cell layers at 80-90 % confluence were washed once with PBS. RNA was extracted using 500µl TRIzol™ added directly to each well of a 12 well plate (Invitrogen Life Technologies, Karlsruhe, Germany), incubated at room temperature for 5 min, and transferred to eppendorf tubes. Chloroform (1/5 volume) was added, mixed and centrifuged at 12.000g for 15 min. The aqueous phase was removed, and the RNA was precipitated with isopropanol. The pellets were washed with ethanol, air dried for 10 min and dissolved in nuclease free water. RNA purity was checked by analysing the A260/A280 ratio and the integrity was evaluated by visualization of the 28S and 18S RNA bands on 1% agarose-gels.

For cDNA synthesis approximately 1µg total RNA was treated with 1 U DNaseI /0.5 µg RNA (15 min, RT). The reaction was stopped with 2 mM EDTA for 10 min at 65°C. 1 µg RNA was incubated in 10 µl with 1 µg of oligo $pd(T)_{16}$ primer for 5 min at 60°C. RNA was reverse transcribed in a final volume of 40 µl containing 1x RT buffer, 10 mM dithiothreitol, 1 mM dNTP, 80 U `RNase out´ ribonuclease inhibitor and 400 U MLV reverse transcriptase. Reactions were carried out for 60 min at 37°C and were inactivated at 70°C for 10 min. All reagents were derived from Invitrogen (Karlsruhe, Germany).

Real time PCR was performed with a Rotor Gene 3000 (-ltf- Labortechnik, Wasserburg, Germany) in a 15µl reaction containing 7.5 µl SensiMix*Plus* SYBR PCR Kit (Quantace, Berlin, Germany), 1µl cDNA and 1µl of each primer (10µM) for 45 amplification cycles (94°C 10sec, 55°C 10sec, and 72°C 10sec). All analyses used three or four biological replicates. Each primer set produced only a single gene product of expected size checked by melt curve analysis and on agarose gels. *Rps6* and *Cxxc1* were used as reference genes, which exhibited no treatment effect. Statistical analysis was performed on standardized CT-values using a two-way ANOVA, followed by a Tukey's post hoc test ($p < 0.05$). Primers were designed using the programme Primer3 [43]. A complete list of all primers, product length and melting temperatures are given in Table 1.

Microarray analysis

Microarray analysis of gene expression profiles used the Affymetrix MOE430A

GeneChip. ET cells were treated with TCDD for 2, 4, and 6hrs. Two biological replicates were analyzed per time-point. cDNA-synthesis, biotinylation and processing of the isolated RNA samples were performed using the Affymetrix cDNA-Synthesis-Kit, one-cycle target labelling and control reagents and the clean-up module (Affymetrix Inc., Santa Clara, US). Labelling and microarray hybridization of 10 µg sample RNA was performed by the *Affymetrix Core Lab Facility* at the Heinrich Heine University (Düsseldorf, Germany) using standard procedures. The resulting CEL files were analysed using R (version 2.4) and the affy package ([16, 17]) from Bioconductor. Expression values were generated using the RMA algorithm. For differential gene expression a 2-fold change in both experiments at a given time point was used. Further statistical analysis, clustering and visualization were done using the TIGR Multi experiment viewer (http://www.tm4.org/mev.html). The number of clusters for the k-means clustering was chosen to provide clearly separated clusters. Euclidean distance and average linkage were used for both cluster algorithms.

Gene ontology analysis

Biological functions ("processes") of differentially expressed genes were classified with the GOToolBox (http://crfb.univ-mrs.fr/GOToolBox/index.php) ([36]). Significance was analyzed using a hypergeometric test and Bonferroni corrected for multiple testing. Functional annotation of the genes based on their common GO entries was done using WPMGA clustering, where at least 5 genes had to share 100% of the GO information. Significant results were Bonferroni corrected.

Identification of statistically over-represented short sequence motifs in gene regulatory regions

Identification of over-represented sequence motifs in AhR regulated genes were analyzed as previously described[32]. Briefly, gene regulatory regions spanning -10.000 bp to +5.000 bp relative to the transcription start site (TSS) were obtained from the UCSC Genome Browser based on their mature RefSeq mRNA accessions. Human genome assembly hg18 (published March 2006) and murine genome assembly mm8 (published march 2006), were stored in the Gene Regulatory Subsystem of the toxicogenomic information management system (TIMS) dbZach (http://dbzach.fst.msu.edu), to facilitate analysis[7]. All short sequence motifs (5-10 nucleotides) were identified using a sliding window method [50]. An empirical Bayes

implementation of the Wilcox's Rank Sum Test was used to identify over-represented 5-10 nucleotide motifs in one population (AhR-target genes) compared to randomly selected regulatory regions. This method computes the posterior probability, which means the likelihood of that result occurring (i.e. a posterior probability of 0.90 means that there is a 90% probability that the result is true)[13, 14].

The resulting motifs were aligned and clustered using ClustalW to derive consensus sequence motifs. Each cluster contained at least 4 unique sequence motifs. T-Reg Comparators constructed position weight matrices (PWMs) from the clusters[42]. Annotation to transcription factor binding sites, stored in TRANSFAC[38] and JASPAR[44] was performed under default settings (checking in both orientations, with a dissimilarity cut-off of 0.8). Note that based on the conservation of the stored reference sites multiple hits are possible for a tested PWM.

Computational scanning for transcription factor binding site abundance in human and murine promoters

Degenerate transcription factor binding sites (e.g., those with few highly conserved regions) map to numerous short sequence motifs and clusters. Identification of these sites will occur within promoter regions due strictly to chance. To identify and eliminate these degenerate sites, all of the previously generated PWMs were used to calculate matrix similarity scores on the regulatory regions from the UCSC genes. Sites with a matrix similarity score > 0.85 were included in further analyses. Frequency of a motif more than five times per 1.000 nucleotides disqualified it from further analysis. Conserved TRANSFAC sites were discriminated based on their reference sequence. Discriminated TRANSFAC sites were highly similar to the JASPAR sites indicating higher confidence in the results.

Results

ET cells express functional AhR

ET cells express functional AhR with nuclear shuttling occurring as early as 30 min following 5nM TCDD exposure (Fig 1a), and decreasing over time (Supplementary Figure 1). Treatment induced Cyp1a1 and Cyp1b1 from 2-6hrs, as shown by quantitative Real Time PCR (QRT-PCR; Fig 1b), with decreasing transcript levels at later time-points. Treatment did not result in an observed decline in mitochondrial functioning as measured by the MTT assay, suggesting no change in cell viability (Supplementary figure 2).

Global gene expression analysis and clustering

Affymetrix MOE 430 gene chips probed gene expression at 2h, 4h and 6h following 5 nM TCDD or vehicle treatment. Two-fold up- or down-regulation was used as the filtering threshold for gene expression due to the low sample size. Hierarchical clustering of the resulting expression profiles clearly distinguished between treatment and solvent control (data not shown). TCDD treatment altered the expression of 201 transcripts, with 158 up-regulated and 43 down-regulated. Eight clusters best represented the patterns by *k*-means clustering (Fig 2b, Table 2). The qRT-PCR reproduced the microarray data with a correlation coefficient of R^2=0.8325 (see supplementary figure 3).

GO analysis identified "regulation of cellular physiological process", "regulation of metabolism", and "negative regulation of apoptosis" as over-represented. Analysis of the individual gene clusters mapped this category to cluster 7 ("immediate up-regulation followed by gradual return to baseline expression"). Further analysis revealed 21 classes of biological processes (e.g. cell death, transcription and control, cellular metabolism, cell communication) in the clusters (supplementary table 2b).

TCDD downregulated AhR expression at 2 and 4h; however, treatment upregulated the AhR gene battery, including Cyp1a1, Tiparp (Fig 2a, Cluster 1), Cyp1b1 (Cluster4), Nqo1, and Aldh3a1 (Cluster5). TCDD caused differential expression of genes involved in cell adhesion (CD44, Nope, Cspg2, Boc), and migration and cell-cell communication (Grem1, Gja1, Spsb1, Rasl11b, Irs1, Edg2, Phip, Rgs4, Cmkor1, Mt1 and 2). In addition, treatment differentially expressed a

variety of transcription factors, including the krupple like factor 5, 9, and 10, AhR, NFκB inhibitor zeta (Nfkbiz), Nfat5, Id1 and 4, Nrip, Mll5, Fosl, Nr3cl, Creb3l1, Nsbp1 and several zinc finger motifs.

Identification of over-represented promoter elements

A search for regulatory elements and transcription factor sequence motifs in the regulatory regions (RR; from -10.000 to +5.000bp relative to the TSS) of AHR-modulated genes identified 6.754 potential transcription factor binding elements. ClustalW clustered these over-represented elements into 439 clusters, each containing at least 4 elements. T-Reg converted these clusters into PWMs. TRANSFAC and JASPAR mapped and annotated the PWMs to 178 unique motifs, representing 52 factors from JASPAR, 114 from TRANSFAC and 12 previously undescribed sequences (Table 4 and Supplementary Table 3). The workflow of this analysis is depicted in figure 3.

Several PWMs matched a large number of the queried word clusters, e.g. Freac-7 (59), Gklf (128), CDXA_2 (116), AP1_Q6_01 (66), EN_1 (65), STAT5A (98) STAT6 (116), and YY1_06 (62). The multiple hits reflect a high degeneracy of the respective binding sites. Only transcription factor binding sites with conserved sequences provide meaningful results in this kind of analysis. Conserved transcription factor binding sites were distinguished by scanning for their respective abundance in all human and murine regulatory regions. An abundance threshold of no more than 5 hits per 1.000 bp was used to identify 12 sites, including the Arnt-bHLH site. Several sites comprised mixtures of a single TF with varying interaction partners. Five TFBS fitted to the Rel half site, while three others matched the MADS. The search also identified binding sites for ROR-α , Pax-4 and RREB-1. A full account of the abundance for each factor on human, murine promoter regions, and control random sequences, is available in supplementary table 4.

Twenty-five conserved transcription factor binding sites were identified from TRANSFAC. Again, the positive controls AhR and AhR-ARNT are present, suggesting high validity of the results. The search also identified binding sites for Pax4 and Hif1, also identified from JASPAR. In addition other factors like the Ebox-motif, comprising Myc-Max, too, the estrogen receptor, as well as glucocorticoid receptor binding sites are included in this list. Table 5 lists all conserved transcription factor binding sites.

Co-treatment analysis of ET cells with $CoCl_2$ and E2

QRT-PCR analysis of genes, associated with Hif1a and ER binding sites, that responded to TCDD tested if co-treatment with either $CoCl_2$ or E2 would interact with gene expression. For instance, $CoCl_2$ quickly and strongly induced Hmox1, a Hifα target gene, while TCDD induced it only moderately. However, co-treatment reduced the Hmox1 expression at 2 h, while significantly enhancing it at 6h compared to the single treatments. TCDD and $CoCl_2$ both induced Vegf, another Hif1a marker gene. Co-treatment resulted in a TCDD-like Vegf transcriptional profile (Fig. 4a and c). Although TCDD strongly upregulated Aldh3a1 (15-fold at 2h, 17 fold at 4h and 35 fold at 6h) and Gpc1 (4.2/3.3/13-fold), cotreatment did not.

E2 treatment alone showed no significant modulation of expression levels of the studied genes by QRT-PCR. Co-treatment induced the expression of CYP4501A1 and Aldh3 beyond that seen in TCDD alone (Fig 4d). However, co-treatment significantly decreased the expression of Cpox, Ndrg, Maff, Hmox1, and Lif compared to TCDD alone.

Discussion

Many chemicals and drugs can change gene expression in thymocytes and thymic epithelium, leading to immunotoxic injury. Cortical and medullary thymus epithelial cells (TEC) form microenvironments to facilitate thymocyte maturational steps in a tight reciprocal relationship with thymocytes[19]. TCDD exposure results in cortical lymphodepletion and thymocyte maturation interference, at least in part mediated by a disrupted crosstalk with epithelial cells in the cortex [11, 29]. Gene expression profiles of total thymus and thymocyte subsets treated with TCDD revealed TCDD-susceptible genes [15, 35]. However, no such profiles exist for TEC. Identifying relevant target cells (here for TCDD immunotoxicity) and applying microarray analysis to them are essential for any meaningful interpretation of data. In previous studies, we and others had analyzed the transcriptomes of fetal and adult thymocyte subsets, correlating results to TCDD-induced immunotoxic phenotype [15, 35]. Here, we extend these studies to the thymus cortical epithelium. TCDD treatment of ET cells, representing cortical TEC, elicited changes in the expression of 201 genes. The regulated genes exhibited eight general expression patterns, with about half of the genes returning to baseline levels by 6hrs. The functions encompassed by these 201 genes included xenobiotic metabolism, negative regulation of apoptosis, regulation of cellular physiological process, regulation of metabolism, transcription and control, cell adhesion, and cell communication. As expected, TCDD altered the expression of the AhR gene battery, including Cyp1a1, Cyp1b1, Nqo1, Tiparp, and Aldh3a1. The strong TCDD induced expression of CYP1a1 within the thymus, is probably irrelevant for thymic atrophy, as the thymus from Cyp1a1$^{-/-}$ mice atrophies following high TCDD exposure, while hepatotoxicty or wasting syndrome are refractory ([52]). Cyp1b1$^{-/-}$ mice on the other hand are partially protected from thymic atrophy induced by BaP pointing to an important role of Cyp1b1 in thymic atrophy[52]. Interestingly, IL6 RNA decreased in ET cells, and concomitantly RNA of its potential functional inhibitor SOCS3 increased. Mainly produced by TEC, IL-6 is critical in the thymus, fostering proliferation, differentiation and/or survival of both TEC and thymocytes[34]. Within two hours TCDD induced the gene for leukemia inhibitory factor (LIF), which contributes to thymus atrophy via induction of corticosteroids[45]. Likewise, TCDD induced Krüpple-like factor (Klf) 9, a transcriptional repressor of ERα signalling and mediator of cell proliferation[54]. Klf9 expression retains a quiescent status in B cells[18]. A recent report indicates that TCDD induces the Klf2 regulon in thymocytes[39].

Klf2 is another member of the krüpple like factors, correlated to differentiation and proliferation events. It is therefore intriguing to speculate on a similar role for Klf9 in the thymus epithelium, e.g. as a gate-keeper of transcription. TCDD modulated Foxc1 (down) and Foxq1 (up) in ET cells. Fox genes belong to the family of winged-helix transcription factors, and can alter cell fate, including tumorigenesis and cancer. Transcription of the gene for nuclear factor of activated T cells (NFAT), a regulator of cytokine genes important for thymocyte development, increased early by TCDD treatment[1, 2]. Indeed, transcription factors were the third largest group of TCDD-modulated genes Taken together, analysis of the tiered list of gene expression changes and its temporal dynamics in ET cells revealed that TCDD modulated genes, which conceivably contribute to the well-known immunotoxic TCDD-phenotype of atrophy and skewed maturation.

Note, that little overlap exists between gene induction in ET cells and thymocytes [15, 36], reaffirming that TCDD mediates gene changes in a highly cell-specific manner. Cell-specific responses to stimuli result from epigenetic factors ([25]), the presence and recruitment of transcriptional co-factors ([22]), and transcription factor cross-talk ([4, 23, 27]). Interestingly, literature screening showed that many of the TCDD-modulated genes tie into other signaling pathways than the AhR system, such as the estrogen-signaling pathway or the hypoxia induced signaling pathway (e.g. Aldh4 Socs3, IL-6, Hmox1, NFAT, LIF). Both estrogen levels and oxygen pressure are relevant for thymus function and cellularity[21, 58], and evidence for cross talk with both pathways (and others) is increasing in the literature. For instance, DREs share the E-box motif, enabling binding of the ER. On the protein level, the AhR competes for ARNT with Hif1α and other ARNT-binding proteins[10]. A comprehensive analysis of Hep3B AhR-HIF modulated transcriptomes suggested competitive and additive HIF-AhR cross talk in genes with regulatory regions that contain specific motifs and architectures[37]. Cross talk of signaling pathways may occur through, steric hindrance, direct protein-protein interaction (shown for the estrogen receptor and AhR), competition for co-regulators, and competition for transcription factor binding sites (TFBS)[22] (REF, REF). The complexity of higher organisms requires a number of versatile transcription factors. For instance, transcription factors constitute approximately 10% of the mammalian genome, 5% in *Caenorhabditis elegans* and 2-3% in yeast ([55]). Computational methods identified 37 over-represented TFBS's, within the regulatory regions (-5,000bp relative to the TSS through 5'-UTR) of the TCDD-regulated genes,

including the DRE, ER, HRE, and NF-κB. This may suggest that cross talk goes beyond Ahr-HIF or AhR-ER protein competition and involves other co-factors, response elements or promoter context. Rather, the relative enrichment of putative transcription factor binding sites within 1,000bp of the TSS, combined with putative DRE clustering within -1500 to +1500 relative to the TSS, suggests an increased likelihood of TFBS cross-talk[6, 45].

Co-treatment studies of TCDD with $CoCl_2$ or E2 followed up these results to identify possible cross talk in ET cells between the AhR, Hif1a, and ER for nine selected genes demonstrated cross-talk by qRT-PCR, with examples of genes experiencing enhancement and repression of expression. Competition for Arnt may modulate the difference following $CoCl_2$ and TCDD co-treatment. The effects of TCDD and $CoCl_2$ treatment on the nine genes analyzed were mainly additive, while E2 and TCDD-E2 combinations tended to decrease transcription of the selected genes. This is curios, as $CoCl_2$ stabilizes HIF1α, and again suggests that a protein-protein competition of the AhR with Hif1α may not be the main factor in the cross talk of these pathways. The divergent direction of TCDD and E2 on ET cell transcriptomes, exacerbated even in the co-treatments, needs further exploration. Reduced proliferation of immature thymocytes and enhanced emigration accompany estrogen mediated thymic atrophy ([58, 59]). The mechanisms by which prolonged estrogen exposures, such as estrogen therapy and pregnancy, impair thymus function have not been well defined. The data presented here indicate that the mechanism of estrogen and TCDD mediated thymic involution differ. Estrogen is used in long-term treatment for e.g. hot flushes in menopausal women; possible exacerbating effects on the aging thymus, will need to be addressed. To date, there is limited information on whether estrogenic endocrine disruptors modulate the immune system of aged individuals[8, 24].

TCDD is a strong and persistent ligand of the AhR. However, many other ligands, including abundant food constituents such as flavonoids, can trigger AhR signalling, and lead to endocrine disruption and physiological changes. The kinetics and dynamics, and indeed the normal background exposure is not known.

In conclusion, thymic cortical epithelial cells demonstrate TCDD-mediated transcriptional changes *in vitro*. These changes included the AhR gene battery, transcripts related to thymic physiology, TCDD induced thymotoxic effects, and

transcription factors. Many of these genes contained an over-representation of DREs and other transcription factor binding sites within the gene regulatory regions. A number of genes demonstrated cross talk following co-exposure of TCDD and $CoCl_2$ or E2. Understanding the extent and nature of the cross talk between AhR signaling and other pathways is important in understanding and possibly manipulating these pathways for risk-management or therapy.

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Figure Legends:

Figure 1: AhR shuttles to the nucleus and is transcriptionally active in cortical thymic epithelial cell culture.

a) ET cells were grown on chamber slides and treated with 0.05% 1,4-dioxan as solvent control or 5 nM TCDD, diluted in dioxan. After 1h the cells were fixed as described in Materials and Methods and stained for AhR protein content. b) Quantitative RT-PCR for Cyp1a1 (black bars) and Cyp1b1 (white bars) was performed in biological triplicate and normalized to RPS6 and Cxxc1 as reference gene. Significance was calculated using ANOVA followed by Tukey's post hoc test (* $p < 0.05$, ** $p < 0.01$ and *** for $p < 0.001$)

Figure 2: Kinetics of differential gene expression in ET cells treated with TCDD

a) Heatmap of differentially expressed genes after 2, 4, and 6h of treatment with 5 nM TCDD or solvent control (n=2). The heatmap shows the hierarchical clustering of the genes, using a weighted average linkage and the mean Euclidean distance generated by TIGR MeV 4.0. b) K-means clustering of time dependent differentially expression. The differentially expressed genes were clustered according to 8 groups returned from an implementation of K-means clustering, using the weighted average linkage and the mean Euclidean distance, in TIGR MeV 4.0. The light line represents the average for all genes in each category.

Figure 3: Scheme for identifying over-represented transcription factor binding sites in TCDD regulated genes.

A computational search identified and clustered over-represented word motifs in regulatory regions (-10.000 bp to + 5.000 bp around TSS) of TCDD modulated genes in ET cells. 178 of the resulting 439 word clusters annotated to known transcription factor binding sites from TRANSFAC and JASPAR, and of these the 37 highly conserved sequences were included in further analyses.

Figure 4: Cross talk of hypoxic and estrogenic signaling with the TCDD response.

Gene expression of nine genes by qRT-PCR in ET cells; ET-cells were treated for 2, 4 or 6 h with (a) 5 nM TCDD, 100µM $CoCl_2$ or a cotreatment of both (n=6); panels (b-d) show the additive or subtractive change of transcription for combined treatments

(b) TCDD minus $CoCl_2$ (c) TCDD minus cotreatment (d) $CoCl_2$ minus co-treatment changes; (e) Expression changes after treatment with 5nM TCDD, 10nM E2 or a cotreatment of both for 2, 4 or 6 h (n=4); panels (f-h) show the additive or subtractive change of transcription for (f) TCDD minus E2, (g) TCDD minus co-treatment, (g) E2 minus co-treatment. All values show log2-fold differential expression; red colour indicates treatment-mediated increase, green colour decrease of transcription..

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Table 1: Quantitative RT-PCR Primers

Gene	Forward*	Reverse	Reference mRNA	Entrez GeneID	Size [bp]	Product Location# Start	End
Aldh3a1	cccctggcactctatgtgtt	gagacctcaccaggcaagag	NM_007436	11670	217	61030714	61031573
Centd2	gtgggcaatgtcaaggaagt	tgagtgcttctgtccacacc	NM_001040111	69710	346	108537170	108540430
Cpox	aggatgctgtccatttccac	gccccgatcatacaacagat	NM_007757	12892	35	58674553	58678091
Cxxc1	cagacgtcttttgggtcca	agacctcatcagctggcac	NM_028868	74322	157	74380020	74380500
Cyp1a1	tccttgcatgtccatgtttc	tgcataagcaaaatacagtcca	NM_009992	13076	344	57551162	57551505
Cyp1b1	gacccggatgttttgtgaat	catgttgagcagcaaaagaa	NM_009994	13078	232	80106832	80107063
Ga17(Eif3m)	tccatgggatggacaagaat	tcgccttccaaaaatttgac	NM_145380	98221	376	104845951	104853464
Gpc1	tgctgcctgatgactacctg	tgagcacatttcggcaatag	NM_016696	14733	282	94751654	94752595
Hmox1	cacgcatatacccgctacct	tctctgcaggggcagtatct	NM_010442	15368	362	77620998	77623802
Hspa1b	tgttgctgacgaagatgaag	aggtcgaagatgagcacgtt	NM_010478	15511	235	35095354	35095588
Lgals3	gagcttatcctggctcaac	gttattgtcctgcttcgtgt	NM_010705	16854	323	47999969	48004385
Lif	ggcaacctcatgaaccagat	agtggggttcaggaccttct	NM_008501	16878	264	4168294	4169115
Maff	tggctgtggatcccttatct	cttctgcttctgcagctcct	NM_010755	17133	248	79187429	79188063
Mt1	ggtcctctaagcgtcaccac	aggagcagcagctcttcttg	NM_013602	17748	191	96703127	96704013
Ndrg1	tcttcggcaaggaggagata	catcttgagcagggtggtct	NM_008681	17988	275	66768760	66771810
Rps6	attcctggactgacagacac	gttcttcttagtgcgttgct	NM_009096	20104	247	86500749	86501906
Socs3	ggaccaagaacctacgcatc	cgcccccagaatagatgtag	NM_007707	12702	249	117829031	117829279
Tiparp	tggagtaacttcagccaact	aaataactgccttgtccaaa	NM_178892	99929	372	65356530	65357207
Txnl1	aatgaggacgcagacattcc	cgcctcctcaaaatccatag	NM_016792	53382	288	63833811	63839036
Vegfa	atcttcaagccgtcctgtgt	aatgctttctccgctctgaa	NM_009505	22339	244	46158037	46162397

*All reactions were performed at 55°C with routine melting temperature analysis as described in Material and Methods

Product location was determined by Blat search using the UCSC Genome Browser

Table 2: **Kinetic of differential gene expression in the ET, cortical epithelial cell line**

Probe Set ID	Gene Symbol	RefSeq Transcript ID[#]	log2 fold regulation by TCDD			Cluster[*]
			2h	4h	6h	
1422217_a_at	Cyp1a1[#]	NM_009992	5,55	6,46	5,43	Cluster1
1426721_s_at	Tiparp	NM_178892	3,74	2,75	3,22	
1452160_at	Tiparp	NM_178892	4,21	2,90	3,58	
1452161_at	Tiparp	NM_178892	3,66	2,05	3,14	
1415973_at	---	---	-0,34	-1,03	-0,52	
1433581_at	1190002N15Rik	NM_001033145	-0,63	-1,16	-0,28	
1427202_at	4833442J19Rik	NM_177101	-0,30	-1,42	0,00	
1449252_at	9030611O19Rik	NM_027828	-1,01	-0,92	-0,87	
1450716_at	Adamts1	NM_009621	-1,20	-1,24	-0,72	
1422631_at	Ahr	NM_013464	-1,70	-1,08	-0,08	
1426942_at	Aim1	XM_001000734 /// XM_894444	-0,37	-1,21	-0,46	
1423890_x_at	Atp1b1	NM_009721	-0,22	-0,90	-1,07	
1439036_a_at	Atp1b1	NM_009721	-0,26	-1,08	-0,92	
1452336_at	BC027382	---	-0,11	-1,03	-0,42	
1422912_at	Bmp4	NM_007554	-0,66	-1,31	-0,42	
1426869_at	Boc	NM_172506	-0,53	-1,00	-0,47	
1460259_s_at	Clca1 /// Clca2	NM_009899 /// NM_030601	-0,23	-1,19	0,17	
1419463_at	Clca2	NM_030601	-0,29	-1,00	-0,26	
1417625_s_at	Cmkor1	NM_007722	-0,57	-0,44	-1,03	
1436736_x_at	D0H4S114	NM_053078	-0,09	-0,85	-1,12	
1450839_at	D0H4S114	NM_053078	-0,02	-0,71	-1,29	
1417143_at	Edg2	NM_010336	-0,23	-1,16	-0,38	
1427225_at	Epn2	NM_010148	-0,10	-1,08	-0,33	
1419486_at	Foxc1	NM_008592	-1,13	-0,61	-0,17	
1427126_at	Hspa1b	NM_010478	-0,01	0,05	-1,15	Cluster 2
1427127_x_at	Hspa1b	NM_010478	-0,08	0,05	-1,06	
1425895_a_at	Id1	NM_010495	-1,53	-0,10	-0,46	
1423259_at	Id4	NM_031166	-1,34	-0,07	-0,20	
1423062_at	Igfbp3	NM_008343	-0,38	-0,79	-1,01	
1450297_at	Il6	NM_031168	-1,39	-0,30	-0,79	
1423104_at	Irs1	NM_010570	-1,09	-0,73	-0,37	
1416029_at	Klf10	NM_013692	-1,10	-0,05	-0,13	
1422771_at	LOC670044	XM_978692	-1,27	-0,56	-0,37	
1424826_s_at	Mtss1	NM_144800	-0,69	-1,19	-0,55	
1434036_at	Mtss1	NM_144800	-0,65	-1,51	-0,57	
1416474_at	Nope	NM_020043	-0,11	-1,14	0,07	
1436970_a_at	Pdgfrb	NM_008809	-0,25	-1,27	-0,21	
1416846_a_at	Pdzrn3	NM_018884	-0,43	-1,32	-0,38	
1416687_at	Plod2	NM_011961	-0,25	-1,04	-0,70	
1423854_a_at	Rasl11b	06 /// XM_915727 /// XM_982940 /// XI	-0,25	-1,29	-0,46	
1416286_at	Rgs4	NM_009062	-1,45	-1,20	-0,88	
1416701_at	Rnd3	NM_028810	-1,02	-0,50	-0,16	
1416779_at	Sdpr	NM_138741	0,15	-1,21	-0,12	
1421849_at	Stag2	NM_021465	-0,18	-0,41	1,10	
1434089_at	Synpo	19 /// XM_981156 /// XM_981197 /// XI	-0,46	-1,01	-0,45	
1424634_at	Tceal1	NM_146236	0,15	-1,26	0,19	
1423835_at	Zfp503	NM_145459	-1,30	-0,73	-0,26	
1423836_at	Zfp503	NM_145459	-1,01	-1,00	-0,30	
1449314_at	Zfpm2	NM_011766	-0,81	-1,38	0,21	
1425082_s_at	9030416H16Rik	M_900536 /// XM_900542 /// XM_9005	-0,25	-0,01	1,02	Cluster 3
1419734_at	Actb	NM_007393	-1,02	-0,13	0,30	
1450897_at	Arhgap5	NM_009706	0,02	-0,18	1,42	
1437696_at	BC049807	NM_001002008	-0,16	-0,76	1,00	
1450950_at	Cspg6	NM_007790	-0,23	-0,01	1,15	
1422795_at	Cul3	NM_016716	-0,22	-0,13	1,01	
1424324_at	Esco1	'XM_919569 /// XM_919574 /// XM_91	-0,25	0,04	1,02	
1418942_at	Ift74	NM_026319	-0,05	-1,04	0,57	
1436766_at	Luc7l2	NM_138680	-0,24	0,06	1,18	
1436767_at	Luc7l2	NM_138680	-0,13	-0,22	1,15	

