CONTACT HYPERSENSITIVITY TO NICKEL: EXPERIMENTAL INDUCTION OF ALLERGY AND TOLERANCE, RESPECTIVELY

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TO MY FAMILY

"Two roads diverged in a wood, and I took the one less travelled by. And that has made all the difference"

ROBERT FROST

ZUSAMMENFASSUNG

C57Bl/6 Mäuse, die in konventioneller, d.h. mit Nickel-haltigem Edelstahl versehenen Umgebung gehalten werden, werden als Ni^{low} Mäuse bezeichnet. Sie können durch die Injektion von Nickelionen (Ni) allein nicht sensibilisiert werden. Sie können jedoch sensibilisiert werden, wenn Ni in 1% H_2O_2 ($NiCl_2/H_2O_2$) injiziert wird, wobei H_2O_2 als endogenes Adjuvans betrachte werden kann.
Der Literatur zufolge führt die orale Applikation von Ni zur Induktion von Toleranz. In dieser Arbeit habe ich dieses Modell dahingehend modifiziert, um die Abhängigkeit der oralen Toleranz gegen Ni von der applizierten Dosis genauer untersuchen zu können.
Tiere, die über einen Zeitraum von 4 Wochen 10 mM $NiCl_2$ im Trinkwasser erhalten haben (bezeichnet als Ni^{high} Mäuse), können nicht mehr durch die Injektion von Ni allein oder $NiCl_2/H_2O_2$ sensibilisiert werden, und ihre Ni-reaktiven T-Zellen sind anergisch und höchst immunsuppressiv. Im Gegensatz zu Ni^{high} und Ni^{low} Mäusen, können $Ni^{very\ low}$ Mäuse, die in einer Metall-freien Umgebung gezüchtet und gehalten werden, durch die Injektion von Ni allein, also ohne zusätzliches Adjuvans, sensibilisiert werden. Diese Unterschiede in der Empfindlichkeit für die Sensibilisierung gegen Ni folgen der Hierarchie der suppressiven Kapazität der Ni-spezifischen T-Regulator (Treg)-Zellen: Ni^{high}> Ni^{low}> $Ni^{very\ low}$. Während Ni-spezifische Treg-Zellen in $Ni^{very\ low}$ Mäusen vollständig fehlen, existieren in Ni^{low} Mäusen Treg-Zellen der $CD4^+$ Subpopulation, in Ni^{high} Mäusen hingegen sind sowohl $CD4^+$ als auch $CD8^+$ Treg-Zellen vorhanden. Interessanterweise können die Ni-spezifischen $CD4^+$ Treg-Zellen die Sensibilisierung durch Ni alleine verhindern, nicht jedoch die Sensibilisierung durch $NiCl_2/H_2O_2$. Um die vollständige Sensibilisierung von Ni^{low} und $Ni^{very\ low}$ Mäusen durch $NiCl_2/H_2O_2$ zu verhindern, sind sowohl $CD4^+$ als auch $CD8^+$ Ni-spezifische Treg-Zellen von Ni^{high} Mäusen erforderlich, die miteinander kooperieren.
Durch die orale Aufnahme von Ni entwickeln Ni^{high} Mäuse nicht nur Treg-Zellen, sondern auch tolerogene Antigen-präsentierende Zellen (APZ). Diese APZ können ebenfalls die Toleranz auf Ni^{low} Empfänger übertragen, aber im Gegensatz zu Treg-Zellen, deren suppressive Fähigkeiten über einen Zeitraum von mindestens 20 Wochen Bestand haben, ist die Tolerogenität der APZ nur von kurzer Dauer. Durch serielle adoptive Transfers konnte gezeigt werden, daß die Ni-Toleranz „infektiös“ von T-Zellen bzw. APZ des Spenders auf den entgegengesetzten Zelltyp des Empfängers, also APZ bzw. T-Zellen, übertragen wird. Aus diesen Beobachtungen kann geschlossen werden, daß die Treg-Zellen und tolerogenen APZ, die durch orale Applikation von Ni in Ni^{high} Mäusen induziert werden, Teil eines positiven Rückkopplungsmechanismus sind, der der Erhaltung und Verstärkung der Toleranz dient. Demzufolge besteht eine Dosis-Wirkungsbeziehung zwischen der täglichen oralen Aufnahme von Ni und dem Phänotyp und der Effektivität Ni-spezifischer Treg-Zellen.
Zusätzlich zu den oben beschriebenen *in vivo* Experimenten habe ich die proliferativen T-Zellantworten gegen Ni *in vitro* untersucht. Unerwarteterweise proliferieren die Lymphknotenzellen von Ni^{low} Mäusen, die mit "Nicht-Ni"-Antigenen stimuliert wurden, *in vitro* bei Restimulation mit Nickel.
Die beobachtete Proliferation ist jedoch eher der in vitro Stimulation Nickel-spezifischer T-Zellen, als der Kreuzreaktion zwischen Ni und anderen Antigenen zuzuschreiben.