Probe Set ID	Gene Symbol	RefSeq Transcript ID	log2 fold regulation by TCDD			Cluster
			2h	4h	6h	
1426817_at	Mki67 /// LOC63877	692 /// XM_133912 /// XM_989637 ///	-0,16	-0,42	1,38	
1452377_at	Mll1	M_916038 /// XM_990843 /// XM_9908	-1,04	-0,27	0,72	
1434704_at	Mll5	---	-1,33	-0,26	0,69	
1438999_a_at	Nfat5	NM_018823 /// NM_133957	-0,92	0,21	1,52	
1449089_at	Nrlp1	NM_173440	-1,05	-0,43	0,58	
1418152_at	Nsbp1	NM_016710	-0,13	0,11	1,16	
1418524_at	Pcm1	NM_023662	-0,24	0,10	1,08	
1436908_at	Pcm1	NM_023662	-0,17	0,01	1,02	
1423432_at	Phip	XM_895482 /// XM_895496 /// XM_89	-0,12	-0,12	1,11	
1435341_at	Ppig	899890 /// XM_909264 /// XM_921212	-0,46	0,13	1,12	
1423444_at	Rock1	NM_009071	-0,62	-0,10	1,18	Cluster 3
1423445_at	Rock1	NM_009071	-0,62	0,07	1,27	
1450994_at	Rock1	NM_009071	-0,72	0,13	1,30	
1421871_at	Sh3bgrl	NM_019989	-0,22	-0,50	1,11	
1428107_at	Sh3bgrl	NM_019989	-0,22	-0,35	1,19	
1427275_at	Smc4l1	NM_133786	-0,19	-0,57	1,33	
1452197_at	Smc4l1	NM_133786	-0,08	-0,43	1,42	
1451063_at	Stxbp4	NM_011505	-0,29	0,06	1,06	
1456112_at	Tpr	NM_133780	-0,71	-0,56	1,31	
1434392_at	Usp34	---	-0,58	0,08	1,06	
1438714_at	Zfp207	NM_011751	-0,25	-0,19	1,16	
1426472_at	Zfp521	NM_144515	0,06	-0,06	1,13	
1426708_at	Antxr2	NM_133738	1,36	1,35	0,93	
1427912_at	Cbr3	NM_173047	0,98	1,62	0,56	
1424638_at	Cdkn1a	NM_007669	2,65	1,56	0,39	
1422492_at	Cpox	**NM_007757**	2,25	2,51	2,00	
1422493_at	Cpox	**NM_007757**	2,46	1,45	1,60	
1416612_at	Cyp1b1	NM_009994	1,62	1,18	1,37	
1416613_at	Cyp1b1	NM_009994	1,93	1,48	1,69	
1419431_at	Ereg	NM_007950	1,53	1,71	0,94	
1417487_at	Fosl1	NM_010235	1,30	1,58	0,36	Cluster 4
1417488_at	Fosl1	NM_010235	1,21	1,91	0,12	
1420499_at	Gch1	NM_008102	1,15	2,01	0,64	
1418936_at	Maf1	**NM_010755**	1,68	1,47	0,15	
1423413_at	Ndrg1	**NM_010884**	1,17	1,61	0,26	
1416543_at	Nfe2l2	NM_010902	1,45	1,15	0,94	
1417262_at	Ptgs2	NM_011198	1,07	1,73	0,77	
1418666_at	Ptx3	NM_008987	1,25	1,40	0,88	
1427233_at	Tshz1	M_888252 /// XM_917016 /// XM_9775	1,22	1,15	0,91	
1450004_at	Tslp	NM_021367	1,18	1,20	0,86	
1418752_at	Aldh3a1	**NM_007436**	1,56	1,94	2,25	
1417225_at	Arl6ip5	NM_022992	0,63	1,30	1,37	
1429555_at	Cldnd1	NM_171826	0,94	1,24	1,67	
1431293_a_at	Cldnd1	NM_171826	0,97	1,17	1,65	
1431293_a_at	Cldnd1	NM_171826	0,97	1,17	1,65	
1419295_at	Creb3l1	NM_011957	0,95	1,85	1,31	
1417389_at	Gpc1	**NM_016696**	1,33	2,21	2,05	Cluster 5
1425357_a_at	Grem1	NM_011824	0,55	1,25	1,19	
1433803_at	Jak1	NM_146145	0,68	0,71	1,48	
1433805_at	Jak1	NM_146145	0,70	0,97	1,52	
1423627_at	Nqo1	NM_008706	0,75	1,32	1,52	
1460303_at	Nr3c1	NM_008173	0,95	0,72	1,30	
1428114_at	Slc14a1	NM_028122	0,43	1,56	1,57	
1454967_at	---	---	0,06	0,31	1,04	
1434941_s_at	2610101J03Rik	M_130548 /// XM_918330 /// XM_9912	-0,11	0,26	1,28	
1434942_at	2610101J03Rik	M_130548 /// XM_918330 /// XM_9912	-0,17	0,58	1,60	
1419577_at	A530089I17Rik	NM_133999	0,35	0,66	1,02	
1437382_at	Acvr2a	NM_007396	-0,38	0,55	1,13	Cluster 6
1421058_at	Adh7	NM_009626	0,37	0,83	1,34	
1418979_at	Akr1c14	NM_134072	0,61	0,55	1,07	
1419562_at	Birc6	NM_007566	-0,32	0,41	1,02	

Probe Set ID	Gene Symbol	RefSeq Transcript ID	log2 fold regulation by TCDD			Cluster
			2h	4h	6h	
1449217_at	Casp8ap2	NM_011997	-0,13	0,35	1,02	
1421694_a_at	Cspg2	M_912905 /// XM_922540 /// XM_9940	0,13	0,53	1,51	
1427256_at	Cspg2	M_912905 /// XM_922540 /// XM_9940	-0,09	0,40	2,23	
1426438_at	Ddx3y	NM_012008	0,02	0,58	1,43	
1428011_a_at	Erbb2ip	NM_001005868 /// NM_021563	0,08	0,72	1,20	
1418253_a_at	Hspa4l	NM_011020	0,34	0,76	1,63	
1449010_at	Hspa4l	NM_011020	0,30	0,84	1,07	
1450738_at	Klf21a	NM_016705	0,56	0,52	1,13	
1415855_at	Klhl	NM_013598	-0,17	0,35	1,07	
1448117_at	Klhl	NM_013598	-0,46	0,42	1,10	
1455434_a_at	Kiln1	NM_008477	0,04	0,78	1,59	Cluster 6
1424609_a_at	LOC432823	NM_001013789	0,12	0,05	1,03	
1417355_at	Peg3	NM_001010988 /// NM_008817	0,06	0,55	1,48	
1433924_at	Peg3	NM_001010988 /// NM_008817	-0,16	0,97	2,06	
1425514_at	Plk3r1	NM_001024955 /// NM_011085	0,18	0,66	1,20	
1450690_at	Ranbp2	NM_011240	-0,30	0,37	1,18	
1418968_at	Rb1cc1	NM_009826	-0,04	0,37	1,11	
1452276_at	Smarcad1	NM_007958	-0,26	0,15	1,24	
1426271_at	Smc5l1	NM_153808	0,04	0,32	1,16	
1424573_at	Tmed5	NM_028876	-0,08	0,38	1,20	
1426949_s_at	Tpr	NM_133780	-0,22	0,22	1,02	
1434842_s_at	Upf3b	NM_026573	-0,19	0,12	1,38	
1417791_a_at	Zfml	NM_008717	-0,13	0,27	1,09	
1423306_at	2010002N04Rik	NM_134133	1,25	0,64	-0,12	
1421031_a_at	2310016C08Rik	NM_023516	1,03	0,47	0,02	
1418250_at	Arl4	NM_025404	1,23	1,05	0,30	
1421392_a_at	Birc3	NM_007464	1,12	0,24	0,45	
1416250_at	Btg2	NM_007570	1,14	0,41	0,16	
1448272_at	Btg2	NM_007570	1,31	0,53	0,24	
1421679_a_at	Cdkn1a	NM_007669	1,23	0,27	-0,14	
1434045_at	Cdkn1b	NM_009875	1,36	0,35	0,34	
1428306_at	Ddit4	NM_029083	1,08	0,70	0,07	
1422735_at	Foxq1	NM_008239	1,13	0,64	0,35	
1429692_s_at	Gch1	NM_008102	1,36	0,87	0,28	
1419080_at	Gdnf	NM_010275	1,36	0,45	0,93	
1422264_s_at	Klf5	NM_010638	1,20	-0,02	0,52	
1428288_at	Klf5	NM_010638	1,53	0,57	0,71	
1428289_at	Klf5	NM_010638	1,41	0,65	0,70	
1456341_a_at	Klf5	NM_010638	1,67	0,61	0,75	Cluster 7
1421207_at	Lil	NM_001039537 /// NM_008501	1,32	0,78	0,11	
1418288_at	Lpin1	NM_015763 /// NM_172950	1,15	-0,03	-0,02	
1449893_a_at	Lrig1	NM_008377	1,10	0,61	0,36	
1418192_at	Mnt	NM_010813	1,12	0,06	-0,03	
1423379_at	Nfatc4 /// LOC67202	NM_023699 /// XM_990636	1,05	0,47	-0,04	
1417483_at	Nfkbiz	NM_030612	1,00	0,10	0,20	
1448568_a_at	Slc20a1	NM_015747	1,34	0,93	0,17	
1416576_at	Socs3	NM_007707	1,37	0,72	0,30	
1455899_x_at	Socs3	NM_007707	1,94	0,70	0,25	
1456212_x_at	Socs3	NM_007707	1,91	0,66	0,27	
1420150_at	Spsb1	NM_029035	1,08	0,17	-0,05	
1417447_at	Tcf21	NM_011545	1,11	0,77	0,70	
1452385_at	Usp53	NM_133857	1,80	0,46	0,52	
1428402_at	Zcchc3	NM_175126	1,01	0,73	0,37	
1451508_at	1700108L22Rik	NM_001040399	0,36	1,00	0,32	
1453004_at	3110004L20Rik	NM_001033167	0,41	1,13	0,35	
1434239_at	AA408556	NM_199447	-0,01	1,01	0,09	
1421002_at	Angptl2	NM_011923	0,41	1,01	0,60	
1451533_at	BC022687	NM_145450	0,45	1,10	0,44	Cluster 8
1452483_a_at	Cd44	I01039150 /// NM_001039151 /// NM_0	0,13	1,26	0,24	
1460177_at	Cndp2	NM_023149	0,45	1,00	0,58	

Probe Set ID	Gene Symbol	RefSeq Transcript ID	log2 fold regulation by TCDD			Cluster
			2h	4h	6h	
1420498_a_at	Dab2	I01008702 /// NM_001037905 /// NM_0	0,15	1,21	0,60	
1417903_at	Dtna5h	NM_018769	0,76	1,01	0,78	
1415800_at	Gja1	NM_010288	0,65	1,24	0,11	
1448239_at	**Hmox1**	**NM_010442**	0,03	1,45	-0,12	
1419647_a_at	Ier3	NM_133662	0,78	1,54	0,59	
1451021_a_at	Klf5	NM_009769	0,55	1,01	0,34	
1422557_s_at	Mt1	NM_013602	-0,48	1,43	-0,24	
1428942_at	Mt2	NM_008630	-0,25	1,04	-0,05	
1450976_at	Ndrg1	NM_010884	0,70	1,56	0,17	Cluster 8
1456174_x_at	Ndrg1	NM_010884	0,94	1,24	0,23	
1450977_s_at	Ndrg1 /// Ndr1	NM_008681 /// NM_010884	0,67	1,18	0,12	
1420760_s_at	Ndr1	NM_008681	0,97	1,20	0,28	
1423269_a_at	Nedd4l	NM_031881	0,15	1,08	0,33	
1418835_at	Phlda1	NM_009344	0,67	1,12	0,37	
1430780_a_at	Pmm1	NM_013872	0,64	1,08	0,77	
1420401_a_at	Ramp3	NM_019511	0,55	1,43	0,65	
1419082_at	Serpinb2	NM_011111	0,65	1,35	0,72	
1416041_at	Sgk	NM_011361	0,97	1,24	0,55	
1448742_at	Snai1	NM_011427	0,06	1,15	0,13	

* Cluster derived from hierarchical clustering. Cluster numbers are equal to those in Fig. 2b

Reference sequence number corresponding to the Affymetrix ProbeSet ID.

§ Genes written in bold were used for validation by qRT-PCR

Table 3 **Characteristics of the clusters used for gene ontology analysis**

Cluster[†]	Input gene numbers[‡]	Non redundant genes[#]	Annotated genes[*]	Non annotated genes[*]
All transcripts	201	163	116	47
1	4	2	2	0
2	46	40	29	11
3	32	26	17	9
4	18	15	13	2
5	14	10	9	1
6	31	26	15	11
7	30	24	19	5
8	26	25	16	9

[†] Cluster of genes as derived from the K-means clustering performed on the genes differentially expressed by TCDD

[‡] Number of transcripts falling in this cluster

[#] For a number of genes several hybridization sequences, as derived from different uni-gene clusters are spotted onto the MOE 430 A array used in this analysis.

[*] For a large number of genes no functional annotation is available, thus giving them no gene ontology annotation. Thus the numbers represent the transcrpits with or without a functional gene ontalogy annotation

Table 4: **Analysis of over-represented motif elements and functional annotation of the resulting clusters to TRANSFAC and JASPAR**

Identification of over-represented sequences

Step	Number of motifs
Word-Search Algorithm[†]	6920
Word-Cluster[‡]	439

Functional annotation

Step	Number of motifs
Total matches[#]	1638
JASPAR	52
TRANSFAC	114
Unknown	12

[†] Number of statistical overrepresented genes in TCDD regulated genes

[‡] Number of PWM derived from clustering of the over-represented words

[#] Matches of PWM matrices as derived from T-REG comparator. Based on the sequence conservation in the queried TFBS multiple hits are possible

Table 5 **Annotation of the over-represented word cluster to the JASPAR and TRANSFAC 7.0 transcription factor binding sites transcription factor binding sites**

Transcription Factor†	Source	Accession Number‡	Length	Consensus sequence	# of Word-Cluster
AGL3 MADS	JASPAR	MA0001	10	CCATAWATAG	10
ARNT	JASPAR	MA0004	6	CACGTG	2
c-REL	JASPAR	MA0101	10	NGGGNTTTCC	15
Dorsal_1 REL	JASPAR	MA0022	12	SGGGTTTTTCCS	3
Dorsal_2 REL	JASPAR	MA0023	10	GGGGWTTTCC	2
MEF2 MADS	JASPAR	MA0052	10	CTATTTATAG	1
NF-kappaB REL	JASPAR	MA0061	10	GGGAMTTYCC	6
p65 REL	JASPAR	MA0107	10	GGGAATTTCC	1
Pax-4 PAIRED-HOMEO	JASPAR	MA0068	30	GAAAAATWNNCNNNNNNNNNNCNCNCCMC	14
RORalfa-1	JASPAR	MA0071	10	ATNNAGGTCA	12
RREB-1	JASPAR	MA0073	20	CCCCAAACCACCCCCMCCCN	11
SQUA MADS	JASPAR	MA0082	14	MCAAAAATRGWAAN	2
AHR_01	TRANSFAC_PUBLIC	M00136	18	CCCCNGNCTNGCGTGAGA	1
AHRARNT_01	TRANSFAC_PUBLIC	M00235	16	KNNKNNTYGCGTGCMS	2
CP2_02	TRANSFAC	M00947	15	GCTGGNTNNNNCTNG	6
E2A_Q2	TRANSFAC	M00804	14	NCACCTGNCNCNGN	2
EGR_Q6	TRANSFAC	M00807	11	GTGGGGGCGRS	2
ER_Q6	TRANSFAC_PUBLIC	M00191	19	NNAGGNNANNNTGACCYNN	2
ERR1_Q2	TRANSFAC	M00511	14	NNNTNAAGGTCANN	1
GR_Q6	TRANSFAC_PUBLIC	M00192	19	NNNNNNCNNTNTGTNCTNN	1
GR_Q6	TRANSFAC_PUBLIC	M00192	19	NNNNNNCNNTNTGTNCTNN	1
HIF1_Q3	TRANSFAC	M00797	14	GNNKACGTGCNGNN	1
HNF1_Q6	TRANSFAC	M00790	18	NRGTTAATNATTANCNNN	1
HOXI3_01	TRANSFAC_PUBLIC	M00023	30	TGCNNNNWNCCYCATTAKTNNNNNNNNYCN	4
KROX_Q6	TRANSFAC	M00982	14	CCCGCCCCCGCCCC	2
MYC_Q2	TRANSFAC	M00796	7	CACGTGS	1
MYCMAX_03	TRANSFAC	M00615	20	NNNNNNNCACGTGNNNNNNN	2
MYOD_Q6_01	TRANSFAC	M00929	18	NNGNNNCAGGTGNNNNNN	3
MYOGNF1_01	TRANSFAC_PUBLIC	M00056	29	CASCTGTNNNNTTTGGCACNSNGCCARNN	1
NF1_Q6_01	TRANSFAC	M00806	17	NTGGNNNNNNGCCAANN	3
PAX4_01	TRANSFAC_PUBLIC	M00373	21	NGNNGTCANGCGTGNNSNNYN	1
PAX8_B	TRANSFAC_PUBLIC	M00328	18	NCNNTNNTGCGTGANNNN	1
PIT1_Q6	TRANSFAC	M00802	18	NATTCATAATTATNNNNA	2
PPAR_DR1_Q2	TRANSFAC	M00763	13	TGACCTTTGNCCN	1
PPARG_03	TRANSFAC	M00528	17	AACNRGGNCAAAGGTCA	1
SP3_Q3	TRANSFAC	M00665	14	ASMCTTGGGSRGGG	7
VDR_Q3	TRANSFAC	M00444	15	GGGTNARNNGGGWSA	6

† Identification of the transcription factor in JASPAR of TRANSFAC 7.0

‡ T-REG comparator internal reference number for the respective transcription factor binding site

1638 individual word clusters were annotated using T-REG comparator. Multiple hits point to a low degree of sequence conservation in the respective PWM matrix.

Figure 1

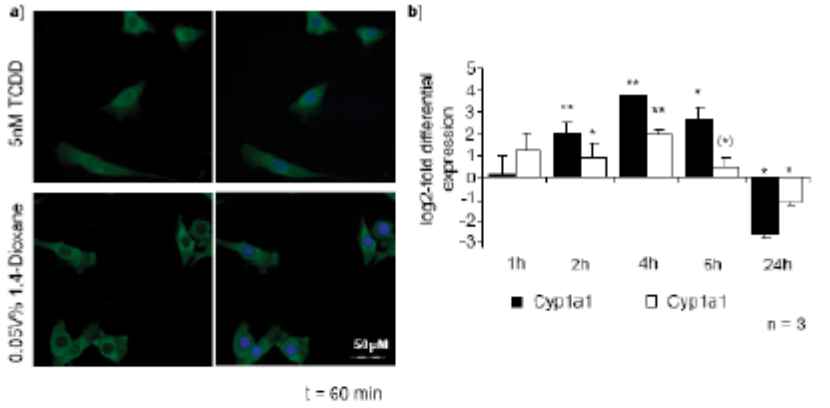

a]
5nM TCDD
0.05V% 1.4-Dioxane
50µM
t = 60 min
b]
log2-fold differential expression
5
4
3
2
1
0
-1
-2
-3
1h
2h
4h
5h
24h
Cyp1a1
Cyp1a1
n = 3

Figure 2

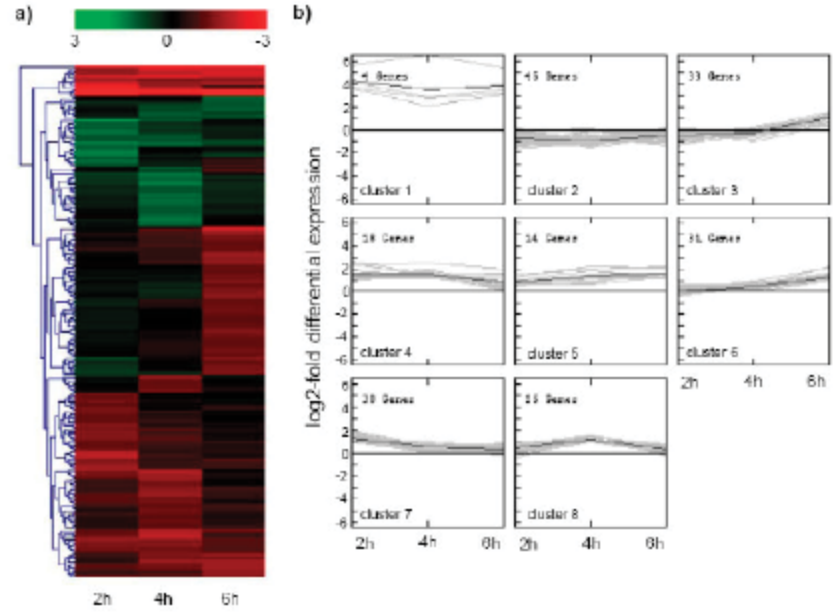

a)
3
0
-3
2h
4h
6h
b)
log2-fold differential expression
cluster 1
cluster 2
cluster 3
cluster 4
cluster 5
cluster 6
cluster 7
cluster 8
2h
4h
6h

Figure 3

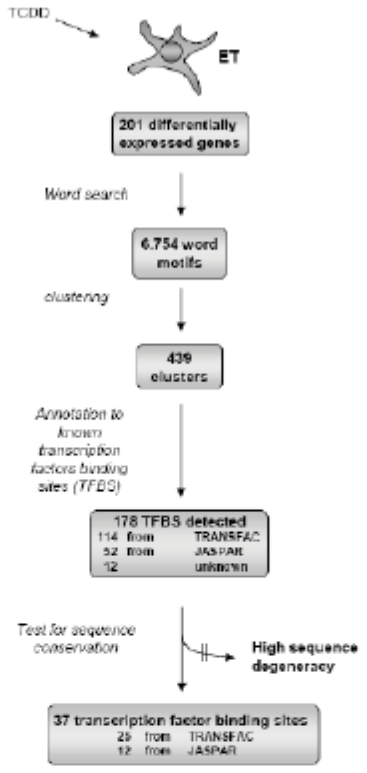
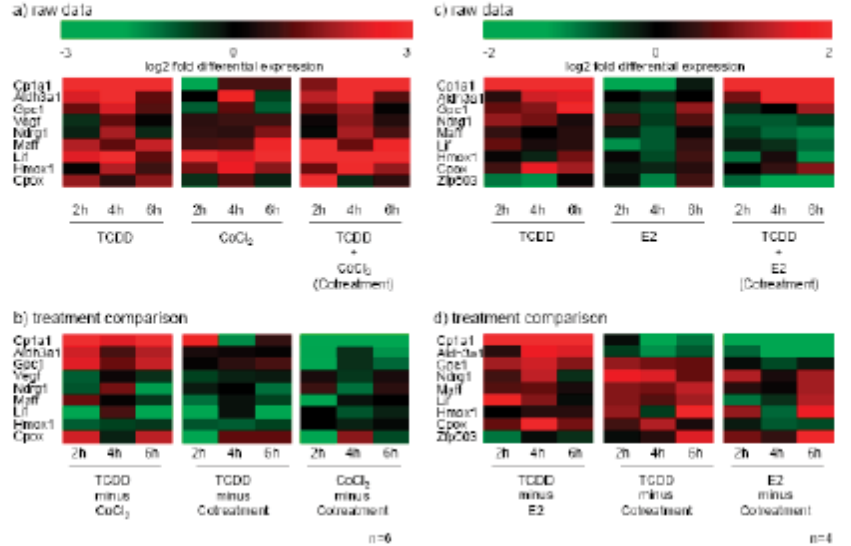

TCDD
ET
201 differentially expressed genes
Word search
6.754 word motifs
clustering
439 clusters
Annotation to known transcription factors binding sites (TFBS)
178 TFBS detected
114 from TRANSFAC
52 from JASPAR
12 unknown
Test for sequence conservation
High sequence degeneracy
37 transcription factor binding sites
25 from TRANSFAC
12 from JASPAR

Figure 4


a) raw data
-3
0
3
log2 fold differential expression
Cpt1a1
Aldh3a1
Gpc1
Vegf
Ndrg1
Maff
Lif
Hmox1
Cpox
2h 4h 6h
TCDD
CoCl2
TCDD + CoCl2 (Cotreatment)
c) raw data
-2
0
2
log2 fold differential expression
Zfp503
TCDD
E2
TCDD + E2 (Cotreatment)
b) treatment comparison
TCDD minus CoCl2
TCDD minus Cotreatment
CoCl2 minus Cotreatment
n=6
d) treatment comparison
TCDD minus E2
TCDD minus Cotreatment
E2 minus Cotreatment
n=4

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Detection of a novel population of fetal thymocytes characterized by preferential emigration and a TCRγδ+ T cell fate after dioxin exposure

Marc Majora[a], Markus Frericks[a], Vladimir Temchura[a], Gaby Reichmann[b], Charlotte Esser[a,*]

[a]Institute for Environmental Medical Research (IUF) at the Heinrich-Heine University of Düsseldorf, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany

[b]University of Düsseldorf, Institute for Medical Microbiology, Düsseldorf, Germany

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Abstract

T cell maturation into TCRαβ^{+} or TCRγδ^{+} cells from common immature CD4^{-}CD8^{-} (DN) precursors occurs in the thymus, and is controlled through ordered regulation of genes. The aryl hydrocarbon receptor (AHR), a latent cytoplasmic transcription factor, affects thymocyte maturation and differentiation at several stages, also including DN cells. We analyzed in murine fetal thymus organ cultures (FTOC) the outcome of AHR-signaling and found a higher frequency of DN TCRγδ^{+} cells in the presence of the AHR-activating ligand TCDD. We detected a novel population of CD25$^{low/lo}$CD44^{hi} cells associated with preferential emigration and a TCRγδ^{+} T cell fate of thymocytes. Sorted DN TCRγδ^{+} emigrants could proliferate if IL-2 was available. Moreover, they suppressed the proliferation of co-cultivated, activated CD4^{+} T cells. Gene expression profiles of purified DN emigrants from TCDD*FTOC revealed 295 modulated genes, 10% of which are genes of the immune system. For instance, RAG-1, TdT, and Gfi-1 were downregulated, yet genes indicative of mature thymocytes were upregulated. In conclusion, we have detected changes in the differentiation programme of fetal DN thymocytes after ligand-activation of the AHR. In particular, we observed a higher frequency of DN TCRγδ^{+} cells with high emigration potential, and possible regulatory functions.

Keywords: Thymus; Lineage commitment; Emigration; TCDD; Arylhydrocarbon receptor

Abbreviations: AHR, aryl hydrocarbon receptor; DN, CD4^{-}CD8^{-} double negative; DP, CD4^{+}CD8^{+} double positive; DRE, dioxin-responsive element; FTOC, fetal thymus organ culture; SP, single positive; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD*FTOC, FTOC treated with TCDD.

* Corresponding author. Tel.: +49 211 3389 253; fax: +49 211 3190 910.
E-mail address: chesser@uni-duesseldorf.de (C. Esser).

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1. Introduction

T cells develop in the thymus from precursors along a tightly controlled, sequential differentiation pathway. Timing and control of lineage commitment towards the two functionally distinct T cell subsets, bearing either the TCRαβ or γδ, are not yet clear. The earliest precursor cells in the thymus are c-kit$^+$CD44$^+$ CD25$^-$ CD4$^-$ CD8$^-$ (DN1[4] cells). They upregulate CD25 to become c-kit$^+$CD44$^+$CD25$^+$CD4$^-$ CD8$^-$ (DN2) cells. DN2 cells are still capable of developing into thymic dendritic cells and, albeit with poor efficiency, into NK cells [1,2]. Rearrangement of the γ,δ, or the β TCR genes, and thus the commitment to the T cell lineage, starts at the transition from the DN2 to the DN3 stage [3,4]. DN2 cells can be divided into IL-7R$^-$ and IL-7R$^+$ cells. The latter have a higher potential towards TCRγδ$^+$ cells, and TCRγδ$^+$ cells are dependent on IL-7/IL-7R and its intracellular signaling by Jak3 [3,5–7]. Rearranged β chains can be found in TCRγδ$^+$ cells, and rearrangement of the TCRγ and TCRδ loci is found in most TCRαβ$^+$ cells [8], (i.e. lineage decision seems to be independent of TCR expression). Yet, rearrangement and expression of the wrong TCR in finally committed cells leads to apoptotic cell death [3,9]. Apparently, the αβ and γδ precursors are not sequentially related, but seem to be alternatives, first developing side by side, then finally separating. Signaling via Jak3 is critical for development of TCRαβ$^+$ as well as TCRγδ$^+$ cells, but is continuously required for the maintenance of the γδ T cell lineage [6]. Additionally, a bias for the αβ T cell lineage is in part due to compromised survival and expansion of TCRγδ$^+$ cells, apparently caused by bcl-2 deficiency [7]. In contrast to the adult thymus, TCRγδ$^+$ cells predominate in the fetal thymus; the shift from pre- to post-natal development is characterized by successive waves of distinct Vγ genes used in rearrangement. The regulation of these developmental changes are not quite clear, albeit Notch1 is essential during a defined developmental window [10].

The AHR is a latent ligand-activated transcription factor, which is abundant in the thymus, thymus stromal cells, and many other tissues [11,12]. The AHR is an orphan receptor of the basic helix–loop–helix Per/ARNT/Sim homology (PAS-bHLH) family, whose members are involved in e.g. rhythm coordination, neurogenesis, and lineage specific transcription [11]. Similar to steroid receptors and many orphan receptors, the AHR becomes activated by specific low molecular weight ligands. A well-studied function of the AHR is ligand-dependent induction of the enzymes, which catalyze oxidative biotransformation of non-polar substances, such as aromatic hydrocarbons. The most efficient AHR-activating ligand is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a planar aromatic hydrocarbon, which has been extensively used to study the receptor and its toxicological implications. TCDD is produced accidentally during industrial processes, including the production of Agent Orange, the herbicide used during the Vietnam War. TCDD mediates its many toxic effects, including cancer, via the AHR. It is still considered a pollutant and health risk of considerable concern. Observations in AHR-deficient mice suggested a role for the AHR in the development of the immune system, the liver, vascular remodeling, and aging processes [13]. The large number of AHR target genes, which are involved in cell differentiation, cell cycling, cell activation, and the recent identification of possible endogenous ligands [14–17] support a role for the AHR in cell functions not connected to its well-known role in biotransformation of external substances.

Whether or not a gene becomes a direct target of the activated AHR is dependent on the presence of so-called DREs in its promoter, and on cell type and cell differentiation stage, i.e., overall accessibility of a particular locus [18]. In addition activation of the AHR can directly target genes via secondary effects. Genes inducible, whether directly or indirectly, by the AHR in thymocytes are, among others, Notch-1, IL-2, bcl-2, CD44 and adseverin [19–22] all of which are known for their role in thymocyte differentiation and survival.

Activation of the AHR changes thymocyte differentiation pathways at several checkpoints [23–25]. As early as 1992, a preferential generation of TCRγδ$^+$ cells was noted in thymocytes of fetuses treated in utero with an AHR ligand; a block at the transition phase from CD4$^-$ CD8$^+$CD24$^+$ to CD4$^+$CD8$^+$ was suggested [26]. Further studies showed that in particular the proliferation of CD24$^+$ DN3 and DN4 thymocytes is diminished within 9–12 h after injection of TCDD into C57BL/6 mice, probably causing the well-known thymus atrophy within 10 days [27]. We

recently found that fetal CD44^{+}TCRαβ^{-} DN cells preferentially emigrate in FTOC treated with TCDD [28]. In agreement with that, studies in one line of AHR-deficient mice revealed a reduced peripheral lymphocyte count, indirectly suggesting participation of the AHR during emigration of T cells from the thymus [13]. The differentiation stage of these aberrantly emigrated fetal DN cells, or any possible function, e.g. in connection to the well-established systemic immunosuppression after AHR overactivation are unknown.

We analyze here the impact of TCDD on the differentiation, function, and gene expression profile of DN fetal thymocytes and thymic emigrants with a particular emphasis on TCRγδ^{+} cells.

2. Material and methods

2.1. Fetal thymus organ cultures (FTOC)

Fetal thymus lobes from gestational day 15 C57BL/6 mice were cultivated for up to twelve days as described previously [29]. 10 nM 2,3,7,8-TCDD was added throughout the culture. Control cultures received solvent only. Emigrants were collected by gently rinsing them off the lobes on the culture filters with PBS as described before [28]. Thymocytes were obtained by homogenizing the lobes.

2.2. In vivo treatment of mice

Pregnant dams were injected intraperitoneally with 5 μg/kg TCDD diluted in olive oil on gestational day 15. Control mice received olive oil. After birth, pups were sacrificed and thymus, spleen and blood were collected. Erythrocytes were lysed in spleen and blood cell preparations.

2.3. Immunofluorescent staining, flow cytometric analysis and cell sorting

The following antibodies were obtained from Pharmingen (Heidelberg, Germany): anti-CD3 (clone 145-2C11), anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD24 (clone M1/69), anti-CD25 (clone PC61), anti-CD44 (clone IM7), anti-CD45RB (clone 16A), anti-CD62L (clone MEL-14), anti-CD69 (clone H1.2F3), anti-c-kit (clone 2B8), anti-TCRβ (clone H57-597), anti-TCRγδ (clone GL3).

Sorting of cells was done by magnetic cell sorting with MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). DN emigrants or thymocytes were obtained by depletion of CD4^{+} and CD8^{+} cells using anti-CD4 and CD8 microbeads. TCRγδ^{+} and TCRγδ^{-} DN emigrants were purified by incubation with anti-TCRγδ^{biotin} Ab, followed by labeling with anti-biotin microbeads before sorting. As APC irradiated Thy1.2^{+} depleted splenocytes were used. Splenic CD4^{+} T cells were also sorted with MACS. Sort purity was controlled flow cytometrically.

Ab-labeled cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, USA), using live scatter gates. Data analyses were performed with CellQuest™ or WinMDI™ software.

2.4. Co-cultivation experiments

1×10^4 DN emigrants were added to 5×10^4 APCs and 2×10^4 CD4^{+} T cells in a 96-well plate, and cultivated for 90 h. Anti-CD3 antibodies were added at 1 μg/ml. As positive control, cells were stimulated with PMA (10 ng/ml) and Ionomycin (500 ng/ml, Sigma, Taufkirchen, Germany). For some experiments, IL-2 was added to the co-culture at a final concentration of 2 ng/ml. Proliferation was measured by incorporation of ^{3}H-thymidin for the last 20 h of the culture.

2.5. Proliferation measurement with CFSE

Splenic CD4^{+} T cells or DN emigrants were labeled with 0.5 μM CFSE (Molecular Probes, Leiden, The Netherlands). Cells were co-cultivated as described above. After 90 h of cultivation cells were stained for CD4 (CD4^{+} T cells) or CD4/CD8 and TCRγδ (DN emigrants), and the CFSE-pattern was analyzed flow cytometrically.

2.6. RNA-preparation and amplification

Total RNA was isolated with Trizol™ (Invitrogen, Karlsruhe, Germany). Linear polyacrylamid was added as a precipitation aid [30]. RNA was amplified prior to chip-hybridization with the MessageAmp™

Kit of Ambion (Woodward St. Austin, USA). To exclude amplification bias, RNA-species of 8 genes were quantified before and after amplification, and found not to differ (data not shown). RNA was biotinylated with the Enzo Bio Array™ HighYield™ RNA transcript labeling kit (Affymetrix, High Wycombe, UK) and purified.

2.7. Chip-array hybridization

RNA was hybridized to the MO430A gene chip (22,500 genes). Normalization and analysis of microarray data was performed with the *affy-package* in the statistical software R. Expression change was stated if the change in expression was at least 2-fold between control and treatment RNA and changes in expression were identified in two independent experiments. Results were verified by Light-Cycler PCR with 6 selected genes.

2.8. Cytokine ELISA

5×10^4 purified either TCDD or solvent-treated DN emigrants were cultivated with anti-CD3 antibodies [1μg/ml] and IL-2 [2 ng/ml]. Supernatants were collected after 72 h. A sandwich ELISA for IFN-γ (mAB R4-6A2 and XMG1.2^{biotin}) and IL-4 (11B11 and BVD6-24G2^{biotin}) was performed. The minimum sensitivity was 100 pg/ml (IFN-γ) or 20 pg/ml (IL-4).

3. Results

3.1. The output of fetal thymus lobes is biased towards DN cells by TCDD

FTOCs have been used intensively to analyze thymocyte differentiation processes [29,31]. Recently, they were shown to be also a valid system to study emigration [32]. We have used this system and, as we had already reported in a previous paper, the absolute output of fetal thymic emigrants is reduced after TCDD-treatment by about 50% [28,33]. As shown in Fig. 1 and Table 1, the output of cell subsets varied. Surprisingly, at day 6 of FTOC not only SP cells emigrated as might be expected [32], but also DP cells. Indeed, DP emigrants predominated, even more in control FTOC than in TCDD*FTOCs. In order to rule out that the appearance of DP emigrants is an artifact of our culture system, we injected pregnant mice i.p. with 5 μg/kg body weight TCDD or solvent on gestational day 15. We analyzed the distribution of CD4 and CD8 subsets in thymus, spleen and peripheral blood of one day old pups. While spleens of these mice contained virtually no DP cells (yet some CD4 and CD8 SP cells), we found that 43% of the lymphocytes in the blood were DP in control mice (Table 2). This result confirms and extends work of Bonomo et al. who detected significant numbers of DP cells in the peripheral lymph nodes of 3 day old mice, and showed that they were thymus derived [34]. 15% DP cells were found in the blood of those pups exposed in utero, paralleling the reduced frequency of DP cells in the thymocytes found in TCDD*FTOC (compare Fig. 1). Overall, CD4/CD8 subpopulations differ substantially between control FTOC and TCDD*FTOC. In the latter, DP cells were much rarer in frequency, as well as in absolute numbers, among thymocytes and emigrants. On the other hand, DN cells were much more abundant in frequency. Considering that the absolute number of thymocytes was reduced by 60% upon TCDD treatment throughout the culture (Table 1), we normalized the number of emigrants in relation to the total thymocyte number (per 1×10^5 thymocytes) to obtain the effective thymic output. As shown in Fig. 1, the effective thymic output of DN cells is always higher in TCDD*FTOC compared to the control. The difference was most drastic on day 6, but DN cells also remained the dominant emigrants in TCDD*FTOC at the later points in time. Even at day 12, when CD4 and CD8 cells were the major emigrant subpopulations in control FTOC (in frequency and number), they were found at suspicious abundance in TCDD*FTOC (Fig. 1). The effect is not caused by augmented apoptosis of any CD4/CD8 thymocyte or emigrant subset, as in all cases >80% were negative for annexinV and propidium iodide staining (data not shown). In contrast to the changes found for the emigration of DN and DP cells, the effective thymic output of CD4 or CD8 cells was not affected upon TCDD treatment throughout the culture.

Thus, apart from SP also DN and DP cells emigrated from fetal thymus. Moreover, emigration of DN cells is promoted by AHR signaling. The

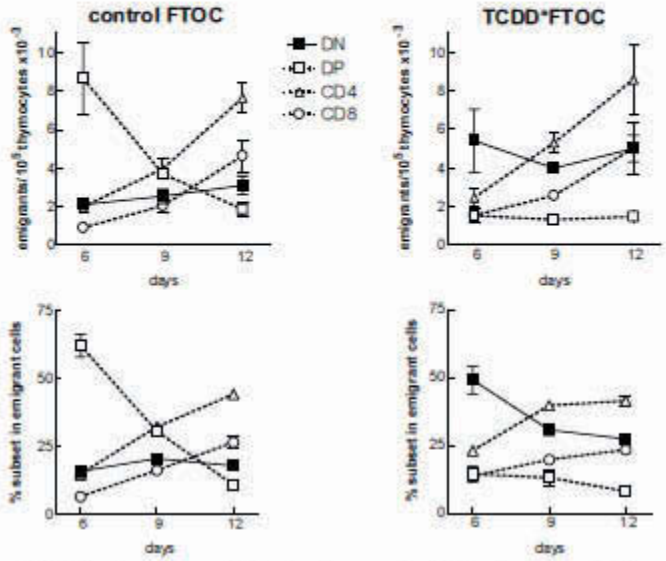

Fig. 1. Overactivation of the AHR leads to an increased effective thymic output of fetal DN thymic emigrants. Fetal thymus organ cultures from gestation day 15 thymi were exposed to 10 nM TCDD/solvent or solvent alone. After 6, 9 or 12 days thymocytes and emigrants were isolated and stained for CD4 and CD8. The percentages and absolute numbers of the different subsets were determined. Due to the fact that TCDD decreases the number of thymocytes and emigrants by 60%, and that emigration directly correlates with the number of thymocytes, the effective thymic output was calculated to normalize the number of emigrants per 1×10^5 thymocytes. The data represent the results of three independent experiments.

effect is significant as calculated by student's *t*-test ($p=0.022$ (day 9) or $p=0.0017$ (day 12) for the effective thymic output of DN emigrants in TCDD*FTOC vs. control FTOC).

3.2. TCDD and TCRγδ cell development

Early studies suggested the induction of a block in thymocyte development by TCDD at the DN stage

Table 1
Absolute numbers of CD4 and CD8 subpopulations in thymocytes and emigrants per thymus lobe*

		Total		DN		DP		CD4		CD8	
		control	TCDD	control	TCDD	control	TCDD	control	TCDD	control	TCDD
TL	d6	57.0±10.4	19.0±3.7**	7.3±2.7	2.8±0.4	35.4±6.9	7.1±2.2**	8.4±1.7	3.6±0.9*	6.0±1.5	5.4±0.8
	d9	51.0±8.3	18.0±2.4**	3.9±0.5	2.0±0.1**	24.5±6.7	2.3±0.3**	14.2±1.0	6.2±1.5**	8.8±0.6	7.5±0.7
	d12	38.0±5.0	14.0±2.1**	4.2±0.4	2.2±0.6*	13.3±2.4	1.2±0.2**	9.9±1.2	4.6±0.8**	10.3±1.2	5.9±0.6**
Emigrants	d6	7.5±1.3	1.9±0.3**	1.2±0.1	0.9±0.2	4.8±1.2	0.3±0.1**	1.1±0.0	0.4±0.1***	0.5±0.1	0.3±0.1*
	d9	6.2±0.7	2.4±0.5**	1.3±0.2	0.7±0.1*	1.9±0.1	0.2±0.0***	2.0±0.3	1.0±0.2*	1.0±0.1	0.5±0.1*
	d12	6.4±0.4	2.7±0.5**	1.1±0.1	0.7±0.0**	0.7±0.2	0.2±0.0*	2.8±0.3	1.1±0.2**	1.7±0.2	0.6±0.1**

* Fetal d15 thymi were cultivated with TCDD or solvent. After 6, 9 or 12 days emigrants and thymocytes were isolated and stained for CD4 and CD8. Absolute numbers of the cell subsets were calculated based on the percentages and total numbers of thymocytes and emigrants. Numbers are given as cells per lobe ×104 (±SD) from n=3 independent experiments. Statistical calculations were performed by using Student's t-test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to the corresponding control).

Table 2
Frequency of DP cells in thymus, blood and spleen of one day old mice[a]

Tissue[b]	%	
	Control	TCDD
Thymus	89	85
Spleen	43	15
Blood	<1	<1

[a] Pregnant mice were injected intraperitoneally with 5 μg/kg TCDD diluted in olive oil on gestational day 15. Control mice received olive oil only. One day after birth neonates were sacrificed and thymus, spleen and blood was collected. After elimination of erythrocytes and treatment with Fc-block cells were stained with anti-CD4 and CD8 antibodies and frequencies of DP cells were determined.

[b] Tissues were pooled for analysis from $n=3$ (controls) and $n=5$ (TCDD) mice.

[26]. Recently, we have shown that fetal DN emigrants do not contain significant amounts of TCRαβ$^+$ cells after 6 days of FTOC [33]. Because the fetal thymus is an important source of TCRγδ$^+$ cells [35], we determined whether the high frequency of DN emigrants isolated from TCDD*FTOC is due to TCRγδ$^+$ cells. As shown in Fig. 2, a high percentage of DN cells were indeed TCRγδ$^+$ T cells, inside the lobes or outside, respectively. The frequency was always significantly higher in TCDD*FTOC than in control FTOCs (Fig. 2A), going up to 70% by day 9 and 12 of culture. However, in absolute numbers, emigrated DN TCRγδ$^+$ cells did not differ between TCDD*FTOC and control FTOC (Fig. 2B). Considering (a) that the effective output of DN cells is increased in TCDD*FTOC, and (b) that the frequency of TCRγδ+cells among DN emigrants from TCDD*FTOC is always higher compared to the control FTOCs, our findings reflect a preferential output of DN TCRγδ+cells in fetal thymi exposed to an AHR activating ligand.

3.3. TCDD confers a novel CD25$^{low/int}$CD44^{hi} phenotype to both TCRγδ and TCRγδ$^+$ DN thymocytes and emigrants, indicating high potential for emigration

We analyzed the expression pattern of TCRγδ, CD25 and CD44 of highly purified DN thymocytes or emigrants from TCDD*FTOC and control FTOC. Control FTOC yielded the distribution known from many publications of cells from the DN1 to DN4 stages, with a majority of cells in DN3 and DN4. In addition, we found a population characterized as CD25$^{lo/int}$CD44^{hi} with low frequency in DN TCRγδ cells from control FTOC, yet high frequency in cells from TCDD*FTOC (compare Fig. 3A vs. B). This population is distinct from CD25$^+$ CD44$^+$ DN2 cells, and could be found in all FTOCs, albeit at different frequencies. In the following, we refer to this population as DN1b. As shown in Fig. 3A and B, exposure to TCDD induced a partial developmental block after the DN1b stage, leading to the accumulation of TCRγδ thymocytes with this phenotype (38.4% in TCDD-treated thymocytes vs. 5.8% in control). The absolute number of DN1b cells was also increased by TCDD (Fig. 4), i.e., the high frequencies do not result from a shift in other subpopulations.

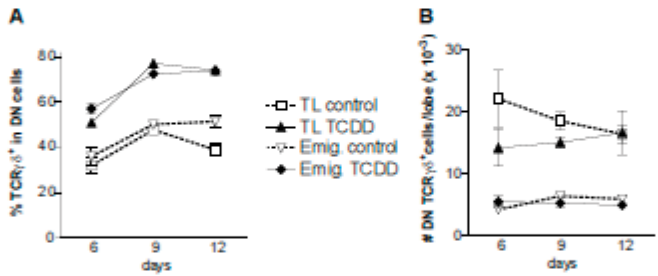

Fig. 2. Frequency (A) and absolute cell count (B) of DN TCRγδ$^+$ emigrants in FTOC, incubated either with 10 nM TCDD or solvent alone. At the indicated days thymocytes and emigrants were isolated, counted and stained for CD4, CD8 and TCRγδ. TL: cells within the thymic lobes. The difference in absolute cell count on day 6 between cells in the TCDD TL vs. control TL is not significant by Student's t-test.

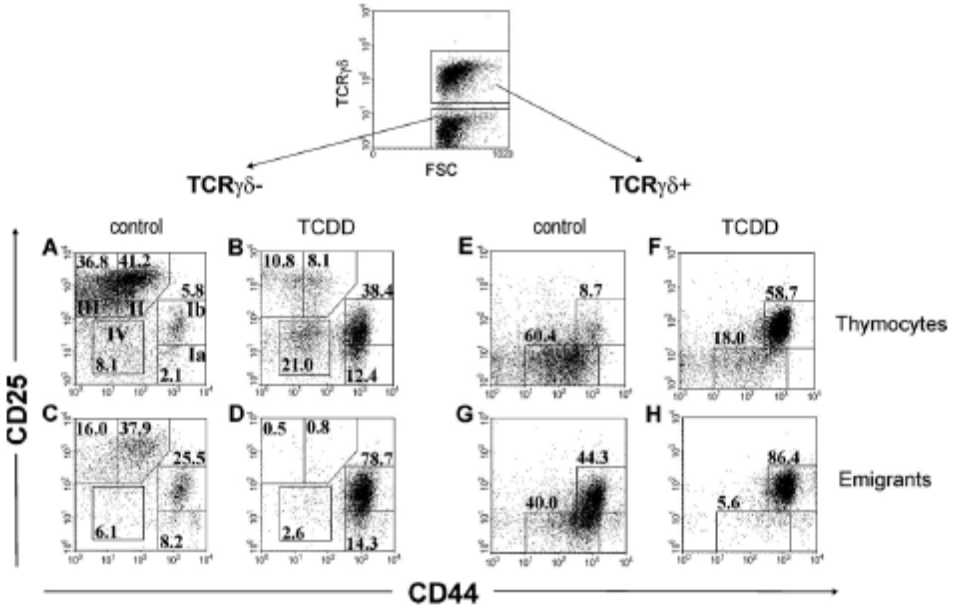

Fig. 3. Overactivation of the AHR leads to the enrichment of DN CD25$^+$CD44$^+$ TCRγδ$^+$ and TCRγδ$^-$ cells in the thymus and facilitates their emigration. Thymocytes from FTOCs, incubated either with TCDD or solvent, were isolated at day 6. DN cells were sorted (purity ≥ 99%), and stained for CD25, CD44 and TCRγδ. Numbers in the plots indicate the percentage of cells found in the respective gate. One representative experiment out of three is shown.

While DN TCRγδ emigrants from control FTOC have considerable amounts (more than 50%) of DN2, DN3, and DN4 cells, in TCDD*FTOC these more mature developmental stages were virtually absent, and nearly 80% were DN1b cells (Figs. 3C,D and 4).

In agreement with data from the literature, we found that most fetal DN TCRγδ$^+$ cells are CD25 CD44$^{lo/int}$ [36,37]. Thus, upregulation of CD25 is unlikely to indicate progression of DN1 cells along the TCRγδ pathway. Rather, the block at the DN1b stage is associated with the potential for emigration, as a large majority of TCRγδ$^+$ and TCRγδ cells found as emigrants were DN1b.

The expansion of pro-thymocytes is driven in a synergistic fashion by the common γ chain, and by c-kit (CD117) [38]. The latter is lost after β-selection. When we analyzed TCRγδ thymocytes and emigrants for c-kit, we found that more DN TCRγδ thymocytes expressed c-kit after TCDD exposure (Fig. 5A). Expression levels were higher after emigration, even in the absence of TCDD (Fig. 5B), reaffirming the block in early DN. It is unclear at which time-point fetal/neonatal TCRγδ$^+$ cells start to re-express c-kit [39]. TCRγδ$^+$ intraepithelial lymphocytes in the gut express c-kit, while splenic TCRγδ$^+$ cells do not. TCRγδ$^+$ cells are c-kit negative in the thymus of newborn mice [39]. In our hands, most TCRγδ$^+$ cells from fetal day 6 TCDD*FTOC (i.e. the time-point correlated to birth) expressed c-kit at intermediate levels in the thymus, and expression levels remained unchanged after emigration (Fig. 5C,D). Only a small percentage of TCRγδ$^+$ cells expressed c-kit at equally high levels as TCRγδ cells.

A similar picture emerges with respect to a number of other markers of maturity, i.e. DN TCRγδ cells express markers characteristic of immature DN cells, whereas expression patterns characteristic of an activated, mature phenotype (see Table 3) are found

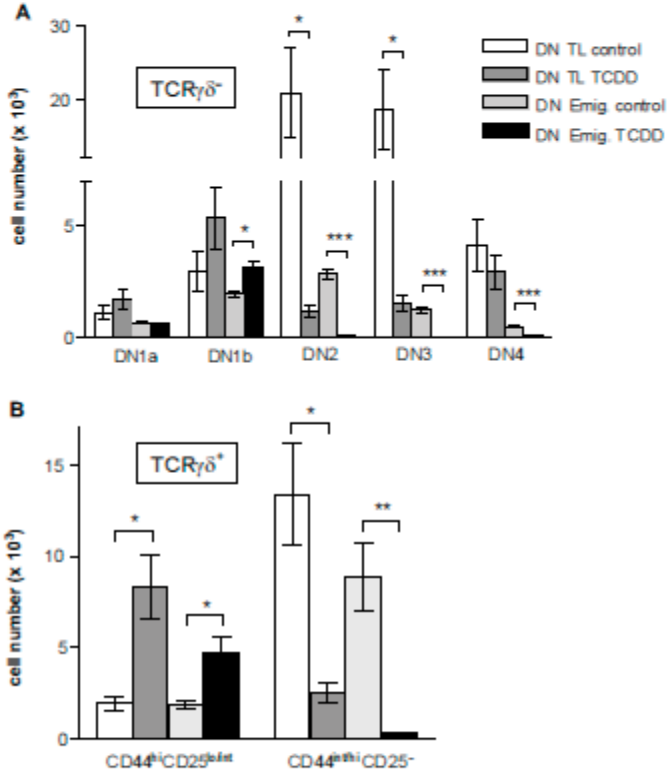

Fig. 4. Absolute numbers of the different CD25 and CD44 expressing populations among DN TCRγδ⁻ and TCRγδ⁺ cells. Thymocytes and emigrants of control and TCDD*FTOC were isolated after 6 days and frequencies of CD4 and CD8 expressing cells were determined by FACS analysis. DN thymocytes and emigrants were sorted and analyzed for expression of CD25, CD44 and TCRγδ. Percentages were used to calculate absolute numbers of the subpopulations of TCRγδ⁻ cells. Bars show mean values ± SEM of three independent experiments. Statistical calculations were performed by using Student's *t*-test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to control).

at higher frequencies on DN TCRγδ^{l} cells. For instance, expression of CD24, a marker for immature DN thymocytes [40], was decreased drastically on DN TCRγδ cells by TCDD, and was virtually absent on DN TCRγδ^{l} thymocytes and emigrants. Likewise, the frequency of CD62L and CD45RB expressing DN cells decreased in TCDD*FTOC, in both thymocytes and emigrants. On the other hand, expression of the early activation marker CD69 was upregulated by TCDD, especially pronounced on TCRγδ^{l} emigrants. Thus, while DN TCRγδ thymocytes had the phenotype of immature cells as expected, TCDD altered this phenotype, towards the characteristics of mature cells. With respect to DN TCRγδ^{l} cells, the low expression of CD62L, and the high expression of CD69 by TCDD in the emigrants is noteworthy.

3.4. Emigrated DN TCRγδ^{l} cells possess suppressive capacities through competition for IL-2

Suppression of both cellular and humoral immune responses is one of the hallmarks of TCDD-mediated

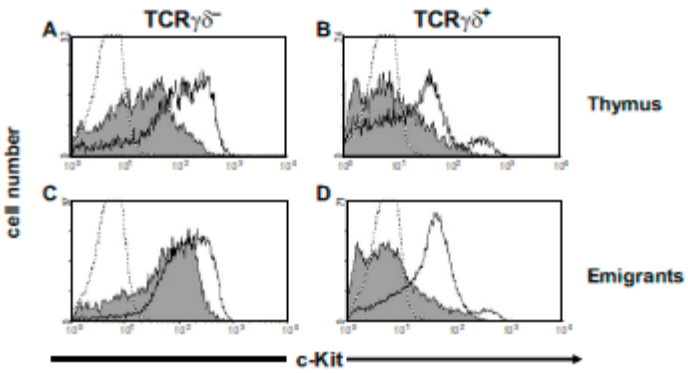

Fig. 5. c-kit expression of DN TCR-γδ+ and TCR-γδ− cells from day 6 FTOCs. Isotype control (dotted line), cells from control FTOC (shaded grey area), cells from TCDD exposed FTOC (black line). The experiment was repeated twice.

activation of the AHR. The underlying mechanism is largely unknown. Because some mature subpopulations of DN cells have regulatory capacities [41], we wanted to analyze if the fetal DN emigrants also possess a suppressive potential in co-cultivation with activated CD4+ T cells. While DN emigrants were unable to proliferate in the presence of APCs, anti-CD3 and even PMA and ionomycin, cultivation with CD4+ T cells together with APCs and anti-CD3 unexpectedly led to a significant increase, rather than a decrease, in total proliferation (Fig. 6A). Similar increased proliferation was found irrespective of whether DN emigrants isolated from TCDD*FTOC or from control cultures were used. To analyze which population in the co-culture, DN or CD4+ T cells, proliferated, we labeled CD4+ T cells with CFSE and cultivated them with DN emigrants, and vice versa. While anti-CD3/APC activated CD4+ T cells divided well in the absence of DN emigrants in the culture (> 60% undergo 6 divisions and more), addition of DN emigrants reduced CD4+ T cell proliferation by 1–2 rounds within the culture period of 90 h. Vice versa, CFSE-staining revealed that DN emigrants proliferated only in the presence of activated CD4+ T cells. Thus, while the overall outcome of co-cultivation was increased proliferation, activated CD4+ T cells were indeed inhibited in growth by the DN emigrants, which were responsible for the observed increase in proliferation in co-culture (data not shown). Addition of IL-2 could replace CD4+ T cells, and TCRγδ+ proliferated equally well (Fig. 6A). As might be expected considering the high frequency of TCRγδ+ cells in DN emigrants, they produced IFN-γ, but little IL-4 if cultivated in the presence of anti-CD3 and IL-2

Table 3
AHR overactivation induces an activated phenotype on DN thymocytes and emigrants[a]

Activation marker	DN TL control		DN TL TCDD		DN emig. control		DN emig. TCDD	
	TCRγδ+	TCRγδ−	TCRγδ+	TCRγδ−	TCRγδ+	TCRγδ−	TCRγδ+	TCRγδ−
CD69	30	19	66	37	33	15	81	59
CD24	46	89	4	27	17	63	0	4
CD45RB^{high}	4	0.4	6	5	3	8	7	14
CD45RB^{low}	28	84	6	22	18	68	4	28
CD62L	38	77	3	25	25	62	13	6

[a] Fetal d15 thymi were cultivated with TCDD or solvent. After six days emigrants and thymocytes were isolated and stained for CD4, CD8, TCRγδ in addition to one of the above mentioned markers. Values represent the percentages of TCRγδ+ or TCRγδ− cells positive for the expression of the respective marker. The experiment was done twice with similar results. Only results of one experiment are shown.

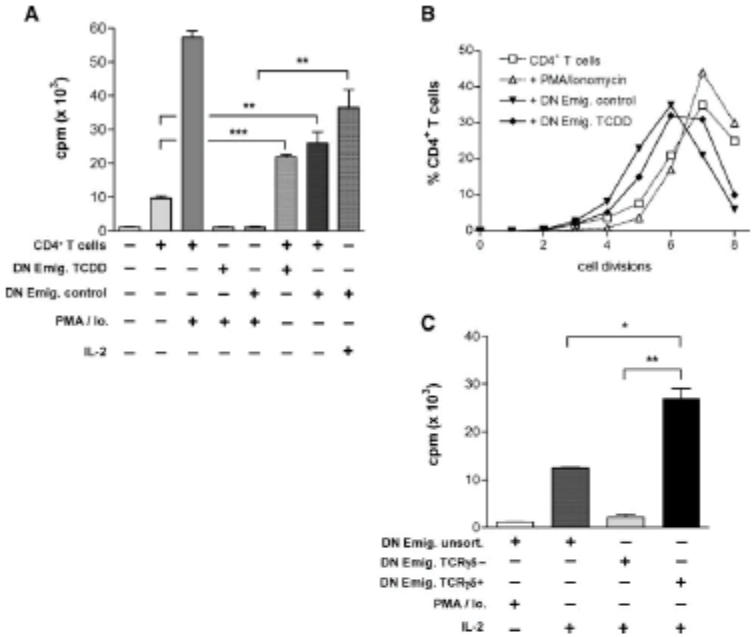

Fig. 6. Proliferation of DN emigrants in co-culture with CD4⁺ splenic T cells. DN cells were sorted with magnetic beads. Cells were cultivated for 90 h together with CD4⁺ splenic T cells. APCs and anti-CD3 Ab [1 μg/ml] were always present. PMA [10 ng/ml] / Ionomycin [0,5 μg/ml], or IL-2 [2 ng/ml] were added as indicated. Bars show mean values ± SEM. Statistical calculations were performed by using Student's *t*-test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to control). (A) ^3H-thymidine incorporation is shown as a measure for proliferation. The ^3H-thymidine was added for the last 20 h of the culture. (B) CD4⁺ T cells were stained with CFSE before co-cultivation, and the percentage of cells, which had divided for a given number determined. (C) Proliferation of sorted DN TCRγδ⁺ and TCRγδ⁻ emigrants. One representative experiment out of three is shown. All samples were tested in triplicate.

for 72 h. DN emigrants from TCDD*FTOC produced slightly, but significantly more IFN-γ and about four times more IL-4 than DN emigrants from control FTOCs (Fig. 7), probably reflecting the higher percentage of TCRγδ$^+$ cells in this population (Fig. 2).

Because DN emigrants contained both TCRγδ$^+$ and TCRγδ$^-$ cells, it was important for us to determine if only one of these populations possesses the property to grow and inhibit proliferation of CD4$^+$ T cells. We therefore separated these subpopulations and analyzed their proliferation by adding APC, anti-CD3 Ab and IL-2. As shown in Fig. 6C, TCRγδ$^-$ emigrants showed virtually no proliferation, while the TCRγδ$^+$ fraction proliferated strongly. Also, analysis of unsorted DN emigrants after 90 h of co-culture with CD4$^+$ T cells revealed, that at the end of the culture all of the proliferating DN emigrants expressed the TCRγδ (data not shown).

In conclusion, DN TCRγδ$^+$ emigrants can be induced to proliferate in the presence of activated CD4$^+$ T cells or IL-2, independently of costimulation by APCs and they are capable of suppressing the proliferation of activated CD4$^+$ T cells.

3.5. Gene expression profile of DN emigrants from fetal thymi after long-term signaling mediated by TCDD

The AHR is a transcription factor, which has been shown to target many genes of different functional

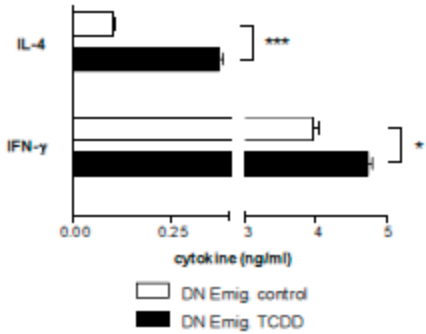

Fig. 7. Cytokine production by activated DN emigrants. Fetal d15 thymi were incubated with either TCDD or solvent. After six days, DN emigrants were isolated and 5×10^4 cells incubated with anti-CD3 Ab [1 μg/ml] and IL-2 [2 ng/ml]. Supernatants were tested after 72 h for IFN-γ and IL-4 with ELISA. Bars show mean values ± SEM. Statistical calculations were performed by using Student's *t*-test (*$p \leq 0.05$, ***$p \leq 0.001$ compared to control).

groups. Gene expression changes are cell type and cell stage specific [22]. In order to define the expression profile of the thymocyte subpopulation under consideration here, we sorted DN emigrants from TCDD*FTOC and control FTOC and analyzed their RNA content on Affymetrix™ gene chips. Choosing a twofold change in expression as cut-off-threshold level, we identified 295/22,500 transcripts (i.e. 1.31% of the transcriptome) as differentially modulated in TCDD exposed DN emigrants. A selection of regulated genes is shown in Table 4. Additionally, the results were verified for some genes by using Light-Cycler PCR. It has to be mentioned here that it is quite difficult to compare the results of microarrays and Light-Cycler due to the technical differences of the methods leading to more or less pronounced deviations. The genes we found are involved in the control of various cellular functions including cell adhesion, cell cycle, metabolism, intracellular signaling, cell growth/development, and transcription. One of the largest single "functional" group of genes regulated in the DN emigrants from TCDD*FTOC, comprising almost 10%, are transcripts of the immune system. The genes include CD-markers for lymphocytes, chemokines, interleukins, integrins, or recombination associated genes (Table 4). Although the cut-off threshold was chosen as two-fold, the majority of genes related to the immune system was modulated more strongly, i.e. at least three-fold. Particularly striking was the downregulation of RAG (recombinase-activating gene)-1 by TCDD, which was confirmed by PCR. Concomitant to this, we also detected a striking downregulation of the terminal deoxynucleotidyl transferase (TdT). Both genes are necessary for gene rearrangement of the TCR-genes. Likewise, expression of c-myb, a gene whose presence is required for the transition from DN3 to DN4 thymocytes [42], was downregulated approximately 30-fold by activation of the AHR. Gfi-1, a putative transcriptional repressor involved in the maturation of immature thymocytes [43], was downregulated in DN emigrants from TCDD*FTOC as well. Notch-1, which might have been expected to be changed, was expressed but not modulated between emigrants from TCDD*FTOC and control FTOC (data not shown).

Detectable also as changes in surface expression, the thymocyte maturation marker genes for c-kit and CD24 were up- or downregulated, respectively (compare Tables 3 and 4). In contrast, IFN-γ expression was downregulated in DN emigrants from TCDD*FTOC compared to control FTOC albeit the protein was secreted more strongly in cultivated DN emigrants cells (compare Fig. 7).

Entering and going through the cell cycle are important checkpoints in thymocyte differentiation. Not surprisingly, in DN emigrants from TCDD*FTOC also genes controlling the cell cycle, were targets of the AHR, e.g. retinoblastoma (Rb)-related p130 (2.0/2.1-fold up), cdc6 (3.7/3.2-fold down), E2F transcription factor 6 (2.0/2.6-fold down), or cyclins B1 and E2 (> 3-fold down). It is important to note, though, that a computer analysis of 1500 base pairs upstream of the transcription start sited ("promoter") revealed that only about 50% of the modulated genes had a nominal dioxin-responsive element in that region, suggesting secondary signaling effects of TCDD (Table 4).

4. Discussion

The thymus is a sensitive target for TCDD-mediated toxicity. Besides the two well-known effects thymus atrophy and systemic immunosuppression, also differentiation of thymocytes is affected, leading

Table 4
TCDD-mediated gene regulation in DN emigrants[a]

Function[b]	Gene name	Fold change				Full length DREs[c]
		Microarray		Light-Cycler		
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	
Immune system	CD24	−11.5	−9.2	−5.2	−7.9	0
	CD28	−3.0	−2.7	–	–	1
	CD160	2.6	2.9	–	–	1
	c-Kit	3.8	2.8	–	–	0
	c-myb	−25.0	−36.6	–	–	0
	Gfi1	−2.6	−7.8	−3.4	−4.0	1
	ICOS	2.3	3.3	–	–	ND
	IFN-γ	−6.7	−3.7	–	–	1
	IL-22	8.8	9.9	9.9	6.1	ND
	NK cell lectin like receptor B1B	2.6	3.7	–	–	ND
	NK cell lectin like receptor B1D	2.2	5.5	–	–	ND
	RAG-1	−66.7	−86.3	−16.6	−3.3	0
	TdT (terminal deoxynucleotidyl transferase)	−24.2	−82.0	−12.8	−7.5	1
Cell cycle control	Cell division cycle 6 (Cdc6)	−3.7	−3.2	–	–	0
	Cyclin B1	−3.7	−3.4	–	–	ND
	Cyclin E2	−4.1	−3.2	–	–	ND
	E2F6 transcription factor	−2.0	−2.6	–	–	0
	Growth arrest specific 5	−2.3	−2.1	–	–	0
	p130	2.0	2.1	–	–	1
AHR gene battery[d]	AHR-Repressor	14.3	13.4	–	–	0
	CYP1A1	2.8	4.4	16.5	20.8	3
	CYP1B1	4.3	18.7	–	–	3
Housekeeping genes	HPRT	0	0	0	−0.1	ND
	RPS6	0	0	0.1	0.1	ND

[a] Fetal d15 thymi were treated with TCDD or solvent. After six days emigrants were isolated and sorted for DN cells by magnetic depletion of CD4 and CD8-expressing cells. RNA of DN emigrants was isolated and prepared as described above. Gene expression profile was analyzed by using Microarray technique and verified for selected genes by Light-Cyler PCR where indicated. Numbers represent the change in expression induced by TCDD.

[b] Genes were grouped according to function.

[c] Gene sequences were analyzed for DREs up to −1500 base pairs relative to the transcription start site by using Tess Transfac 4.0 software. The corresponding sequence motif was "KNNKNNTYGCGTGCMS" as annotated in the Transfac 4.0 databank. ND: not done.

[d] AHR battery refers to the genes of xenobiotic metabolism which are known targets of the AHR. CYP1A1 was found to be inducible in a number of various cell types, including thymocytes and T cells.

to reduced numbers of DP cells and skewing towards $CD8^{+}$ cells [28,44]. Recently, it has been shown that DN thymocytes are direct targets for TCDD resulting in thymus atrophy [27]. Here we show that TCDD does not only interfere with the development of fetal DN thymocytes, but also changes their differentiation and emigration pattern. To our surprise the majority of the emigrating cells from control FTOC were DP rather than SP. We could also detect significant amounts of DP cells in the blood of neonate mice. We did not find DP in spleen, though; possibly this is due to the lack of some unknown surface molecule necessary to enter the spleen. Conceivably, TCDD as a activator of the pleiotropic transcription factor AHR may also change such surface molecules, albeit we have not investigated this in this study. Bonomo et al. showed that DP cells disappear from the LNs of young mice within the first three weeks of life and suggested that they could be involved in autoimmunity, because they were not deleted in male anti-H-Y TCR transgenic mice [34].

TCDD exposure resulted in strong decrease of DP emigrants in FTOC as well as in neonate mice. While any possible physiological role of thymic emigrant DP cells is not understood, regulatory capacities have been described for DN cells. Emigration of DN cells

was enhanced by TCDD. Many of these DN cells were TCRγδ$^+$ T cells, which had a particular high potential to emigrate. This confirms and extends older studies, where a block in the very immature DN cells and skewed generation of TCRγδ$^+$ T cells in fetal thymi isolated from mice treated with TCDD in utero had been reported [26]. A distinct phenotype of CD25$^{lo/int}$CD44^{hi} expression (DN1b) was found in DN thymocytes from TCDD*FTOC, but even more pronounced among the emigrants. Indeed, almost 60% of the TCRγδ$^+$ thymocytes and 90% of the emigrants showed this phenotype. This suggests an early developmental block at the DN1b stage and the concomitant emigration of these cells. Presence of c-kit on most DN cells and the shutdown of RAG-1 and TdT expression in the emigrants further support this idea. Until now it is unclear at which stage separation of the TCRγδ$^+$ from the TCRαβ$^+$ lineage occurs. Our data suggest that the commitment towards the TCRγδ$^+$ lineage already takes place at the DN1b stage, and that TCDD can induce a shift towards this lineage. An increased frequency of TCRγδ$^+$cells with high CD44 expression was also detected in adult mice exposed to TCB, another AHR ligand [45], indicating that TCDD acts via activation of this transcription factor. The CD25$^{lo/int}$CD44^{hi} phenotype was not only found among DN TCRγδ$^+$ but also on TCRγδ$^-$ cells from TCDD*FTOC. Additionally, TCRγδ$^-$ cells contained increased percentages of B220$^+$ and NK1.1$^+$ cells in comparison to the control (data not shown), indirectly confirming the data of Laiosa et al.; they showed decreased proliferation and numbers of DN T cell progenitors [27]. The DN1b phenotype has not been observed in either adult or fetal DN thymocytes (whether gated on lineage negative cells or just CD3$^-$ cells) [39,46]. We could detect it, because we gated on DN plus TCRγδ$^+$ cells. Moreover, it is known that the fetal thymus in culture produces generally more TCRγδ$^+$ than the adult thymus in vivo [32], making detection of this population feasible. However, DN1b cells have been observed in Gfi-1 deficient mice and in transgenic mice expressing 'dominant interfering Myb' and Pim1 [42,43]. Both mouse strains have a block in the DN to DP transition, and c-myb and Gfi-1 are necessary for β-selection. The CD25$^{lo/int}$CD44^{hi} population in Gfi-1$^{-/-}$ mice contained a higher percentage of IL-7Rα$^-$ cells; IL-7Rα$^+$ expressing cells have a much higher potential to develop into TCRγδ$^+$ T cells. Taken together with our data, we think that the CD25$^{lo/int}$ CD44^{hi} phenotype is indicative of TCRγδ$^+$T cells destined to emigrate out of the fetal thymus. In the Gfi-1 gene upstream region (1500 bp) we identified a putative dioxin-responsive element, and Gfi-1 is a target of the activated AHR in DN cells, as is c-myb. Both genes were strongly downregulated in DN cells by TCDD. The phenotype of Gfi-1$^{-/-}$ mice resembles in several aspects the phenotype of TCDD-exposed mice: decreased thymic cellularity, reduction of CD4$^+$ CD8$^+$ cortical cells, accelerated positive selection, and skewed generation of CD8$^+$ thymocytes [19,29,44]. It would be interesting to analyze further a possible relationship between AHR-and Gfi-1-signaling. Additionally, the phenotype found for DN cells isolated from TCDD*FTOC resembles that seen on mature activated T cells, i.e., high expression of CD69 and low expression of CD45RB, CD62L and CD24. This is especially true for the DN TCRγδ$^+$ emigrants. Emigrants are known to undergo phenotypical maturation after having left the thymus. Compared to thymocytes, they gradually downregulate expression of CD24 and CD69 in the periphery. On the other hand, CD45RB and CD62L are continuously upregulated on CD4$^+$ or CD8$^+$ RTEs, respectively [47].

We noted a strong heterogeneity with respect to surface markers among emigrants. This suggests that subsets develop in the thymus and may have different fates in the periphery. For instance, CD62L is associated with preferential homing to the spleen. The lower frequency of this marker on TCRγδ$^+$ after TCDD exposure is particularly noteworthy, as intraepithelial gut lymphocytes (IEL) do not express CD62L, and the fetal thymus is a well-known source for IEL cells. No study on the effects of TCDD on the seeding of the gut lymphoid tissue has been done so far.

One reason for TCDD induced immunosuppression could be the generation and export of regulatory T cells in the thymus. Peripheral TCRγδ$^+$ T cells from adult mice have some immunomodulatory function, especially with respect to inflammatory reactions to bacterial infections. We found that TCRγδ$^+$ T cells, which cannot proliferate, even if stimulated with APC and anti-CD3 Ab or with PMA/Ionomycin, proliferate well in the presence of IL-2. This result is reminiscent of the data by Boursalian et al., who could show that addition of IL-2 could overcome the poor proliferation

of CD4$^+$ RTEs in the presence of anti-CD3 and anti-CD28 Ab [47]. If co-cultivated with activated CD4$^+$ T cells, they competitively impair the proliferation of the latter. Our data suggest to us that TCDD induced immunosuppression might not only be caused by effects on mature T cells in the periphery [48], but might also involve regulatory T cells from the thymus. Further studies will be needed to characterize this modulatory function further.

When analyzing the gene expression profile of DN emigrants from TCDD*FTOC in comparison to control FTOC, we found more than 1% of the entire genome modulated by TCDD. We have not subdivided the DN cells into more subsets, such as TCRγδ$^+$ cells, due to technical restraints and feasibility. Thus, the difference in overall gene expression profile reflects also the overall heterogeneity between DN cells from TCDD*FTOC and from control FTOC. However, the pattern correlates well with the changes and hints (a) to the profound and pleiotropic effects of AHR-signalling on genes accessible in immune cells and (b) will be of help in generating a molecular understanding on (TCDD-driven) thymocyte emigration and cellular differentiation, in particular of TCRγδ$^+$ cells. It is likely that not all genes regulated by TCDD are direct targets, i.e. induced via DREs in their promoters; they can also be modulated by secondary interactions [49]. Modulated genes of interest will also have to be analyzed further on the protein level, since gene transcription and protein production may not strictly correlate, as we already found for IL-4 and IFN-γ.

What is the physiological role of the AHR in thymocyte differentiation? On day 15 of gestation thymus cellularity is drastically reduced in AHR null mice [28], and in young mice the seeding of lymphoid organs is delayed. This has been interpreted as a defect in thymic output and homing of T cells [13,28], and our study helps to explain the phenomenon on a cellular and molecular level. Because the natural ligand is not known, no studies on its role in the early differentiation of thymocytes are available. AHR-deficient mice are refractory to all toxic and adverse effects of TCDD so far, indicating that TCDD acts primarily via AHR-signalling [28,50]. Presumably the natural ligand is necessary for proper thymocyte differentiation, but will be degradable better than TCDD, giving a lever for its cellular control.

We have investigated in parallel the emigration pattern in adult mice in vivo after TCDD-exposure of mice. Also in this setting, DN cells emigrate preferentially, and are capable of suppressing CD4 proliferation in co-culture. However, TCRγδ$^+$ cells are less abundant, reflecting again the special role of the fetal thymus in generating this T cell lineage. Also, as expected due to the different phenotype, gene expression profiles overlap only partially (manuscript submitted).

In conclusion, our results indicate the participation of the AHR in TCRγδ$^+$ T cell development, suggesting its role in lineage commitment occurring between the DN1 and DN2 stage of fetal thymocytes. The emigrated TCRγδ$^+$ T cells have regulatory potential by inhibiting the proliferation rate of activated CD4$^+$ T cells. Moreover, gene expression profile reveals an association between Gfi-1 and c-myb and AHR transcription repressors/ transcription factors.

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Role of the aryl hydrocarbon receptor in thymocyte emigration *in vivo*

Vladimir V. Temchura[1], *Markus Frericks*[1], *Wolfgang Nacken*[2] *and Charlotte Esser*[1]

[1] Institute for Environmental Medical Research (IUF), Heinrich-Heine University of Düsseldorf, Düsseldorf, Germany
[2] Institute of Experimental Dermatology, University of Münster, Münster, Germany

The aryl hydrocarbon receptor (AHR) is a ligand-dependent member of the PAS-bHLH-family of nuclear receptors. Anthropogenic ligands include environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Over-activation of the AHR causes thymus atrophy and immunosuppression. Signaling via the AHR changes the thymocyte differentiation program at several checkpoints, in particular within the $CD4^-CD8^-$ double-negative (DN) thymocyte subset. Here, we show that AHR over-activation led to the preferential emigration of DN thymocytes to the periphery and accumulation in the spleen. Some of these recent thymic emigrants (RTE) had a novel "activated immature" phenotype ($CD3^-TCR\beta^-CD25^{+/int}CD44^-CD45RB^{+/int}CD62L^+$ $CD69^-$ cells). Gene expression profiling of DN RTE revealed 15 genes that were up-regulated more than threefold by TCDD, including the S100A9 gene. Exposure of S100A9 null mice to TCDD showed a role for this protein in AHR-mediated thymic egress.

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Introduction

The thymus provides mature T cells for the peripheral immune system. The majority of thymocytes die within the thymus due to positive and negative selection processes geared to ensure that autoreactive T cells are eliminated and T cells become MHC restricted. In young mice, only about 0.5–1% of thymocytes survive selection, reach the mature $CD4^+$ or $CD8^+$ single positive (SP) T cell stage, and eventually get exported. In older mice, the thymus atrophies and, concomitantly, export of T cells gets considerably lower. Mechanisms of T cell homeostasis ensure that peripheral T cell numbers remain constant [1, 2]. Thymic output in mice is approximately 1×10^6 cells/thymus/day [3]. Very little is known about the final steps that allow or direct thymic T cells to emigrate and join the recirculating peripheral T cell pool. Questions remain open about the stage at which thymocytes emigrate, requirements for cell surface molecule expression, or homogeneity of the emigrating cells, *e.g.*, in terms of sensitivity for tolerization [1, 4–6]. Mechanisms of emigration including down-regulation of tethering adhesion molecules, passive carriage due to local fluid dynamics, chemotaxis, and active cell movement mediated by G-protein-coupled receptor have been experimentally addressed, and all found to at least contribute to emigration [7–10]. Moreover, thymic output differs between adult and fetal cells, as in the fetus γδ T cells emigrate in waves [11], and also emigrated $CD4^+CD8^+$ double-positive (DP) cells are found [12].

The aryl hydrocarbon receptor (AHR) is a nuclear transcription factor and belongs to a family of basic-loop-helix proteins, whose members are involved in *e.g.*,

Correspondence: Charlotte Esser, IUF, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany
Fax: +49-211-3190910
e-mail: chesser@uni-duesseldorf.de

Abbreviations: **AHR:** aryl hydrocarbon receptor · **DN:** $CD4^-CD8^-$ double-negative cells · **DP:** $CD4^+CD8^+$ double-positive cells · **ETO:** effective thymic output · **FTOC:** fetal thymus organ culture · **IC:** intracellular · **RTE:** recent thymic emigrants · **SP:** $CD4^+$ or $CD8^+$ single-positive cells · **TCDD:** 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

rhythm coordination, neurogenesis, and lineage specific transcription [13, 14]. Upon ligand binding the AHR translocates to the nucleus, where it binds to responsive elements in target gene promoters. A number of co-activators are known to be necessary for efficient transcription. Many genes are target genes of the AHR, and transcriptional activation is tightly controlled [15]. Regarding its physiological role, involvement of the AHR in lymphocyte maturation, migration and seeding of lymphoid organs, and in liver development was observed in AHR-deficient mice [16, 17]. The AHR is abundant in thymocytes and thymus stromal cells [18], and its activation in experimental animals leads to thymus atrophy.

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a high-affinity ligand for the AHR. TCDD is a toxic side-product present in "Agent Orange", used in the Vietnam War, and has been of concern for human health for years. TCDD exerts its toxicity via the AHR, and has become a model substance to study the AHR and its associated transcription machinery.

Recently, we have shown that treatment of mouse fetal thymus lobes in organ culture with AHR ligands results in a drastic decrease in the emigration of thymocytes in terms of numbers and type of cells, and in a preferential emigration of $CD4^-CD8^-$ double-negative (DN) cells. This emigration is an active process as it could be blocked by cytochalasin D [19]. As pointed out above, there are differences in emigration patterns between fetal and adult thymus.

In the present study, we therefore extended our analysis to adult animals exposed to TCDD. We labeled thymocytes by intrathymic injection of fluorescein isothiocyanate (FITC), and studied phenotype, preferred migration sites, and gene expression profiles of recent thymic emigrants (RTE) in the periphery. We found that TCDD exposure results in a phenotypically distinct population of DN RTE with an unusual gene expression profile. Moreover, our results indicate the involvement of S100A9, a member of the calcium-binding S-100 protein family, in the molecular process of TCDD-mediated thymic export.

Results

Effective thymic output of DN cells increases by TCDD; kinetic of DN RTE accumulation in the spleen

We injected mice intrathymically with FITC 5 days after TCDD exposure and compared recent thymic emigrants in the spleen, mesenteric lymph nodes (MLN), and blood 48 h later. At this time point the thymus was atrophied by about 70% (Fig. 1). The dose we used does not lead to systemic toxicity in the C57BL/6 mice. Control mice received the solvent corn-oil only. Saline was used for sham injections to control for stress effects of the intrathymic injections. Invariably, $FITC^+$ cells were only detected in mice, which had been FITC injected.

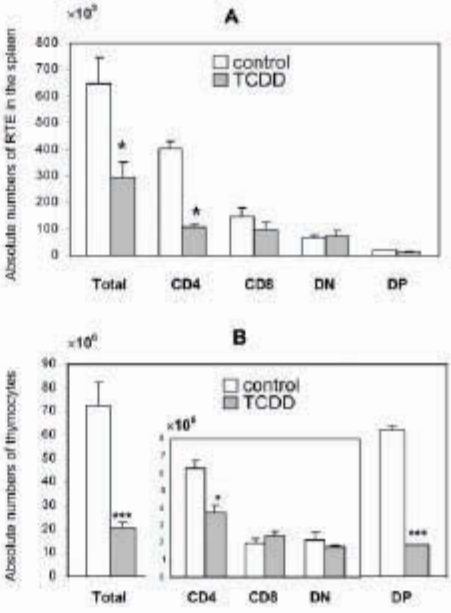

Fig. 1. (A) Absolute number of splenic RTE 48 h after intrathymic injection of FITC. (B) Absolute number of CD4/CD8 thymocyte subpopulations in TCDD treated and oil-treated (control) mice. The number of RTE in each CD4/CD8 subset was calculated from the frequency of $FITC^+$ cells in the spleen and absolute number of spleen cells, and then corrected based on the percentage of FITC-labeled thymic cells. Numbers were determined for each mouse individually. *Statistically significant by Student's *t*-test ($^*p<0.05$, $^{***}p<0.001$). Data are presented as the mean ± SEM. There were at least six mice per each group.

Approximately 90% of RTE in the spleen and the blood of control mice were $CD4^+CD8^-$ or $CD4^-CD8^+$ SP cells and the remaining 10% were mostly $CD4^-CD8^-$ DN cells. In MLN all cells were SP. In contrast, in TCDD-exposed mice we observed higher frequencies of DN RTE in spleen and blood, approximately 25%, and 12% in MLN (Table 1). The frequency of $CD4^-CD8^+$ cells increased slightly simultaneously in spleen, blood and MLN, and $CD4^+CD8^-$ cells decreased twofold. The well-known TCDD-induced shift in CD4/CD8 subpopulations in the thymus is shown for comparison. Note, that the major change in the thymus is a reduction in DP cells (Table 1).

Table 1. CD4/CD8 subpopulation of thymocytes[a)] and RTE[b)] in lymphoid tissues of C57BL/6 mice

	Thymus		Spleen		MLN		Blood	
	Control	TCDD	Control	TCDD	Control	TCDD	Control	TCDD
$CD4^+CD8^-$	8.7 ± 0.3[c)]	17.7 ± 1.0	62.4 ± 1.5	36.0 ± 1.6	66.0 ± 2.1	35.3 ± 9.7	64.0 ± 4.9	32.7 ± 4.1
$CD4^-CD8^+$	2.7 ± 0.3	11.3 ± 0.5	23.4 ± 1.9	33.0 ± 4.2	31.0 ± 2.1	44.3 ± 3.2	27.1 ± 3.8	38.6 ± 2.8
$CD4^+CD8^+$	85.6 ± 1.2	62.8 ± 0.8	3.1 ± 0.4	4.1 ± 1.3	1.0 ± 0.0	3.0 ± 1.4	2.9 ± 1.0	4.7 ± 1.2
$CD4^-CD8^-$	3.0 ± 0.6	7.8 ± 0.4	9.4 ± 1.2	25.5 ± 2.4	2.0 ± 0.1	11.7 ± 3.8	6.0 ± 1.0	23.9 ± 3.9

a) Five days after injection with either oil (control) or TCDD (10 μg/kg body weight), mice were injected with FITC in each thymic lobe. Organs and tissues were removed after 48 h and cells were stained for CD4 and CD8.
b) Percentage of viable cells from each phenotype among the cells stained with FITC over background level (RTE).
c) Mean ± SEM. n > eight mice per group.

An increased frequency of DN RTE may reflect either a changed output from the thymus, or a parallel decrease in the other subpopulations. As shown in Fig. 1 the number of total RTE in TCDD-treated mice was significantly reduced, while no differences in the numbers of DN RTE were observed. The reduction in thymic output parallels the reduction in thymocyte numbers, in particular in DP cells (Fig. 1).

T cell export is known to be a function of thymocyte, rather than peripheral T cell numbers [20]. Effective thymic output (ETO), defined as the ratio of RTE:thymocytes, is thus an appropriate way to describe changes in thymus emigration. No differences in ETO were detectable for total thymic emigrants early after FITC injection. However, 24 and 30 h after FITC injection, ETO was significantly higher in TCDD-exposed mice than in control mice (Fig. 2A). This increase was also observable at 30 h in the blood (data not shown).

At all time points, the ETO was significantly higher for DN RTE of TCDD-exposed mice than for control mice (Fig. 2B). Taken together, these data indicate that the effect is moderate for overall total effective thymic output, but DN cells emigrate at a higher rate and speed by TCDD.

DN RTE from AHR-(over-)activated mice have an immature activated phenotype

The DN cells which emigrate at a higher rate after TCDD exposure might conceivably differ from the normally emigrating DN subset, and we compared their phenotypes 48 h after FITC injection (Fig. 3). At this point the absolute numbers of DN RTE in these groups are the same (Fig. 1), and any deviation in percentage can, accordingly, be taken as a change in absolute cell numbers as well.

It is known that mature B cells are exported among DN RTE [21]. We also detected about 8% of B cells in TCDD-exposed and control mice. We, therefore, analyzed $CD4^-CD8^-CD19^-$ RTE (Fig. 3B). While DN RTE from control mice were mostly $CD3^+$, in the TCDD-treated mice a population of $CD3^-$ DN RTE appeared (Fig. 3C), and the frequency of TCRβ-expressing cells decreased (Fig. 3D). No changes were detected with respect to the export of thymus-dependent NK cells. For $TCR\gamma\delta^+$ cells, we found considerable variations between individual mice: from 9% to 45% (21.8±16.8) in control DN RTE and from 5% to 10% (6.8±2.4) in TCDD-treated animals.

CD45RB is a marker, whose down-regulation indicates activation of peripheral T cells. After TCDD

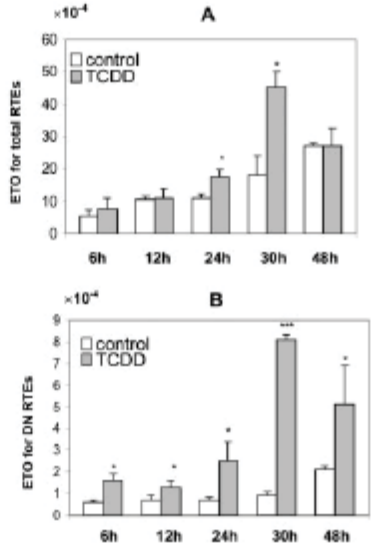

Fig. 2. Kinetics of the effective thymic output of total (A) and DN (B) splenic RTE. ETO (RTE:thymocyte ratio) was determined for each mouse individually based on the absolute number of RTE in the spleen and cellularity of thymus at the respective time point. *Statistically significant by Student's t-test (*$p<0.05$; ***$p<0.001$). Data are presented as the mean ± SEM. There were at least six mice per each group.

exposure, the frequency of CD45RB^{hi} cells decreased, and CD45RB^{int} cells increased compared to DN RTE from control animals (Fig. 3E). We also compared the expression of CD69, a retention marker, and CD62L, a marker for thymocytes destined for export [14, 22]. As would be expected, both control and TCDD-treated splenic DN RTE were CD62L^{hi} and CD69^{-} (data not shown).

Taken together, these data indicate that splenic DN RTE of TCDD-treated animals contain more cells with an immature-activated phenotype than control DN RTE.

Identification of the developmental stage of DN RTE

DN cells can be divided into four differentiation stages (DN1–DN4) according to the expression of CD44 and CD25. The intrathymic late precursor DN3 and DN4 populations are direct targets for the AHR, which inhibits their proliferation [23]. To identify the distribution of DN progenitor cell populations in DN RTE, we compared splenic DN RTE and thymic DN cells with respect to CD44 and CD25 expression (Fig. 4A, Table 2). The CD44/25 profiles of DN thymocytes differed between control and AHR mice, and between thymocytes and emigrants.

Most of the DN RTE in the control mice were CD44^{-}CD25^{-}, and thus belong to DN4. This is congruent with their being CD3^{+} (Fig. 3C). However, approximately 30% of DN RTE in AHR-activated mice were

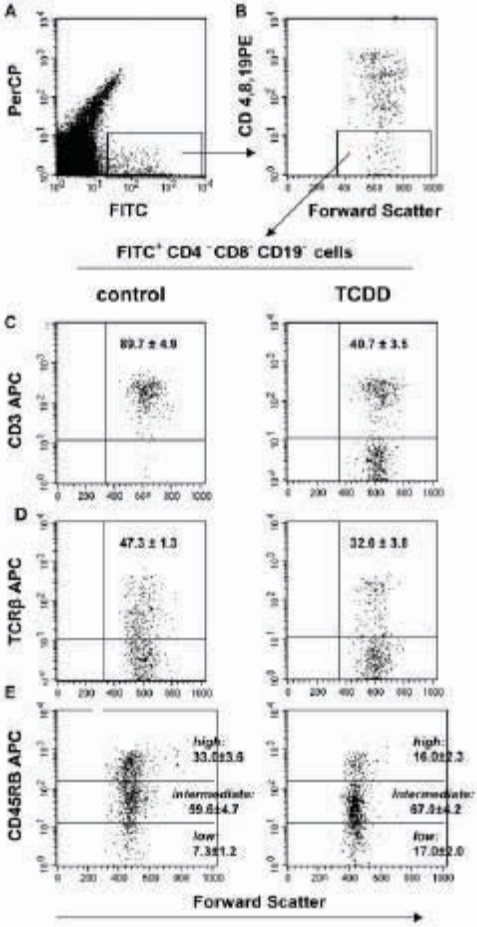

Fig. 3. Comparison of surface markers on splenic DN RTE from control and TCDD-exposed mice. (A) Gate used to identify FITC^{+} cells. Fluorescence at 675 nm ("PerCP") was used to exclude unspecifically fluorescent cells. Only cells negative for CD4, CD8, and CD19 (B) were analyzed further (C–E). Shown are representative dot plots for CD3, TCRβ and CD45RB RTE in control mice (left panel) and TCDD-treated mice (right panel). For each group spleen cells from three mice were pooled. One representative experiment out of three is shown. The mean percentages of the subsets ± SEM are indicated in the plots.

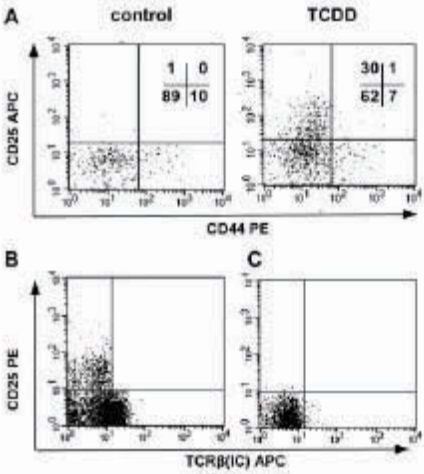

Fig. 4. CD44 and CD25 distributions (A) and TCRβ (IC) expression (B) in DN RTE and after TCDD exposure. CD4^{-}CD8^{-}CD19^{-} cells were isolated from the spleen by depletion with magnetic beads. Purity after depletion was >95%. (A) Cells from control and TCDD-treated mice were stained with antibodies against CD44 and CD25, and FITC^{+} cells were analyzed. (B) Expression of IC TCRβ chain in CD25^{+} DN RTE from TCDD-treated mice, and (C) efficiency of blocking by unlabeled antibodies. One representative experiment out of three is shown. Each group contained cells pooled from three mice.

Table 2. Absolute numbers in DN1–DN4 differentiation stages within the thymus[a] and among RTE in the spleen[b] of C57BL/6 mice

	Thymus × 10^5		Splenic RTE × 10^3	
	Control	TCDD	Control	TCDD
CD44$^+$CD25$^-$ (DN1)	1.19 ± 0.35[c]	2.23 ± 0.09	5.85 ± 1.37	6.75 ± 0.90
CD44$^+$CD25$^+$ (DN2)	0.27 ± 0.06	0.75 ± 0.21	0.78 ± 0.26	1.20 ± 0.23
CD44$^-$CD25$^+$ (DN3)	4.29 ± 0.63	10.58 ± 0.16**	0.87 ± 0.04	19.73 ± 2.03*
CD44$^-$CD25$^-$ (DN4)	16.06 ± 0.95	3.82 ± 0.31**	56.75 ± 1.50	46.95 ± 1.86*

[a] Individual thymi from oil and TCDD-injected sham-operated mice were removed at day 7 after treatment. Gated CD4$^-$CD8$^-$ cells were analyzed for their CD44/CD25 pattern.

[b] Mice were treated either with corn-oil or with 10 μg TCDD/kg body weight, and FITC injected into the thymus 5 days later. After 48 h, CD4$^-$CD8$^-$CD19$^-$ were isolated from the spleen, and analyzed for CD44 and CD25 surface expression. The number of RTE in each CD4/CD8 subset was calculated from the frequency of FITC$^+$ cells in the spleen and absolute number of spleen cells, and then corrected based on the percentage of FITC-labeled thymic cells.

[c] Means ± SEM. *$p<0.05$; **$p<0.001$ by Student's t-test.

CD44$^-$CD25$^{+/lo}$ in the spleen (Fig. 4A). This DN3-like phenotype was absent in control mice.

DN3 cells can be divided further into two subsets: larger cycling cells with a high proportion of in-frame β-rearrangements (termed 'L' cells) and the pre-selected small resting cells, mostly without rearrangements ('E' cells) [24]. According to their forward scatter characteristics (data not shown) and lack of intra- and extracellular expression of TCRβ chain (Fig. 4B, C), the CD44$^-$CD25$^+$CD3$^-$CD45RB$^{+/lo}$ DN3-like RTE from AHR-activated mice are E cells, suggesting a premature export of these cells.

Gene expression profiles of DN thymocytes and RTE

The AHR is known to target genes involved in cell differentiation, activation and cell cycling [15]. Transcriptional activation by the AHR is cell and even cell-stage specific, and only few genes (in particular *CYP450 1B1*) seem to be inducible in all AHR-containing tissues [15]. We sorted DN RTE and thymocytes from AHR-activated and control mice, and analyzed their gene expression profiles on Affymetrix™ gene chips. The results for DN RTE of TCDD-exposed versus control mice are shown in Table 3. Choosing a 3-fold difference as our cut-off threshold, we identified 15 genes as up-regulated. No genes were down-regulated at least 3-fold. *Adseverin* and *CYP4501B1*, two known AHR-target genes were found expressed at a 11.12-fold and 3.27-fold higher level, respectively. Both of these genes were also up-regulated in DN thymocytes, while the others were not (data not shown).

Direct AHR-mediated induction of transcription has been shown formally only for a limited number of genes, and secondary effects are likely, especially after longer treatment with TCDD [25, 26]. Effects of TCDD begin within hours after exposure. TCDD is degraded and excreted only slowly. Thus, after a single injection, the AHR will remain activated for weeks, and it is likely that the effects we describe here comprise primary and secondary events. The lack of dioxin-responsive elements (DRE) in the promoters in 10 of the 15 genes in Table 3 supports this notion. However, in AHR-deficient mice thymotoxicity is absent, highlighting that the AHR is the primary effector of TCDD-induced effect [27].

S100A8, S100A9, fibronectin 1, small inducible cytokine A9 (CCL9) and small inducible cytokine subfamily, member 2 (CXCL2) were up-regulated in the TCDD-exposed mice. These genes are known to be involved in chemotaxis and cell motility, and thus are candidates for a function in aberrant emigration in the DN3-like RTE.

For the other genes listed in Table 3, a connection with emigration is not apparent. We note though, that haptoglobin and the cathepsins have functions in antigen presentation, and possibly a Th2 to Th1 switch [28, 29]. Also, AHR over-activation led to the expression of genes that are usually not expressed in T cells. C-type lectin superfamily 8 is normally restricted to macrophages (Table 3) [30]. TCDD is known for its multi-faceted toxicity, and the perturbation of cell-specific gene expression may contribute to it.

S100A9-deficient mice show no difference in ETO between TCDD-treated and control groups

S100A8 and S100A9 (alias MRP8, MRP14 or calgranulin B) are calcium-binding proteins related to the transendothelial migration of neutrophils [31]. S100A9 can interact with pertussis toxin-sensitive G-protein-coupled receptors [32], a pathway relevant to thymocyte emigration [7]. Also, S100A9 can interact directly with

Table 3. TCDD-related gene transcription in DN RTE from TCDD-exposed mice[a]

Probeset[b]	Gene name[c]	Induction	Gene product functions[c]	DRE[d]
1448881_at	Haptoglobin	3.14	Serine-type endopeptidase	No
1420394_s_at	C3H gp49B1	3.17	—	No
1423547_at	Lysozyme	3.24	Hydrolase, acting on glycosyl bonds/ antimicrobial peptide	No
1416612_at	CYP4501B1[e]	3.27	Xenobiotic metabolism	Yes
1422557_s_at	Metallothionein 1	3.45	Heavy metal binding	Yes
1451310_a_at	Cathepsin L	3.83	Proteolysis and peptidolysis	Yes
1419394_s_at	S100 A8 (calgranulin A)	3.83	Chemotaxis, calcium ion binding	No
1420804_s_at	C-type lectin, superfamily member 8	4.36	Sugar binding / recognition	No
1426642_at	Fibronectin 1	4.71	Cell adhesion, cell motility,	Yes
1415904_at	Lipoprotein lipase	5.35	Lipid catabolism/ metabolism	No
1448756_at	S100 A9 (calgranulin B)	5.44	Chemotaxis, calcium ion binding	No
1417936_at	Small inducible cytokine A9	5.84	Chemotaxis/signal transduction/immune response	No
1419594_at	Cathepsin G	6.29	proteolysis and peptidolysis	No
1450276_a_at	Adseverin (Scinderin)[e]	11.12	Structural constituent of cytoskeleton/actin binding	No
1449984_at	Small inducible cytokine subfamily, member 2	11.66	Chemotaxis/signal transduction/immune response	Yes

[a] DN RTE were sorted from the spleens of mice exposed to TCDD (10 μg/kg). RNA was isolated and hybridized to Affymetrix™ gene chip MOE 430A. Shown are genes whose transcription was upregulated at least threefold after TCDD exposure.
[b] Identification number
[c] Common name and functional assignment according to the Affymetrix™ 430A gene chip annotation.
[d] The presence or absence of a DRE consensus sequence was analyzed in 2000 bp upstream of transcription start site with the MatInspector program (http://www.genomatix.de/cgi-bin/matinspector/matinspector.pl). Consensus sequence used: 5'-t/gGNGCGTGCc/gAN-3'.
[e] CYP450 1B1 and adseverin are the only genes in this list, which we found also differentially regulated when comparing DN thymocytes from TCDD-treated *vs.* untreated mice. All other genes were up-regulated by TCDD only in RTE.

β2 integrins in forming "activated receptor" epitopes, and plays a role in cytoskeletal reorganization [32, 33]. Both S100A8 and S100A9 RNA were up-regulated in DN RTE of TCDD-exposed mice (see Table 3).

Histological analysis of the thymus from wild-type and S100A9-deficient mice revealed no morphological alterations [32, 33]. We found that the percentage and absolute numbers of CD4/CD8 thymocyte subpopulations also did not differ from wild type (data not shown). Thymus atrophy induced by TCDD treatment was as strong as in wild type, indicating that these mice are comparably AHR sensitive. However, in contrast to the wild type, the number of DN cells in TCDD thymus was significantly ($p<0.05$) less ($2.4 \times 10^6 \pm 0.2 \times 10^6$ in control group *vs.* $1.2 \times 10^6 \pm 0.2 \times 10^6$ in TCDD group). SDF-1 (CXCL12) is known to attract DN and DP cells [9]. We tested the migration capacity towards this chemokine in the transwell migration chemotaxis assay; thymocytes from TCDD-treated wild-type mice had a 3-fold higher migration rate towards SDF-1 (CXCL 12), but this effect was abolished in S100A9$^{-/-}$ mice (data not shown).

DN RTE numbers were 2-fold lower in the spleens of TCDD-treated S100A9$^{-/-}$ mice than in the oil-treated group (Fig. 5A). However, the percentage of this subpopulation was the same between the groups (Fig. 5B). Likewise, there was no significantly increased percentage of DN RTE in MLN and blood after TCDD exposure (MLN: $4.5 \pm 0.5\%$ in control *vs.* $1.7 \pm 0.7\%$ in TCDD; blood: $11.0 \pm 1.4\%$ *vs.* $9.0 \pm 4.2\%$, correspondingly). The ETO of splenic DN RTE was not changed by TCDD, nor were the frequencies in CD25$^+$ RTE (Table 4).

Taken together, in S100A9$^{-/-}$ mice the unusual emigration of DN by TCDD exposure found in wild-type mice was absent.

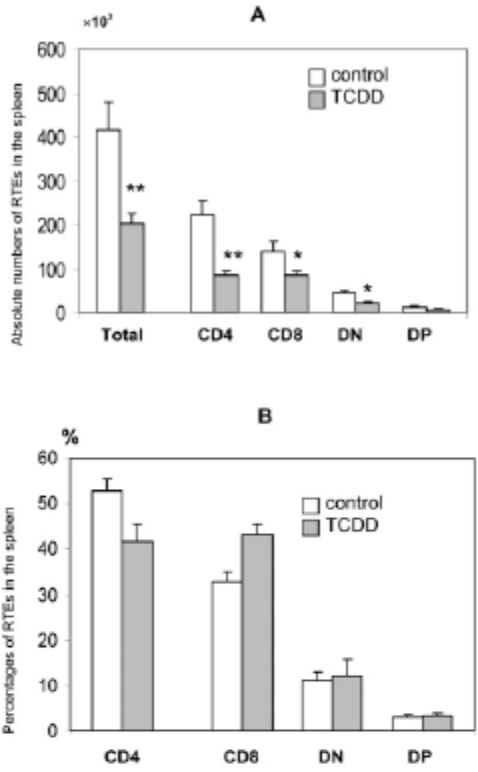

Fig. 5. Absolute number (A) and percentage (B) of CD4/CD8 splenic RTE subpopulations in S100A9-deficient mice 48 h after intrathymic injection of FITC. The number of RTE in each CD4/CD8 subset was calculated from the frequency of FITC⁺ cells in the spleen and absolute number of spleen cells, and then corrected based on the percentage of FITC-labeled thymic cells. Numbers were determined for each mouse individually. *Statistically significant by Student's *t*-test (*$p<0.05$; **$p<0.001$). Data are presented as the mean ± SEM. There were at least six mice per each group.

Discussion

It is well established that TCDD continuously activates the AHR and, in turn, has complex effects on thymocyte differentiation. In particular, studies using fetal thymus organ cultures (FTOC) have shown that intrathymic progenitor cells are direct targets. They get arrested in proliferation [34], which contributes largely to TCDD-induced thymus atrophy. In addition, the differentiation towards mature $CD4^-CD8^+$ thymocytes is accelerated and skewed [35]. Recently, we showed preferential emigration of a novel subset of DN thymocytes after TCDD exposure in late fetal thymus organ culture [19]. Aberrant emigration of DN cells might add to thymus atrophy, and the emigrant cells might also link atrophy to TCDD-induced immunosuppression. However, this putative link has not been addressed experimentally. Enhanced CD8 differentiation and proliferation arrest of immature thymocytes have been demonstrated in fetal and adult thymi. In contrast, AHR-mediated emigration has not.

FTOC represent a valid method to study thymocyte differentiation and emigration [36]; however, emigration patterns of fetal *vs.* adult cells differ. For instance, in newborn mice, as well as in FTOC of corresponding maturity, $CD4^+CD8^+$ cells emigrate [12, 19], while emigrants from adult mice are mostly $CD4^+$ or $CD8^+$ SP T cells. In FTOC, we also found a TCDD-dependent increase in $\gamma\delta$-TCR^+ thymocytes, which might be specific for the fetal situation [37]. Here, using intrathymic FITC labeling, we found that the effective thymus output *in vivo*, calculated from the ratio RTE:thymocytes, was not generally affected by TCDD exposure. The drop in export of cells to the periphery simply reflected the overall thymus atrophy. However, the frequency and absolute number of DN cells among RTE detected in the bloodstream, spleen and MLN was increased within days after systemic TCDD exposure. Thus, in terms of output the situation in the adult animals was similar to the fetal thymus [19].

Emigrants from FTOC were αβ-TCR negative. In adult emigrants, however, they were a mixture of β-TCR negative (70%) and positive (30%) cells. $CD44^{hi}$ cells –

Table 4. DN RTE patterns in S100A9 deficient mice[a]

Treatment	ETO[b] of total splenic RTE $\times 10^{-4}$	ETO of DN splenic RTE $\times 10^{-4}$	% CD25 among DN RTE[c]
Control	54.3 ± 8.2	5.9 ± 0.4	3.4 ± 0.2
TCDD	52.8 ± 2.0	5.9 ± 1.7	3.2 ± 1.2

[a] $S100A9^{-/-}$ mice received either 10 μg/kg TCDD in oil (TCDD), or oil alone (control). After 5 days, mice were with injected intrathymically with FITC. RTE were analyzed 48 h later in the spleen.
[b] Effective thymic output (RTE:thymocyte ratio) was determined for each mouse individually, based on the absolute number of RTE in the spleen and cellularity of thymus.
[c] The frequency of $CD25^+$ cells within the FITC-labeled RTE was determined flow cytometrically.

the majority of FTOC emigrant cells – were not found in adult RTE at all, nor were adult RTE as large as their fetal counterparts. Thus, while thymic egress of DN cells seems promoted by the activated AHR in fetal and adult cells, the emigrating DN cells are themselves different.

What type of cells are DN emigrants *in vivo*? Rare in frequency, they can be $CD3^+TCR\gamma\delta^+$, $CD3^+TCR\gamma\delta^-$, $CD3^+$ NK1.1$^+$ and even B cells [38]. As expected, we find in the control group that the majority of $CD4^-CD8^-CD19^-$ are $CD3^+$, (about 50% of them stain on the surface for TCRβ) and they are $CD62L^{high}$ $CD69^-$ $CD45RB^{int/hi}$, known as indicators of recent emigration [38]. With respect to CD44 and CD25, they belong to the DN4 stage (Fig. 4), *i.e.*, the developmental stage before up-regulation of CD8 and CD4 [39]. In contrast, the DN cells from the TCDD-treated mice are different. Only 40% are $CD3^+$. At the same time, not all of the "TCDD-emigrant" DN cells are $CD44^-CD25^-$, but about 30% still express CD25, *i.e.* ,seem to be of the DN3 stage. However, CD25/CD44 expression might be changed, and no longer be indicative of the maturation stage in the aberrant situation of TCDD treatment. We additionally surveyed the genes known to be expressed in the various thymocyte maturation stages. Unfortunately, no single distinct cell-stage-specific gene expression marker combination is known [40, 41]. We therefore compared the overlap of 75 differentially expressed transcripts between thymocyte differentiation stages, as published in the literature, and the DN cells, identified by us after TCDD exposure. The strongest match in expression profiles is both within the DN3 and DN4 stage (Fig. 6).

In addition, cell size and expression of an intracellular TCRβ chain were used to identify cells that had undergone β-selection. These parameters also indicated that the $CD25^+$DN cells that had emigrated are immature, with a DN3-like stage [24]. Thus, in addition to DN4-like emigrants, which were detectable in the controls, more immature DN3-like cells also emigrate after TCDD exposure, *i.e.*, AHR activation, of the mouse.

Very little is known about the final steps that allow or direct T cells to emigrate. A broad spectrum of possible emigration mechanisms is still under discussion. At the least, emigration seems to be an active process [22, 36]. Regarding the stage of emigration, it has been shown that active emigration is induced within 24 h after positive selection [36]. Our data suggest that active processes mediated by chemotactic- and migration-mediating molecules (CCL9, CXCL2, S100A8, S100A9) are involved. Conceivably, unbalancing the expression of these genes leads to release of immature cells from their normal inhibition to emigrate.

This is the first time that expression of S100A8 and A9 transcription in thymocytes, and its up-regulation by TCDD has been described. Possible associations with activity of integrin β2, known to be stimulated via

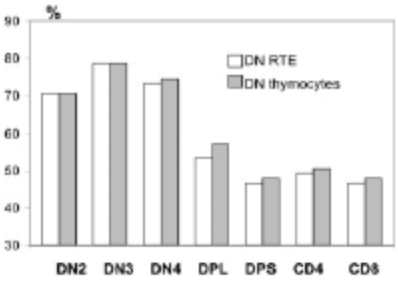

Fig. 6. Comparison of the gene expression profiles of phenotypically characterized differentiation stages in thymocytes and RTE from TCDD-treated mice, with published expression profiles. Transcripts (n=75) shown to be differentially expressed in thymocyte development in a microarray analysis using an Affymetrix platform [40, 41] were randomly chosen and their presence or absence noted in our array data. Since a direct comparison of different array platforms is not possible, we accessed the raw expression profiles from the GEO [46, 47], and normalized this data to our data using the median signal values. This method permits comparing the signal intensities despite different array platforms in both studies. The figure shows the percentages of matching values for different thymic differentiation stages. DPL and DPS stages are according to the nomenclature of [40]

S100A9 [42] in humans, are intriguing, as in our hands we found integrin β2 to be up-regulated after emigration (regardless of TCDD treatment). As has also been described by others, *adseverin* transcription was up-regulated upon TCDD treatment in thymocytes [43]. While the association of adseverin to the TCDD-induced block of thymocyte proliferation has been addressed [34, 43], our data suggest another, additional, role of adseverin in thymocyte egress. Conceivably, changes in actin reorganization can have consequences for active cell movement and thus thymic emigration.

TCDD action on the thymus is cell stage specific, *e.g.*, AHR-mediated IL-2 induction is found in DN but not in DP thymocytes [26], and TCDD blocks proliferation at the DN3 stage [34]. Here, we also describe cell stage-specific effects of TCDD action. The high abundance of the AHR in lymphoid tissues has been considered suggestive of a physiological role of this transcription factor. Studies on AHR-deficient mice have confirmed the role of the AHR in TCDD-mediated immunotoxic effects. Analysis of the physiological role of the AHR is hampered, though, because the real ligand is unknown. Indeed, it is not even clear whether there are one or several ligands, as the ligand binding site is unusually promiscuous.

In conclusion, prospective thymus emigrants are targets of the over-activation of the AHR *in vivo*, and aberrant emigration of DN3-like thymocytes ensues.

S100 A9 is involved in these emigration processes, which may conceivably contribute to the well-known TCDD-induced systemic immunosuppression.

Materials and methods

Experimental animals

C57BL/6 or S100A9$^{-/-}$ mice, 6–8 weeks old, received 10 μg TCDD/kg body weight i.p. (Oekometric, Bayreuth, Germany) diluted in 200 μL corn oil, or received corn oil alone.

Intrathymic labeling of thymocytes

Thymocytes were labeled 5 days after TCDD exposure with FITC *in vivo* as previously described [3]. Briefly, mice were anesthetized, and 10 μL 1 mg/mL FITC or PBS was injected per thymus lobe. Typically, up to 70% of thymocytes were FITC$^+$. The absolute numbers of RTE in the spleen were normalized by the fraction of thymocytes labeled in each particular mouse.

Transwell chemotaxis assay

Chemotaxis was performed as described [44]. Briefly, freshly isolated thymocytes (1×10^6) were placed in a Costar® (Corning Inc. NY) transwell chamber that inserted into culture wells containing hrSDF-1 (100 ng/mL). Cells were incubated for 3 h, and counted by flow cytometry after adding Dynospheres Uniform Microspheres (Bangs Laboratories, Fishers, IN) for calibration.

Enrichment of DN cells

CD4$^-$CD8$^-$CD19$^-$ cells were enriched by depletion of CD4, CD8 and CD19 cells with the AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of sorting was controlled by flow cytometry.

Flow cytometry and cell sorting

Cells were stained with the appropriate antibodies for 10 minutes at 4°C. Data were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Intracellular staining (IC) was done after blocking the corresponding surface molecules with unlabeled mAb according to Levelt *et al.* [45]. DN FITC$^+$ cells were sorted by FACSdiva (Becton Dickinson). All antibodies were purchased from Pharmingen (Heidelberg, Germany). Autofluorescent cells were excluded using FL1/FL3 gating, *i.e.*, only FITC$^+$ PerCP$^-$ cells were analyzed further (Fig. 3A).

Gene expression analysis

Total RNA was isolated with TRIzol™ (Invitrogen, Karlsruhe). RNA was amplified prior to chip hybridization with the MessageAmp™ Kit of Ambion (Woodward St. Austin, USA). Amplification of RNA types was validated by exemplary quantification of unamplified *vs.* amplified RNA of eight selected genes (data not shown). RNA was biotinylated with the Enzo Bio Array™HighYield™ RNA transcript labeling kit (Affymetrix, High Wycombe, UK) and purified.

RNA was hybridized to the MO430A gene chip (Affymetrix). The resulting *.chp files were analyzed with the bioconductor affy package using the RMA (robust microarray analysis) algorithm. Two independent experiments were performed. A threefold expression difference was chosen as cut-off. To be considered, transcripts had to have a present call in the MAS 5.0 algorithm.

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Impairment of maturational competence of cultured Langerhans cells from aryl hydrocarbon receptor (ahr) null mice and suppression of ahr signalling points to its role in LC tolerogenic strategy*

Bettina Jux, Markus Frericks and Charlotte Esser

Div. Molecular Immunology, Institut für umweltmedizinische Forschung (IUF) at the Heinrich-Heine University of Düsseldorf. Auf´m Hennekamp 50, 40225 Düsseldorf, Germany

corresponding author:
Charlotte Esser, Ph.D.
Institut für umweltmedizinische Forschung (IUF)
Auf´m Hennekamp 50
40225 Duesseldorf
Germany
Tel.: +49-2113389253
FAX: +49-2113190910
chesser@uni-duesseldorf.de

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Abstract

Langerhans cells (LC†) are professional antigen-processing cells of the epidermis. Recently, it was suggested that they are tolerogenic, and control adverse immune reactions against low molecular weight chemicals (LMWC). The aryl hydrocarbon receptor (AhR), a ligand activated transcription factor, induces expression of LWMC metabolizing enzymes. Cellular metabolism of LMWC may transform them into haptens, and thus conceivably trigger allergic reactions, including contact dermatitis. We studied whether and how LC possess strategies to avoid this risk.

We found that the AhR is abundantly expressed in LC of C57/BL6 mice. Unexpectedly though, *in vivo* treatment with a strong AhR ligand (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) did not lead to gene induction in LC. The unusually high constitutive expression of the AhR-repressor in LC might explain this finding, and points to a relevant tolerogenic strategy of LC. We further investigated LC in AhR null mice. We found that (i) AhR-deficient LC express only marginal amounts of the AhR-repressor, (ii) AhR deficient LC were impaired in maturation; they remained smaller and less granular, and did not up-regulate expression of co-stimulatory molecules CD40, CD80, and CD24 during *in vitro* maturation, and (iii) their phagocytic capacity did not decrease. GMCSF, needed for LC maturation, was secreted in significantly lower amounts by AhR-deficient epidermal cells (i.e. keratinocytes and LC). Our data suggest that the AhR is involved in LC maturation, both cell-autonomously and through by-stander cells. At the same time, the AhR is part of LC´s risk strategy against unwanted immune activation by potential skin allergens.

† abbreviations used:
AhR: arylhydrocarbon receptor, AhRR: AhR repressor, ARNT: AhR nuclear translocator, LC: Langerhans cells, DC: dendritic cells, TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

with AhR for the binding site of ARNT and forms a transcriptionally inactive complex (3).

It is known that the skin performs active metabolic functions including xenobiotic metabolism. For instance, cytochrome P450 monooxygenase CYP1A1, an important XME and most prominent target gene of the AhR is expressed in the epidermis, more precisely in keratinocytes (4). The skin is also a target organ of the deleterious effects of toxic AhR activation by environmental pollutants, causing human skin diseases like chloracne, hyperkeratosis, and photosensitivity. These diseases occurred after human exposure to the AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (5). The AhR can be activated by a variety of small molecules, including dioxins, furans, flavonoids and tryptophane derivatives (reviewed in 6). However, TCDD, due to its high affinity is still the ligand of choice to study AhR biochemistry and physiological function. The exposure to TCDD leads to a wide range of toxic responses including cancer, teratogenicity and embryotoxicity. In the immune system, abnormalities in development and immunosuppression were observed, either after over-activation (i.e. TCDD exposure) or in the absence of the AhR in gene-deficient mouse strains (7). Recent studies pointed to DC as the direct target of TCDD and congeners, which then mediate the immunosuppressive effects on T and B cells (8-10). With respect to LC, topical application of TCDD induced an increase in the density of LC in hairless mice (11).

It appears self-evident that immune responses against small molecular weight chemicals must be under strict control in the skin, in order to suppress frequent unwanted allergic reactions. Recently, in a shift of paradigm, novel findings have suggested that LC rather than initiating contact sensitivity (12), have regulatory and tolerogenic functions (13). Furthermore, LC were shown to be unable to elicit T cell

responses to certain viral antigens (14,15). The underlying mechanisms are not known.

The skin as a major barrier to the environment comes in contact with a wide range of AhR ligands, which may be immunologically inert themselves, but which could be metabolized into potential haptens by AhR-inducible XME (16). For polyaromatic hydrocarbons, conversion of the parent compound to a reactive metabolite is necessary for the development of contact hypersensitivity (17). We therefore examined gene expression profiles and maturation of LC after over-activation of the AhR and in its absence. We identified as AhR-dependent the secretion of GMCSF, which is necessary for LC survival and development. Our data point to a critical role of the AhR in LC maturation and demonstrate for the first time a mechanism by which LC may remain tolerant against harmless substances.

Material and methods

Experimental animals

C57BL/6 mice were purchased from Janvier Laboratories (Le Genest Saint Isle, France), $AhR^{-/-}$ mice (bred onto the C57BL/6 background) were from Charles River Laboratories (Sulzfeld, Germany). These mice lack a functional AhR, as the bHLH domain is deleted (18). Mice were bred and housed in our own animal facility under specific pathogen-free conditions. 6-10 weeks old female mice received a subtoxic dose of 10 µg 2,3,7,8-TCDD/kg body weight i.p. (LGC Promochem, Wesel, Germany) initially dissolved in DMSO and then diluted in corn oil (Sigma, Taufkirchen, Germany), or vehicle (DMSO in corn oil) alone. Mice were sacrificed by CO_2 asphyxiation for removal of organs. The experiments have been reviewed and permitted in accordance with relevant German animal welfare laws.

Cell preparations

Epidermal cell (eC) suspensions were prepared from ears and dorsal skin of C57BL/6 and $AhR^{-/-}$ mice as described elsewhere with some modifications (19). Briefly, skin was rinsed with 70 % alcohol; ears were split with the aid of forceps, and placed, dermal side down, on a 0.25 % trypsin/PBS solution for 2 hours at 37°C. In the case of dorsal skin, the subcutaneous fat was scraped off with a scalpel before placement on trypsin. Epidermal sheets were then peeled from the underlying dermis and floated in RPMI 1640 supplemented with 10 % heat-inactivated FCS, 2 mM L-glutamine, 5 x 10^{-5} M 2-mercaptoethanol, 0.15 % sodium hydrogencarbonate, 1 mM sodium pyruvate, non-essential amino acids, 100 U/ml Penicillin, and 100 µg/ml Streptomycin (PAA Laboratories GmbH, Cölbe, Germany), hereafter referred to as complete medium. Cells were released by breaking up sheets with forceps and vigorous pipetting. The resulting cell suspension consisted of approximately 90 %

keratinocytes and 1-3 % LC. These cells are referred to as "epidermal cells" in the text.

For cell cultures of epidermal cells, an additional digestion with DNAse I (Invitrogen, Karlsruhe, Germany; 15 min, 130 U/ml) was included. The resulting single cell suspension was cultured in complete medium for various times at a density of $1.5x10^6$ cells/ml in 6-well plates. These cells were either used for FACS-analyses of LC, or for isolation of highly pure LC for molecular analysis. To sort cells, the non-adherent cells were harvested and centrifuged on dense BSA as described (19). The low-density fraction contained 20-35 % LC, which were enriched by FACS sorting of vital MHC-II$^+$ cells using a FACSCalibur (Becton Dickinson, Heidelberg, Germany) to a purity >95 %.

Antibodies, flow cytometry and cell sorting

The following anti-mouse antibodies were obtained from BD Pharmingen (Heidelberg, Germany): Anti-mouse CD11c (clone N418), anti-CD16/32 (clone 2.4G2), anti-CD24 (clone M1/69), anti-CD40 (clone 3/23), anti-CD44 (clone IM7), anti-CD80 (clone16-10A1), anti-CD86 (clone GL1), and anti-I-A/I-E (clone 2G9). Cells were preincubated with unconjugated CD16/32 before staining to block Fc-receptors (except samples, where CD16/32 expression was analyzed). All stainings were performed for 15 min at 4 °C. Data were analyzed on a FACSCalibur flow cytometer with CellQuest software (BD Pharmingen).

Gene expression analysis with microarray

Total RNA was isolated with Trizol™ (Invitrogen) according to the manufacturers' instructions. For microarray analysis mRNA was amplified prior to chip-hybridization using the MessageAmp™ Kit of Ambion (Woodward St. Austin, USA). RNA was biotinylated (Enzo Bio Array™HighYield™ RNA transcript labeling kit (Affymetrix, High Wycombe, UK)) and purified. RNA was hybridized to MOE430A gene chips

(Affymetrix). The resulting *.chp files were analyzed with the bioconductor affy package using the RMA (robust microarray analysis) algorithm. Two independent experiments were performed. A two-fold expression difference was chosen as cut-off. Internal controls on the chip excluded a faulty preparation of the chips. The affymetrix calls are the result of a comparison of perfect match and mismatch intensities leading to the conclusion if a gene is detectable (present) or not detectable (absent). GeneExpressionOmnibus accession number is GSE9506.

Reverse transcription

For cDNA synthesis total RNA was treated with 1 U DNase I/0.5 µg RNA (15 min, RT). The reaction was stopped with 2 mM EDTA for 10 min at 65°C. 0.5 µg RNA was incubated in 10 µl with 1 µg of oligo $pd(T)_{15}$ primer for 5 min at 60°C. RNA was reversely transcribed in a final volume of 40 µl containing 1x RT buffer, 10 mM dithiothreitol, 1 mM dNTP, 80 U ´RNase out´ Ribonuclease Inhibitor and 400 U MLV reverse transcriptase. Reactions were carried out for 60 min at 37°C and were inactivated at 70°C for 10 min. All reagents were purchased from Invitrogen.

PCR

Real-time PCR was performed on a LightCycler™ (Roche Molecular Biochemicals, Swiss), in 20µl final volume, containing 10 µl QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 1 µM of each primer, 2 µl cDNA and RNase free water. Amplification conditions were 50 cycles of 15 sec at 94°C for denaturation, 20 s at 55°C (AhRR) and 58°C (CYP1A1, CYP1B1), respectively, for primer annealing, 30 s at 72°C for elongation and 2 s at 72°C for fluorescence detection.

Sequences of PCR-primers were as follows:

AhRR: 5´-GCCAATGCTGTCTAATGAAG-3´ and 5´-AACAGAGCACCAAGAAAACA-3´; CYP1A1: 5´-TCCTTGCATGTCCATGTTTC-3´ and

5´-TGCATAAGCAAAATACAGTCCA-3´; CYP1B1: 5´-GACCCGGATGTTTTGTGAAT-3´ and 5´-CATGGTGAGCAGCAAAAGAA-3´;

RPS6: 5´-ATTCCTGGACTGACAGACAC-3´and 5´-GTTCTTCTTAGTGCGTTGCT-3´. x-fold expression levels were calibrated to the expression of RPS6 as house-keeping gene in the same sample.

Analysis of AhRR expression in heart, brain, and LC was performed on a Trio-thermocycler (Biometra, Göttingen, Germany). The reaction volume of 50 µl contained 2 µl cDNA (see above), 2.5 U Taq DNA polymerase, 5 µl 10x PCR buffer (NatuTec, Frankfurt, Germany), 0.2mM concentration of dNTPs (NatuTec), and 0.4 µM concentration of each primer (Operon Biotechnologies, Köln, Germany). Amplifications were carried out for 5 min at 94°C, followed by 35 cycles for AhRR and 30 cycles for RPS6 of 1 min at 94°C for denaturation, 1 min at 54°C (AhRR) and 56°C (RPS6), respectively for primer annealing, 1 min at 72°C for elongation and 10 min at 72°C after the last cycle.

Phagocytosis assay

Freshly isolated epidermal cells from wild-type and $AHR^{-/-}$ mice were resuspended in PBS/10 % FCS and incubated with 0.5 mg/ml of FITC-dextran (Mr = 40.000, Sigma) for 45 min at 4°C or 37°C. Cells were washed three times with ice-cold PBS containing 1 % FCS. After staining with PE-conjugated anti-I-A/I-E mAb viable LC were analyzed by flow cytometry. The percentage of MHC+/FITC+ living cells was determined.

GMCSF measurement

Epidermal cells were isolated as described above and seeded at 10^6 cells/ml. Cells were cultured for 48 hours. GMCSF concentrations in the supernatants were determined by ELISA (ebioscience, San Diego, USA) according to the manufacturers' instructions.

Cultivation of epidermal cells with GMCSF

Epidermal cells were seeded out at a concentration of 10^6 cells/ml and cultured with 10 ng/ml GMCSF. After 48 hours cells were harvested and analyzed by flow cytometry.

Statistical analysis

Paired student's t-test or the Mann-Whitney test were used to assess statistical significance of data. P-values below 0.05 were considered as significant.

Results

The AhR is expressed in epidermal Langerhans cells

The AhR is expressed at varying levels in many tissues and cell types, including lymphoid organs like thymus and spleen. The human keratinocyte cell line HaCaT shows a pattern of increased AhR expression correlated to differentiation, and AhR expression in dermal fibroblasts has been described as well. Here, we demonstrate for the first time AhR expression in Langerhans cells. Easily detectable by RT-PCR, LC express the AhR at similar levels as liver and thymus (Fig. 1). Note that epidermal cells (of which ~ 90% are keratinocytes) express the AhR at very high levels.

AhR target genes are induced in epidermal cells after *in vivo* TCDD treatment

We first analyzed whether typical AhR-target genes are induced *in vivo* in skin cells after systemic exposure to the AhR activating ligand TCDD. Mice were injected with a moderate, non-toxic dose of 10 µg/kg body weight TCDD and epidermal cells were isolated after different time points to analyze expression of CYP1A1, CYP1B1 and AhR repressor (AhRR) by RT-PCR. As shown in Fig. 2, no induction of either gene was detectable 3 hours after TCDD injection, whereas CYP1A1 transcription levels increased more than 60-fold after 12 hours. 24 hours after treatment, CYP1A1 in epidermal cells was 78-fold higher compared to untreated animals. Similarly, CYP1B1 was up-regulated 12 and 24 hours after TCDD exposure (10-fold and 22-fold induction, respectively) as well as the AhRR (2-fold and 12-fold). As induction of all genes was most prominent at 24 hours we chose this time point for gene expression profiling of purified LC.

Expression of the AhRR in epidermal LC

Both the AhR and the AhRR transcripts are detectable in LC by chip-analysis (Table I) and RT-PCR. AhR signalling is regulated by the AhRR in a negative feedback fashion. The AhRR competes with the AhR in dimerization with AhR nuclear translocator (ARNT) to form a transcriptionally inactive complex (3). Note that ARNT expression is low in LC (Table I), and AhRR expression is constitutively high in LC (see Table I). Brain and heart are the organs with the highest constitutive expression of AhRR transcripts known so far (21), and AhRR expression in LC was even higher than in those tissues (Fig. 4), and above the expression level of housekeeping gene RPS6. Interestingly, expression is not further increased after TCDD exposure. The high constitutive expression of the AhRR in LC conceivably explains the unresponsiveness of LC on mRNA level towards TCDD. Note that AhRR expression is lower, but not absent in LC of AhR-deficient mice in comparison to wild-type counterparts. (Fig.4 compare lanes 3 and 4), supporting the notion of an interdependency of AhR and AhRR expression levels.

Impaired maturation competence of LC in AhR$^{-/-}$ mice

Dependent on their differentiation and maturation status, LC express surface proteins necessary for stimulation of T cells (e.g. MHC class II, CD24, CD40, CD80 and CD86). LC have been shown to mature during *ex vivo* culture of epidermal cells (19). Therefore we analyzed expression levels of maturation markers on the cell surface of freshly isolated ("immature") LC and on LC after three days of culture ("mature") of the epidermal cells from either wild-type or AhR$^{-/-}$ mice.

Treatment of cells for 10 min with trypsin did not impair antibody staining, indicating that the surface markers described here are not sensitive to the treatment necessary for preparing epidermal sheets/cell suspension (data not shown).

Figure 5 shows representative histograms of the expression of co-stimulatory molecules CD24, CD40, CD80, and CD86 on mature LC from vehicle treated wild-type and $AhR^{-/-}$ mice. In Table IIa the mean fluorescence intensities of these markers, are shown on mature and immature LC, comparing LC from wild-type and $AhR^{-/-}$ mice (Table IIa), and LC from vehicle treated mice and mice with TCDD over-activated AhR (Table IIb and c).

All markers tested, except CD24, had similar basal expression levels on immature LC. CD24 was expressed significantly lower on immature as well as on mature $AhR^{-/-}$ LC compared to wild-type LC. As expected, maturation of LC during *ex vivo* culture of epidermal cells led to strong up-regulation of co-stimulatory molecules CD40, CD80, and CD86 in wild-type LC. MHC II was also up-regulated (data not shown); in accordance with literature data CD24 expression decreased (22). Qualitatively, LC from AhR-deficient mice up-regulated co-stimulatory molecules after three days of maturation in culture as well. Quantitatively though, in comparison to the wild-type mice, the increase was significantly lower during maturation for $AhR^{-/-}$ mice (Table IIa, compare upper and lower row) for CD80. For CD40 a similar trend was seen in three independent experiments, albeit statistical significance was not reached.

Changes in LC maturation after over-activation of the AhR in vivo

We next asked whether strong activation of the AhR by its ligand TCDD also affects the phenotype of immature and mature LC. Expression of co-stimulatory molecules was analyzed 24 hours after *in vivo* exposure of mice with 10 µg/kg body weight TCDD i.p. or solvent alone. As might be expected from the unusual

unresponsiveness of the LC transcriptome to TCDD (see above), the phenotype of LC was not affected by TCDD exposure, i.e. the maturation-associated surface expression of co-stimulatory molecules remained normal (compare rows for "control" with rows "TCDD" in Table IIb). A single exception was the expression of CD86, which increased moderately after TCDD treatment in mature LC of wild-type mice (Table IIb). As expected, LC from AhR$^{-/-}$ mice did not respond at all to TCDD exposure (Table IIc).

Morphology and phagocytic capacity of LC from AhR deficient mice

In addition to the expression of surface markers we analyzed how the AhR affects size and granularity of LC. It is well known that LC change their morphology during maturation, and become larger and more granular (19). Figure 6 shows the relative granularity (C, D) and size (E, F) of immature and mature LC from AhR$^{-/-}$ and wild-type mice, and of the morphology after TCDD-exposure in mice of both genotypes. Congruent with the data on the surface marker expression, also the morphology of LC differed between AhR$^{-/-}$ and wild-type mice after *in vitro* maturation. No significant changes of LC from TCDD-treated compared to control mice were observed. However, in the absence of a functional AhR, immature and mature LC were generally smaller, and remained of significantly lower granularity after maturation.

We analyzed the phagocytic capacity of LC after culture as a marker for functional immaturity. LC from AhR$^{-/-}$ maintained a significantly higher phagocytic activity, indicating again an AhR-dependency of maturation (Fig. 7).

In conclusion, results from surface marker expression, morphology and phagocytic capacity of LC are suggestive of a previously unconsidered role of the AhR in maturation of LC, either cell autonomously or through by-stander effects of AhR$^{-/-}$ keratinocytes in the culture.

GMCSF secretion is diminished in epidermal cells of AhR$^{-/-}$ mice

GMCSF is one of the key cytokines for LC survival and differentiation (23). GMCSF is produced and secreted by keratinocytes in a paracrine fashion, and GMCSF plasma levels are known to be enhanced by TCDD (24). Therefore we analyzed the supernatants of cultured epidermal cells (containing appr. 90% keratinocytes) from AhR$^{-/-}$ and wild-type mice by ELISA. Results are shown in Fig. 8A. Epidermal cells from wild-type mice secreted more GMCSF (mean 40 pg/ml) compared to cells isolated from AhR$^{-/-}$ mice (mean 18 pg/ml). GMCSF is pivotal for up-regulation of CD80 of maturing LC. As described above, AhR$^{-/-}$ LC are impaired in up-regulation of co-stimulatory molecules, including CD80. We cultured epidermal cells from AhR$^{-/-}$ cells in the presence of GMCSF and measured morphology and CD80 expression. As shown in Fig. 7B, addition of GMCSF to cultures completely normalized CD80 expression of AhR$^{-/-}$ LC during maturation in culture. Note that CD80 expression even increased compared to that of untreated wild-type cells, reflecting the higher GMCSF concentration used (10 ng/ml) compared to natural secretion by keratinocytes (40pg/ml, see Fig. 7A). In contrast to CD80 expression, low granularity and size of these cells were not affected by addition of GMCSF to epidermal cell cultures.

Discussion

The skin is metabolically active and epidermal cells express the enzymes necessary for degradation of small molecular weight chemicals, which can be(-come) haptens (25). Many of these enzymes (xenobiotic metabolizing enzymes, XMEs) are under transcriptional control of the aryl hydrocarbon receptor, AhR. We show here for the first time that primary LC express the AhR in the cytosol at high levels. AhR expression in keratinocytes and fibroblasts had also been reported in the literature; upon activation with appropriate ligands, the AhR leads to induction of typical XME genes, *CYP450 1A1* and *CYP450 1B1* in these tissues, (26). Surprisingly, LC were different, i.e., no XME was inducible after exposure *in vivo* to the strong AhR ligand TCDD. Moreover, in contrast to keratinocytes, constitutive expression of XMEs was absent or at least below detection limit (by affymetrix "calls" or RT-PCR) in primary LC, isolated directly from skin.

We here analyzed the effects of AhR over-activation and AhR-deficiency in LC and epidermal cells with a view to potential tolerogenic strategies of LC/the skin. To our knowledge, only one study addressed the effects of AhR-activation on LC until now. In 1989 Puhvel et al. (11) investigated density and morphology of LC in HRS/J mice, a murine model for skin effects of TCDD. They reported that LC from hairless mice were smaller, and had fewer dendritic protusions than controls. Extending these early studies, we show that (i) the AhR is present, but functionally shut down in LC and cannot be activated to induce metabolic enzymes, even by a very strong stimulus, and (ii) that beyond its role in metabolic degradation of chemicals, the AhR appears critical in controlling maturation of LC.

The LC transcriptome was inert to TCDD exposure, although it is known that more than 3000 genes have AhR-responsive elements (so-called DREs) in their promoters (27,28). Such unresponsiveness is in contrast to findings in numerous other cell types, including DC (29). Our finding highlights once again the cell-and context specific use of the AhR (30,31). Transgenic mice with a constitutively active AhR develop skin lesions accompanied by inflammation and immunological imbalances resembling typical atopic dermatitis (32). Obviously, LC need to control strictly the exogenous over-activation of the AhR.

Based on this data it has been suggested that blocking of the AhR signal may help to relieve allergic skin symptoms, as many xenobiotics, including those metabolized via the AhR pathway can exacerbate allergic reactions (25). Our data shows that LC already employ this strategy. We found that they abundantly express the AhR repressor, AhRR, at levels higher than in most other tissues (unpublished observations). Interestingly, normal human fibroblasts also display high AhRR expression and no inducibility of CYP1A1 (33). Repression is thought to work via competition for the AhR dimerization partner ARNT. AhRR expression was not inducible further upon exogenous AhR activation (data not shown). Possibly, this maximal expression is driven by an endogenous ligand, which activates the AhR. 6-formylindolo[3,2-b]carbazole (FICZ), a ligand formed in skin from tryptophane by UV light exposure, would be a candidate (34). Our data are congruent with the hypothesis that LC keep AhR-mediated XME induction low to reduce the risk associated with metabolization of small molecular weight chemicals. However, the surrounding keratinocytes may yet generate haptens or haptenated peptides, which can be taken up by LC, and either channelled into the exogenous MHC-II pathway, or by cross-presentation into the MHC-I pathway (35).

However, the fact that LC express the AhR at all, points to a physiological role of the AhR pathway for LC, independent of XME regulation. As the AhR is known as an inducer of cell differentiation, we analyzed immature and mature LC from AhR-deficient mice in epidermal cell cultures. Three relevant parameters of LC maturation were analyzed, namely up-regulation of co-stimulatory molecules, size and scatter, and phagocytic capacity. Our data provides evidence that in the absence of the AhR in epidermal cells, LC maturation is impaired. Interestingly, the number of LC/mm^2 was unaffected in skin (data not shown).

Note, that AhR deficiency led to low CD24 expression, while AhR over-activation induced CD24 (see also reference 8). A lower constitutive CD24 expression in AhR-deficient mice compared to wild-type mice could reflect the presence of an endogenous ligand (which would drive AhR activity in normal mice), as already suggested above. Considering the LC´s inertness towards AhR-ligands, we think it is likely, that non-ligand-dependent activities of the AhR are more relevant or more prominent in LC. Several authors have suggested functions of the AhR beyond its ligand-activated transcription factor status, for instance by direct contact and interaction with NF-kappaB (reviewed in 36). Thus, we suggest that LC need the AhR for proper maturation, yet simultaneously control its risk of activating metabolizing enzymes after contact with environmental chemicals as potential ligands. While maturation may be influenced by signals from surrounding keratinocytes (which are affected by AhR absence in those cells), the latter appears to be an intrinsic effect in LC as our microarray data suggests.

Up-regulation of co-stimulatory molecules and morphological changes are critical features of LC maturation and their function as potent antigen presenting cells. In particular, the expression of CD24 and CD80, both significantly low in AhR-deficient LC, might reduce their capacity to stimulate Th1 responses. Splenic lymphocytes

from $AhR^{-/-}$ mice produce more IFNγ and IL-12 after ovalbumin immunization than $AhR^{+/+}$ mice (37). Indeed, a number of cytokines can be regulated by AhR signalling, including IL-2 in T cells and TNF-α, and IL-1ß in keratinocytes (38,39). It is therefore not too surprising that epidermal cells secreted less GMCSF in AhR-deficient cultures, in particular as the gene has four putative DREs in its promoter (28). GMCSF is relevant for LC maturation, i.e. CD80 expression levels (40). While the CD80 phenotype of $AhR^{-/-}$ LC could be rescued by GMCSF, the morphological changes persisted. Apparently, other factors are lacking as well in $AhR^{-/-}$ mice, which warrant further investigation.

In conclusion, we provide evidence for a critical role of the AhR in LC maturation and suggest that the AhR is part of the tolerogenic strategy of LC against haptenic chemicals. These findings contribute to a better understanding of allergic skin immune responses, and their medical management.

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Figure legends

Figure 1. *Epidermal LC express the AhR*

RNA from epidermal cells, LC sorted to >95% purity from back skin, thymus and liver was prepared and RT-PCR was performed. Shown is the expression level relative to liver, a tissue known to express AhR highly and robustly.

Figure 2. *Induction of typical target genes in eC after TCDD treatment.*

Mice were injected i.p. with 10 µg/kg TCDD or vehicle (DMSO in corn oil) alone. At the indicated time points epidermal cells were isolated from ears and mRNA expression of CYP1A1 (black bars), CYP1B1 (white bars) and AhRR (hatched bars) was analyzed by semi-quantitative PCR in a light cycler™. Shown is the n-fold induction in cells from TCDD treated mice compared to expression in eC from vehicle treated mice. The bars are representative for 2 independent experiments.

Figure 3 *Transcriptome of LC after TCDD treatment*

A. LC were enriched as described in Material and Methods and sorted by FACS. Viability and purity were controlled by trypane blue staining and FACS analysis. Purity was >95%.

B. Affymetrix Microarray was performed from LC after TCDD and vehicle treatment. Mice were injected i.p. with 10 µg/kg TCDD or vehicle alone for 24h. LC were isolated and purified > 95%. mRNA and microarrays were prepared and analyzed as described in Material and Methods. The scatter-plot shows the average of two

independent LC isolations. The lines indicate where expression falls within the border-threshold of <2-fold expression change between TCDD-treated and control transcription levels.

Figure 4. *LC have a high constitutive expression of the AhRR.*

mRNA was prepared, reversely transcribed, and amplified by PCR for AhRR and the RPS6 house-keeping gene. (1) heart; (2) brain; (3) LC isolated from wild-type mice; (4) LC isolated from AhR ko mice. S, 100 bp marker; N, negative control without DNA in PCR. The picture is representative for 3 independent experiments.

Figure 5. *The expression of co-stimulatory molecules on mature LC is dependent on the AhR.*

WT (white curve) and $AhR^{-/-}$ (grey curve) mice, respectively were injected with vehicle. After 24h mice were sacrificed epidermal cells were isolated as described in Material and Methods and were further cultured for 72h. Cells were harvested, LC were enriched over BSA-density gradient centrifugation, stained with described antibodies and analyzed with FACS. Shown are representative data from three to four independent experiments. Significances were calculated by Student´s t-test.
(*, $p < 0.05$)

Figure 6. *The morphology of LC is dependent on the AhR expression.*

WT and $AhR^{-/-}$ mice, respectively were injected with TCDD or vehicle. Twenty-four hours later epidermal cells were isolated and analyzed in the FACS. An aliquot of cells was ex vivo cultured for 72h and analyzed again. Cells were gated on MHC-II (i.e. for LC) and scatter characteristics analyzed. Data are from at least four

independent experiments. ■ wild-type; ▲ AhR$^{-/-}$ mice Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$)

Figure 7 *LC from AhR$^{-/-}$ mice retain high phagocytic capacity in maturation conditions*
Freshly isolated epidermal cells were cultured at 4° or 37°C with FITC-dextran beads and then stained with anti-MHC-II antibodies. Shown is the percentage of FITC+/MHC-II+ cells after 45 minutes of uptake.

Figure 8 *GMCSF-production in AhR$^{-/-}$ epidermal cells*
Epidermal cells were prepared from back skin and cultured for 48 hours. (A) Supernatants were tested for GMCSF-content by ELISA. (B) GMCSF-was added to epidermal cells from AhR-/- mice, and cells cultured for 48 hours. The mean fluorescence index (MFI) of CD80 was determined.

Table I
Expression of AHR pathway-related genes and typical target genes coding for xenobiotic metabolizing enzymes Langerhans cells

Probe Set ID	Gene	log 2 expression		affymetrix calls[b]
		Control[a]	TCDD	
1422631_at	AHR	8.63	8.41	P
1421721_a_at	Arnt	6.01	5.99	M
1420796_at	AHRR	8.93	8.88	P
1422217_a_at	CYP1A1	5.84	5.76	A
1450715_at	CYP1A2	5.49	5.36	A
1416613_at	CYP1B1	4.37	4.15	A
1421041_s_at	GST α2	4.95	4.93	M
1423436_at	GST α3	4.23	4.29	A
1416368_at	GST α4	6.39	6.40	A
1418752_at	AIDH3 α1	4.11	4.05	A
1423627_at	Nqo1	6.31	6.20	A

a) RNA from Langerhans cells, sorted to >95% purity was prepared and hybridized to an affymetrix MOE430A chip. Data were analyzed as described in material and methods. The average of two independent LC isolations and microarray chip experiments is shown.
b) P = present; M = marginal; A = absent

Table IIa Influence of absence of functional AHR on LC phenotype in immature versus mature cells[a]

	CD16/32		CD24		CD40		CD44		CD80		CD86	
	Immature	Mature	Immature	Mature	Immature	Mature	Immature	Mature	Immature	Mature	Immature	Mature
Wt	14[b]	2[c]	156	122	13	188	n.d.	75	2	99	13	458
AhRko	13	2	**60**[**c]	**56***	9	142	n.d.	86	2	**42***	11	401

[a] Single cell suspension were prepared from ear or skin epidermis, and either immediately ("immature") analyzed by FACS or cultivated for three days to allow maturation of LC ("mature"). See Material and methods for details. Cells were stained MHC II and the indicated surface markers. LC were identified by MHC II expression and analysed for the indicated surface markers. The cell suspension consisted of mainly keratinocytes, some other epidermal cells and 1-3% LC. Scatter characteristics were used to exclude dead cells from the analysis. As controls unstained and isotype control stained cells were used. Cultivation and stainings were done independently 3 to 5 times

[b] channel of mean fluorescence (MFI), determined by FACS.

[c] The significance of values between C57BL/6 mice and AHR$^{-/-}$ mice was calculated by student´s t-test and is indicated by asterix and bold letters. * $p<0.05$; ** $p<0.005$; *** $p<0.0001$. Values differing significantly between immature and mature LC were also calculated and are highlighted in by the grey field.

Table IIb Effect of TCDD exposure on LC phenotype in C57BL/6 mice

	CD16/32		CD24		CD40		CD44		CD80		CD86	
	Immature[a]	Mature	Immature	Mature	Immature	Mature	Immature	Mature	Immature	Mature	Immature	Mature
Control	14[b]	2[d]	156	122	13	188	n.d.	75	2	99	13	458
TCDD	14	3	162	117	14	179	n.d.	84	3	118	13	**549***

for a,b see Table IIa

[d)] The significance of values in LC from TCDD treated and control group mice was calculated by student´s t-test and is indicated by asterix and bold letters. * $p<0.05$; ** $p<0.005$; *** $p<0.0001$. Values differing significantly between immature and mature LC were also calculated and are highlighted in by the grey field.

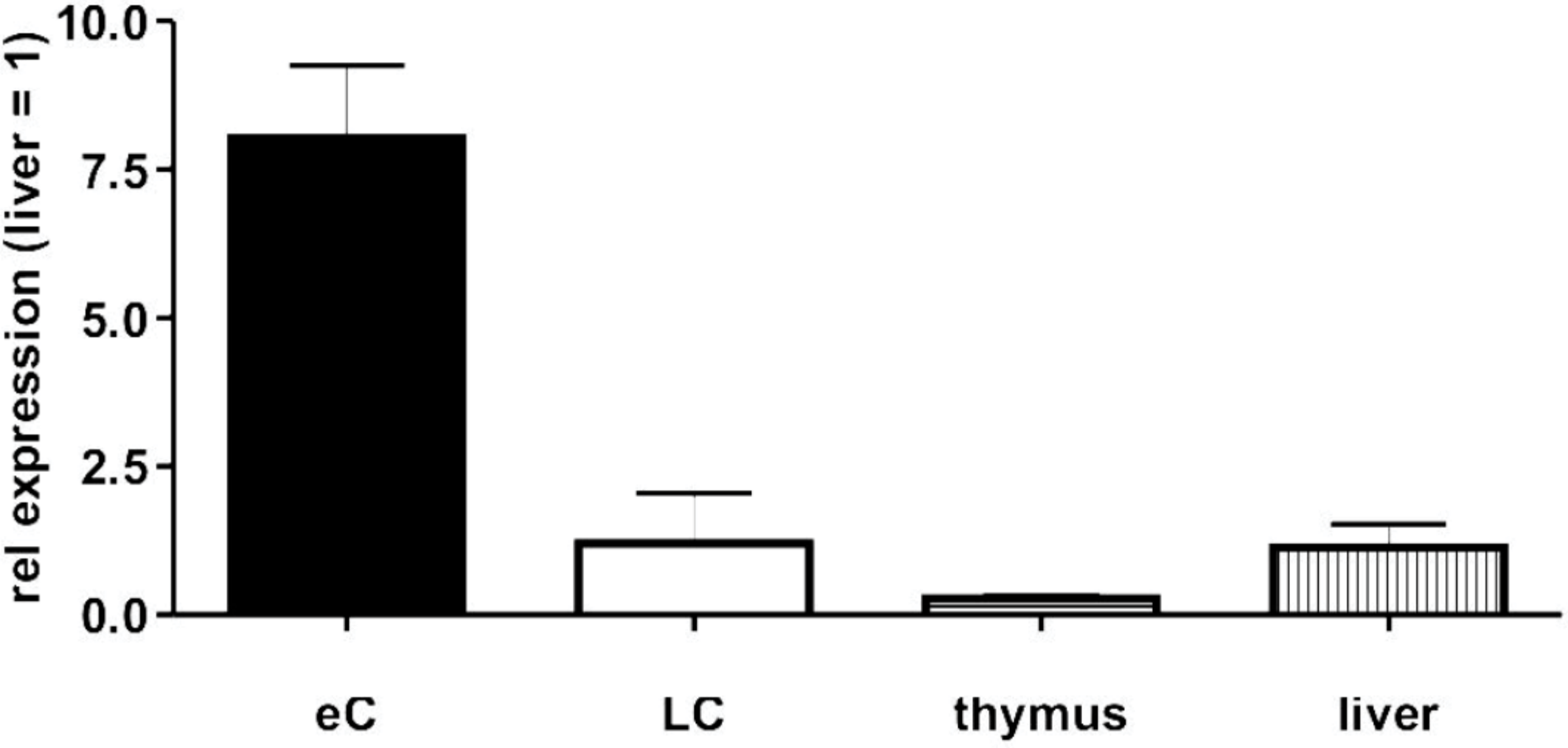
Table IIc TCDD exposure does not affect LC in AHR$^{-/-}$ mice

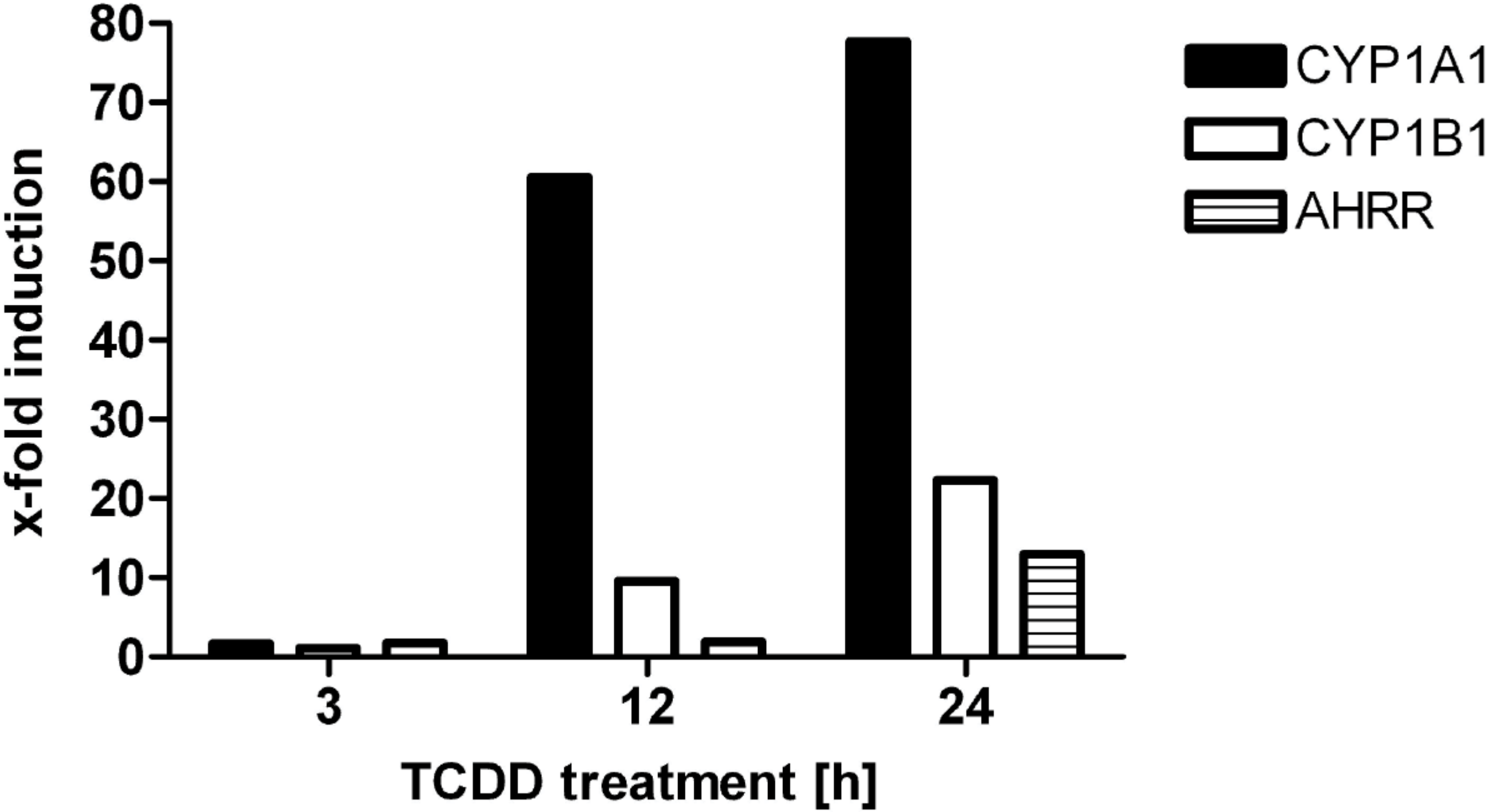
	CD16/32[a)]		CD24		CD40		CD44		CD80		CD86	
	Immature	Mature	Immature	Mature	Immature	Mature	Immature	Mature	Immature	Mature	Immature	Mature
Control	13[b]	2[e]	60	56	9	142	n.d.	86	2	42	11	401
TCDD	14	2	45	47	8	134	n.d.	68	2	54	9	440

For a,b see Table IIa

[e] The significance of values in LC from TCDD treated and control group mice was calculated by student´s t-test and is indicated by asterix and bold letters.
* $p<0.05$; ** $p<0.005$; *** $p<0.0001$. Values differing significantly between immature and mature LC were also calculated and are highlighted in by the grey field.

Fig. 1




80
70
60
50
40
30
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x-fold induction
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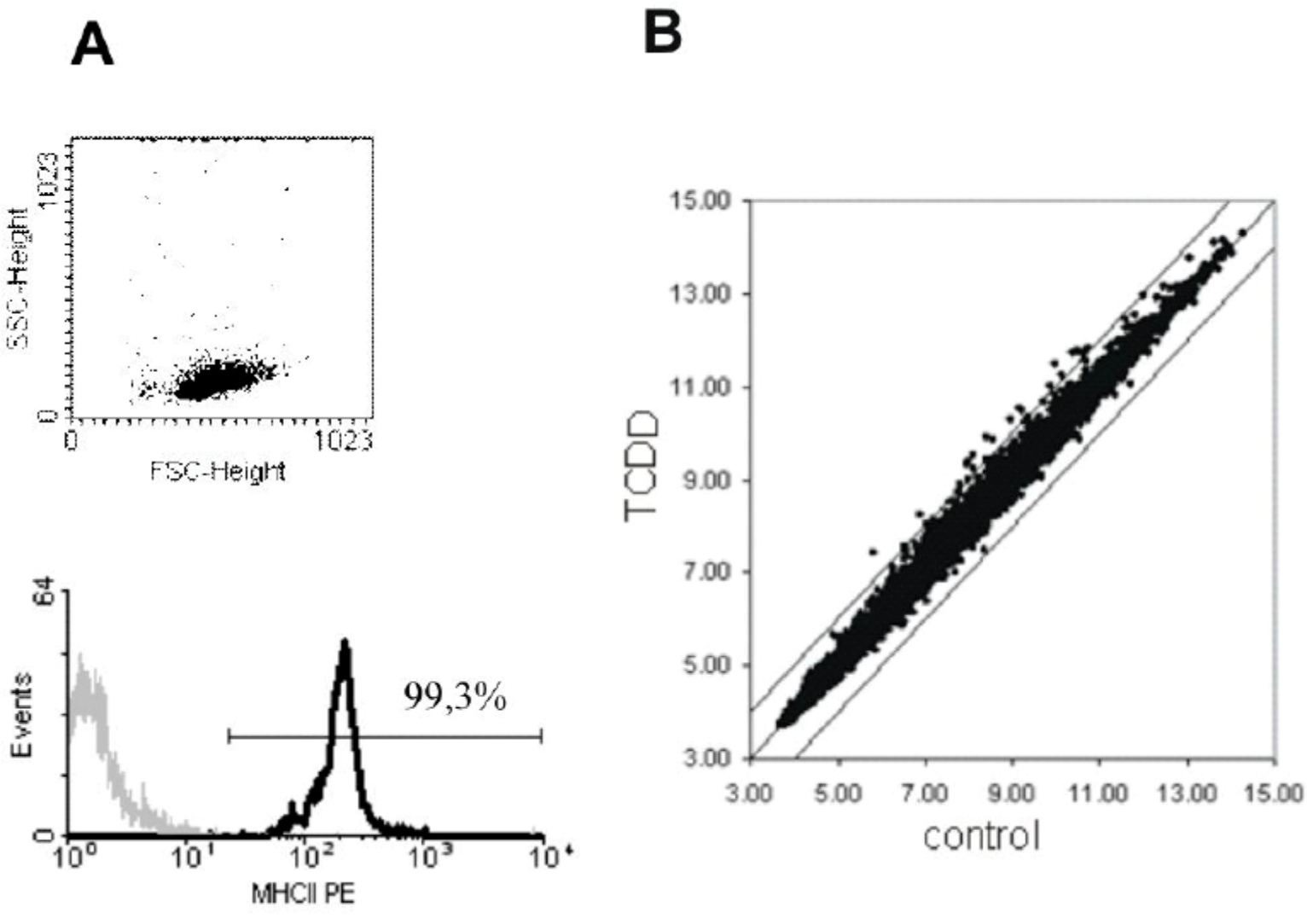
12
24
TCDD treatment [h]
CYP1A1
CYP1B1
AHRR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10^0
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MHCII PE
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control

Figure 4

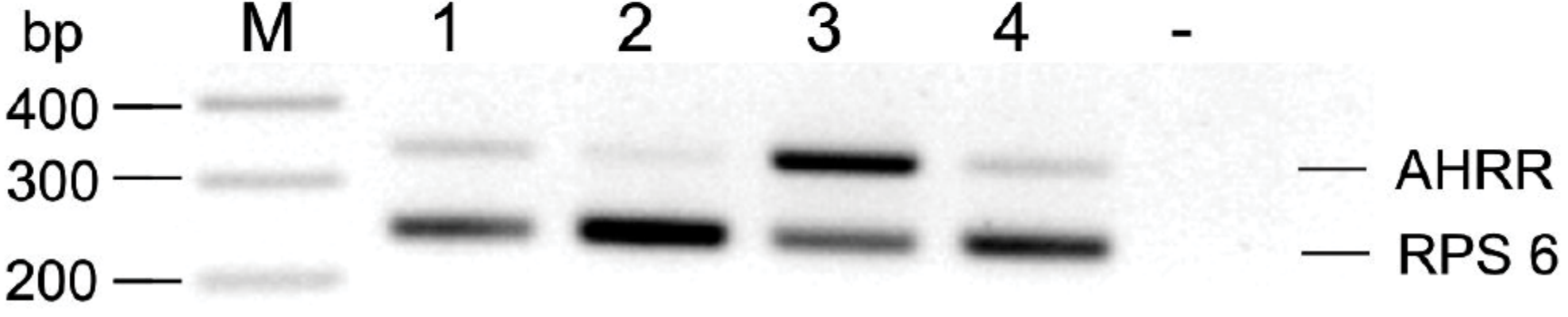
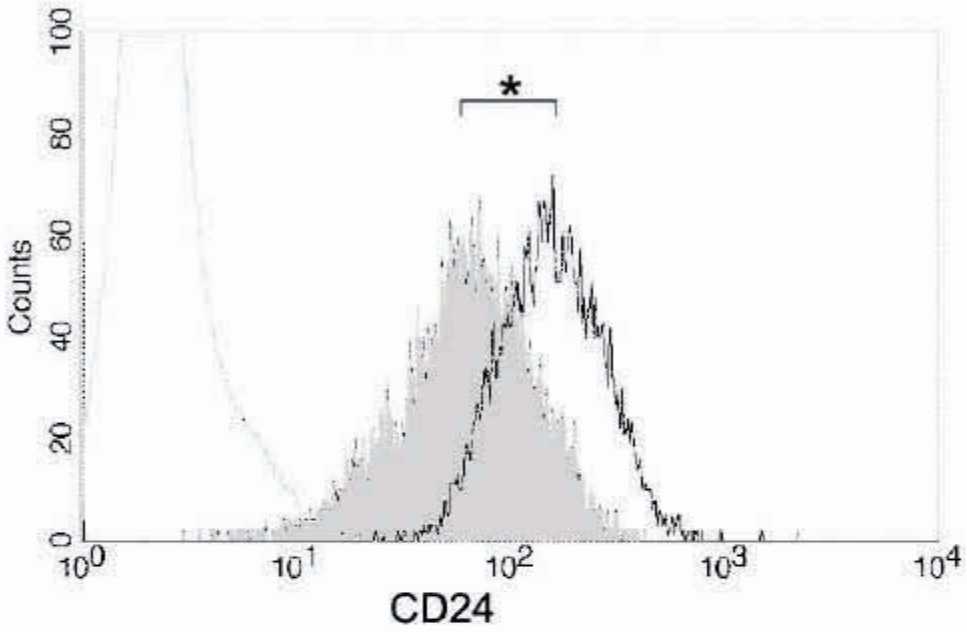


Figure 5


Counts
0
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40
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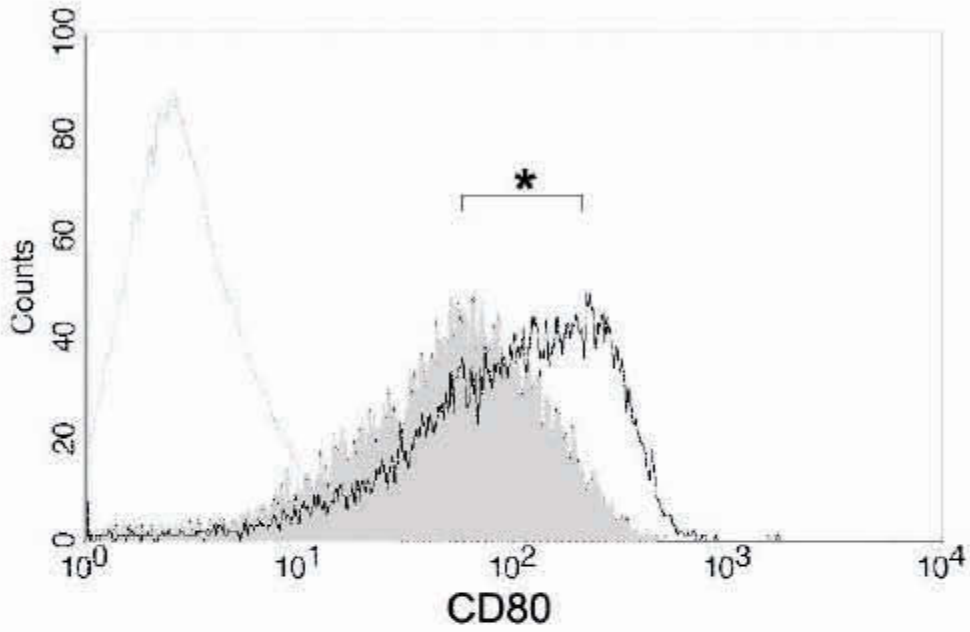
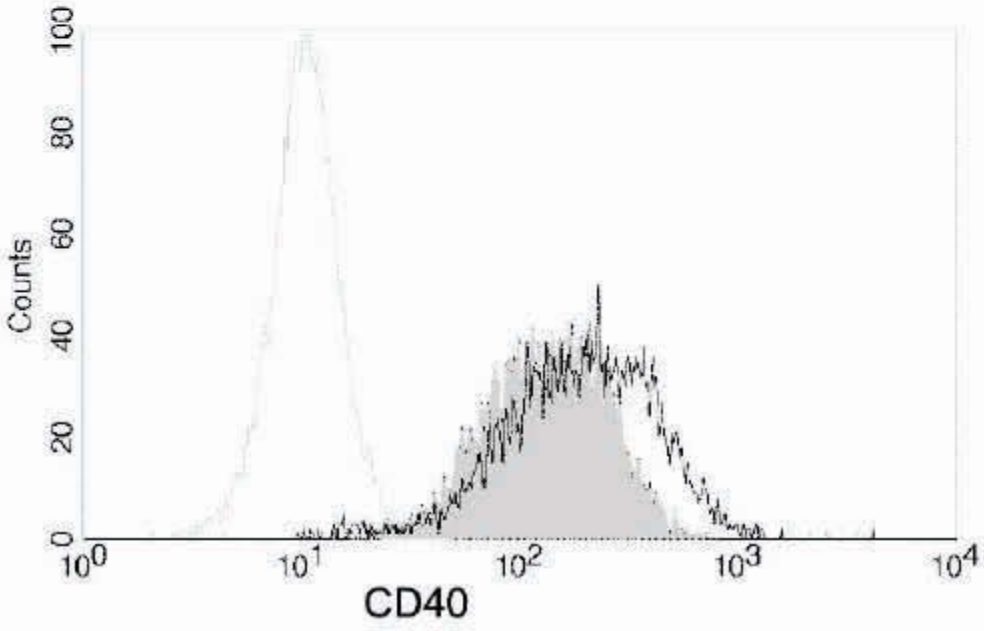
10^4
CD24


Counts
0
20
40
60
80
100
*
10^0
10^1
10^2
10^3
10^4
CD80


Counts
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40
60
80
100
10^0
10^1
10^2
10^3
10^4
CD40

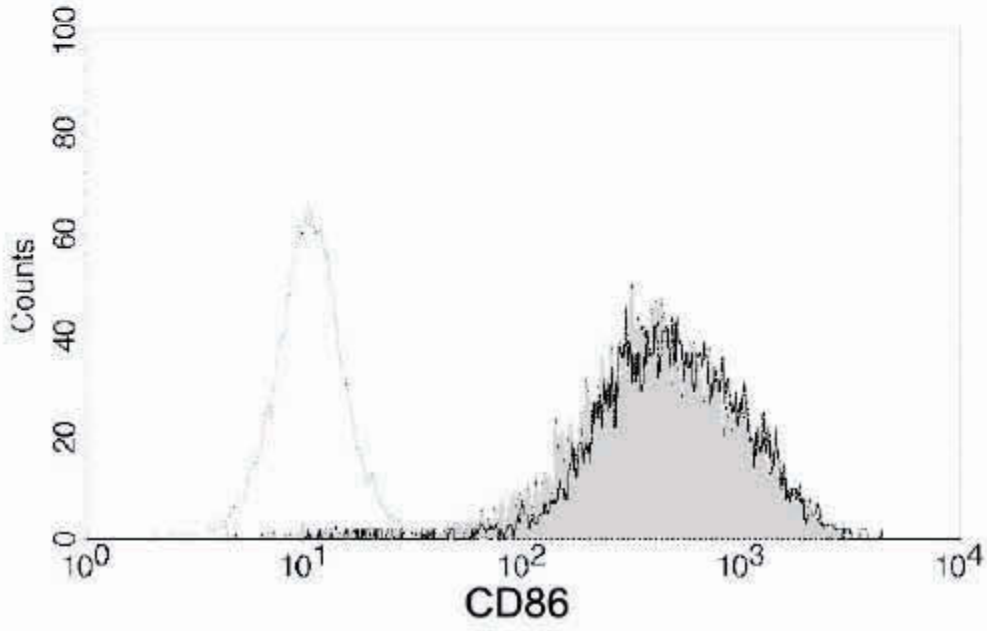

Counts
0
20
40
60
80
100
10^0
10^1
10^2
10^3
10^4
CD86

Figure 6

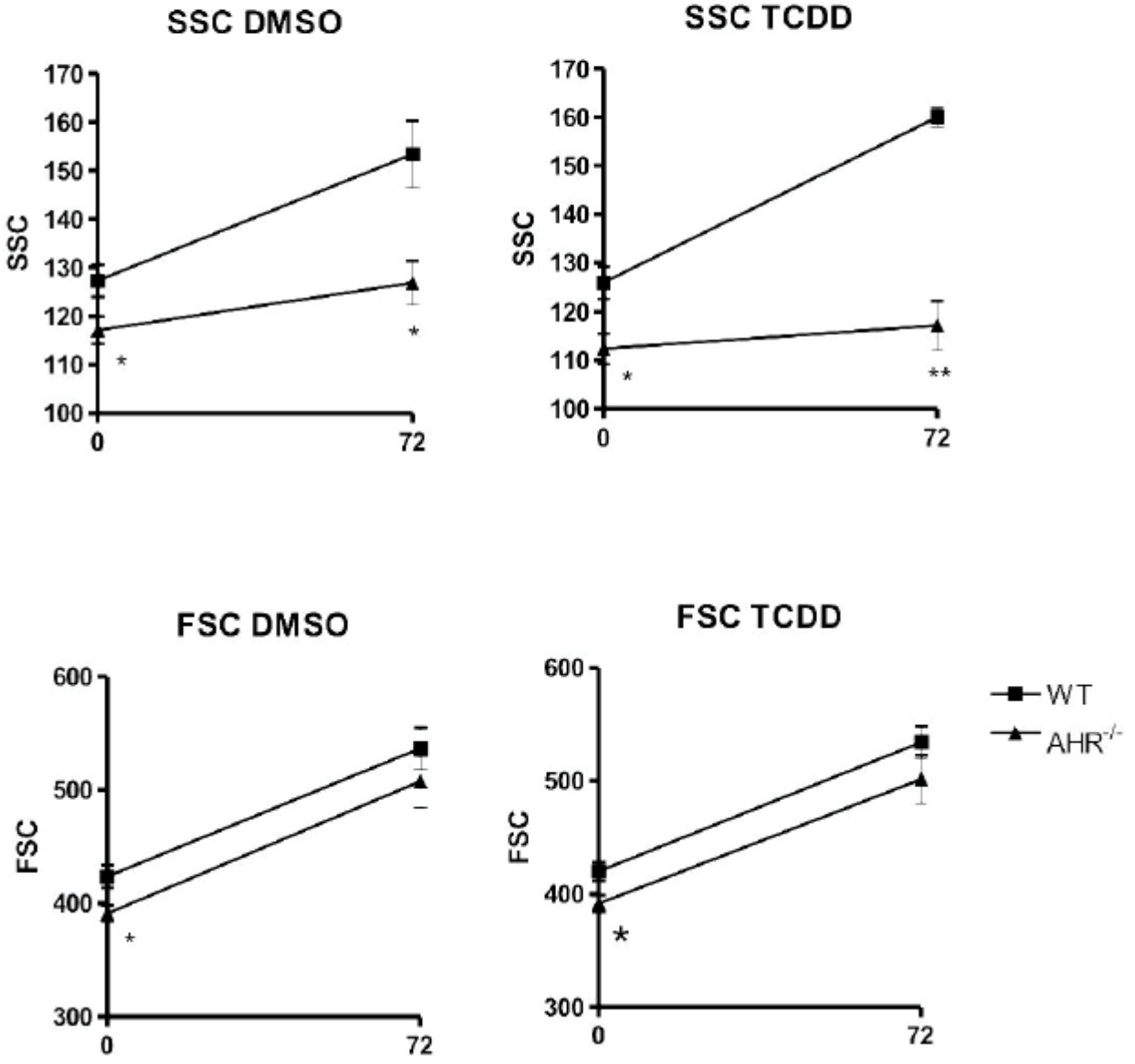

SSC DMSO
SSC TCDD
FSC DMSO
FSC TCDD
SSC
FSC
170
160
150
140
130
120
110
100
600
500
400
300
0
72
*
**
WT
AHR-/-

Figure 7

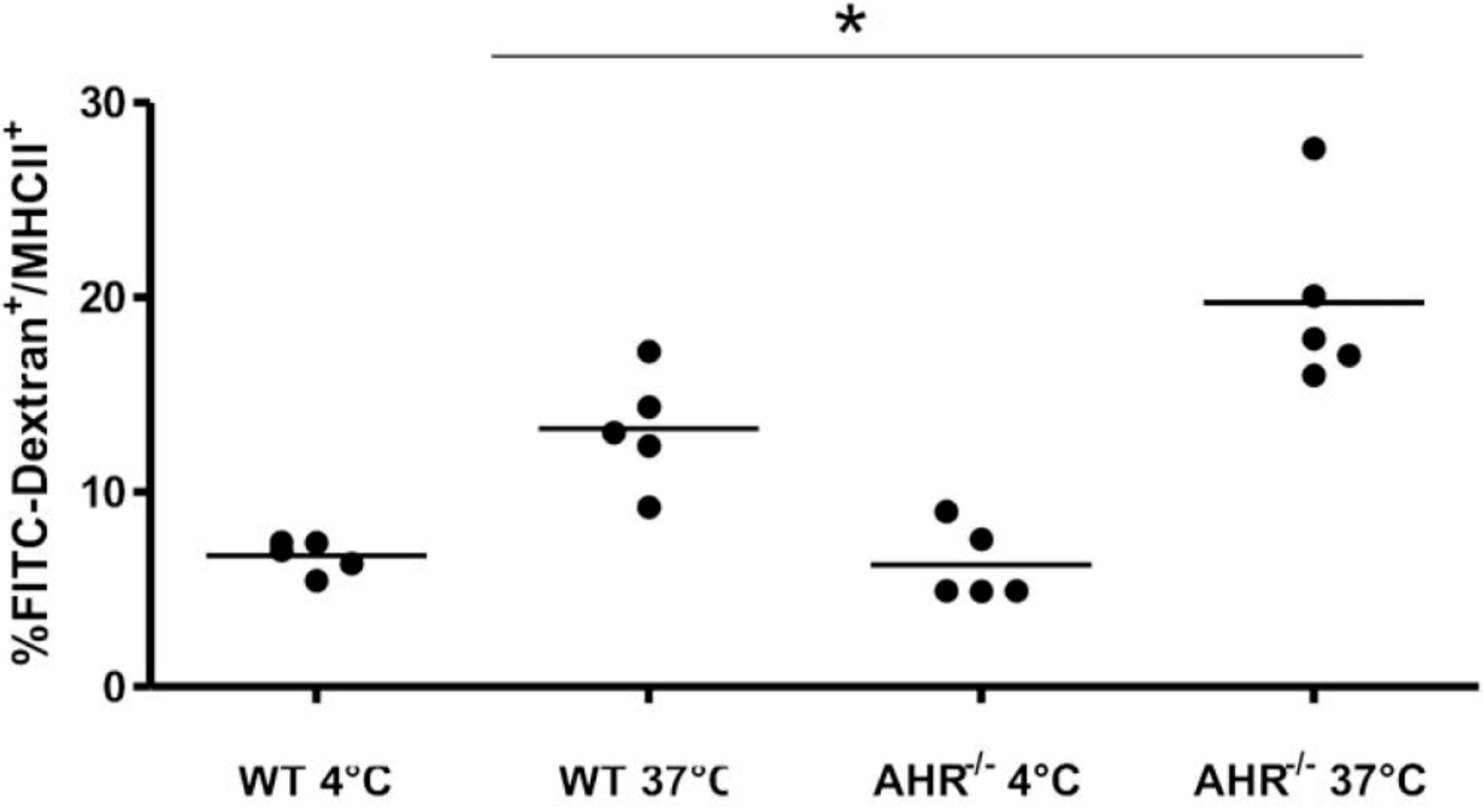
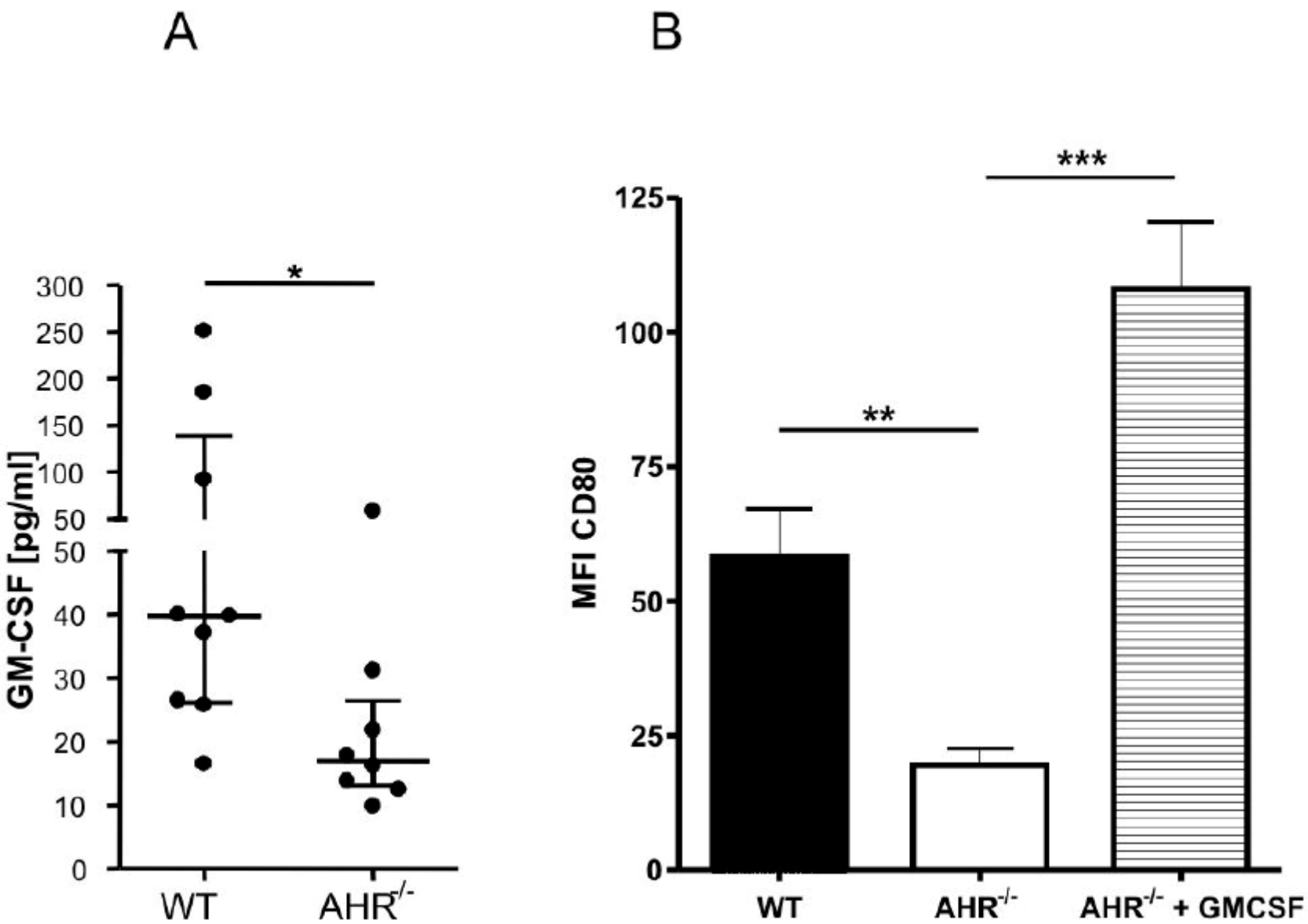


Figure 8


A
B
GM-CSF [pg/ml]
300
250
200
150
100
50
50
40
30
20
10
0
*
WT
AHR-/-
MFI CD80
125
100
75
50
25
0
**
***
WT
AHR-/-
AHR-/- + GMCSF

Anhang B

Inhalt der beigefügten DVD

Zu den als Erstautor veröffentlichten Daten gehören, neben den in Anhang A abgedruckten Veröffentlichungen, auch umfangreiche in elektronischer Form veröffentlichte Daten. Aus diesem Grund wurden die zusätzlichen Figuren und Tabellen, mit Erlaubnis der Verlage, der Arbeit in Form einer DVD beigefügt.
Die DVD enthält darüber hinaus die vollständige Datenbank NC-GED. Dazu gehören die normalisierten Datensätze, eine Auswertung aller zu den jeweiligen Experimenten zählenden Bedingungen differentieller Genexpression sowie eine Dokumentation der Datenbank in englischer Sprache.
Im Folgenden wird der Inhalt der DVD noch einmal in Form der Verzeichnisstruktur dargestellt.

Verzeichnisstruktur der DVD:

Veröffentlichungen

- Erstautorenschaften
  - 2.1.1 - Transcriptional signatures of immune cells in aryl hydrocarbon receptor (AhR)- proficient and AhR-deficient mice
    (Für diese Veröffentlichung erteilte der Verlag keine Erlaubnis zur elektronischen Veröffentlichung, weshalb sie nur in gedruckter Form angehängt wurde)
  - 2.1.2 - Microarray analysis of the AhR system: tissue-specific flexibility in signal and target genes
    Veröffentlichung in elektronischer Form (PDF)
    Elektronisch veröffentlichte Figuren und Tabellen
  - 2.1.3 - Transcription factor crosstalk controls transcriptional response to AhR-over-activation by TCDD in thymic epithelial cells
    Veröffentlichung in elektronischer Form (vorläufiges PDF)
    Elektronisch veröffentlichte Figuren und Tabellen

- Veröffentlichung als Ko-Autor
  - 2.2.1 - Role of the aryl hydrocarbon receptor in thymocyte emigration in vivo
    Veröffentlichung in elektronischer Form (PDF)
  - 2.2.2 - Detection of a novel population of fetal thymocytes characterized by preferential emigration and a TCR γδ+ T cell fate after dioxin exposure
    Veröffentlichung in elektronischer Form (PDF)
  - 2.2.3 - Impairment of maturational competence of cultured Langerhans cells from aryl hydrocarbon receptor (AhR) null mice and suppression of AhR signalling points to its role in LC tolerogenic strategy
    Veröffentlichung in elektronischer Form (vorläufiges PDF)

Datenbank NC-GED

Dokumentation der Datenbank in englischer Sprache (PDF)

- Rohdaten
  - Liste aller Verwendeten Microarrays
  - Normalisierte Expressionsdaten
    - Excel-Datei
      Da jede Excel-Arbeitsmappe auf 255 Spalten beschränkt ist, wuden die 1967 Expressionsprofile auf 8 Blätter verteilt (siehe Dokumentation)
    - Daten als Tab-getrennte Textfiles
      Enthält alle Datensätze in einer Textdatei
      Enthält alle Datensätze auf 8 Textfiles verteilt, deren erste Spalte jeweils der Affymetrix Referenz-ID entspricht
- Weiterführende Analysen
  - Range of Expression
    Diese Tabelle gibt Auskunft über die Expressionsstäreke, gemessen als mediane Fluoreszenzintensität, sowie Informationen über die Varianz der Expressionsstärke
  - Tissue-/cell type specific expression
    Im Rahmen der unter 2.1.2 vorgestellten Arbeit wurde die Expression Tausender Gene in einzelnen Zelltypen und Geweben analysiert. Diese Tabelle gibt für jedes analysierte Gewebe die mediane Fluoreszenz-

intensität, sowie deren Standardabweichung an. Die letzte Zeile gibt Auskunft über die jeweils in dem Gewebe verwendeten Arrays.

- Conditions of differential AhR-Expression

  Im Rahmen der unter 2.1.2. besprochenen Veröffentlichung wurde untersucht, in welchen in der Datenbank enthaltenen GEO-Serien der AhR zusammen mit mindestens 3 potentiellen AhR-Zielgen differentiell exprimiert wird. Hierzu wurde innerhalb der Serie die Bedingungen differentieller Expression identifiziert und jeweils die mittlere Expression sowie deren Standardabweichung berechnet. Anschließend wurde jeweils zwischen den einzelnen Bedingungen die differentielle Expression berechnet.

- Conditions of differential AhR target gene xpression

  Im Rahmen der unter 2.1.2. besprochenen Veröffentlichung wurde untersucht, in welchen in der Datenbank enthaltenen GEO-Serien mindestens 4 potentiellen AhR-Zielgen differentiell exprimiert wird. Hierzu wurde innerhalb der Serie die Bedingungen differentieller Expression identifiziert und jeweils die mittlere Expression sowie deren Standardabweichung berechnet. Anschließend wurde jeweils zwischen den einzelnen Bedingungen die differentielle Expression berechnet.

- Differentielle Expression innerhalb einzelner GEO-Serien

  Die Datenbank enthält 159312 Bedingungen differentieller Expression, also Bedingungen, in denen sich Behandlungen, Differenzierungsstadien oder Gendeletionen unterscheiden. Diese Bedingungen sind jeweils innerhalb ihrer Veröffentlichung aufgeteilt abgespeichert (siehe Dokumentation). Aufgrund der Begrenzungen durch Excel wurden jeweils 10 Experimente in einer Excel-Arbeitsmappe abgespeichert, die durchnummeriert in den in diesem Verzeichnis hinterlegten Ordnern vorliegen.

Anhang C

Zusätzliche Dateien der Paper und die Datenbank NC-GED auf DVD

Danksagung

Ich danke Frau Prof. Dr. C. Esser für die Bereitstellung des Themas und die Betreuung der vorliegenden Arbeit. Unsere anregenden Diskussionen haben die Arbeit in dieser Form erst möglich gemacht.

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Erklärung

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder einer ähnlichen Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 13. Februar 2008