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Comparison of nickel reactivity in Ni^{high}, Ni^{low} and $Ni^{very\ low}$ mice
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ABBREVIATIONS

ACD	allergic contact dermatitis
ACT	ammoniumchloride-tris
ANOVA	analysis of variance
ACAID	anterior chamber associated immune deviation
Ag	antigen
APC	antigen presenting cells
BCR	B cell receptors
BSA	bovine serum avalbumin
CO_2	carbon dioxide
CFA	complete Freund's adjuvant
Con A	concanvalin A
cpm	counts per munite
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
^oC	degrees centigrade
DC	dendritic cell
DTH	delayed-type hypersensitivity
DMSO	dimethyl sulphoxide
DNFB	2,4-dinitrofluorobenzene (Sangers reagent)
DNBS	2,4-dinitrobenzenesulphonic acid
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylene diaminetetraacetic acid
FCS	foetal calf serum
FITC	fluorescein-isothiocyanate
FSC	forward scattered
FoxP3	forkhead box P3
GITR	glucocorticoid-induced tumor necrosis factor receptor
Th	helper T cell
Ni^{high}	high nickel
HAT	hypoxanthin, aminopterin, thymidine
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HT	hypoxanthin, thymidine
H_2O_2	hydrogen peroxide
iDC	immature dendritic cells
IU	international units
i.d.	intradermal
i.v.	intravenously
ILN	inguinal lymph nodes
INF-γ	interferon-gamma
IL	interleukin
i.p.	intraperitoneal
LC	Langerhans cells

Ni^{low}	low nickel
LN	lymph node
LNC	lymph node cells
LTT	lymphocyte transformation test
µCi	microcurie
µl	microliter
µM	micromolar
MIP-2	macrophage-inflammatory protein-2
MHC	major histocompatibility complex
ml	millilitre
MLR	mixed lymphocyte reaction
MEST	mouse ear-swelling test
Nrp1	neuropilin-1
Ni	nickel
$NiCl_2$	nickel chrolide
NFκB	nuclear factor κB
OD	optical density
%	percentage
PBMC	peripheral blood mononuclear cells
PMA	phorbol myristate acid
PBS	phosphate buffered saline
PEG 1500	polyethylene glycol 1500
PLN	popliteal lymph nodes
RANK	receptor activator of NFκB
Treg	regulatory T
rmp	rotations per minute
SSC	sideward scattered
SDS	sodium dodecyl sulphate
SC	standard cocktail
SD	standard deviation
SI	stimulation index
AV-HRP	Streptavidin-Horseradish Peroxidase
H_2SO_4	sulphuric acid
TCR	T cell receptors
TMB	3,3’,5,5’-tetrametylbenzidine
TGF-ß	transforming growth factor-ß
Tr1	T regulatory type 1
TNFR	tumor necrosis factor receptor
TC	tumour cocktail
UV	ultraviolet
$Ni^{very\ low}$	very low nickel
v/v	volume/volume
w/v	weight/volume

CONTENT

1 INTRODUCTION:

1.1 THE IMPORTANT OF IMMUNOLOGY

Historically, the term immunity signified a persons protection from disease especially infectious diseases. The immune system is a collection of cells and molecules which coordinate the immune response to foreign substances. Nowadays immunity is defined as a response to substances including microbes, proteins and polysaccharides without causing physiological or pathological problems to the host.

1.2 INNATE AND ADAPTIVE IMMUNITY

Individuals have various cellular and biochemical defense shields which protect the host against foreign substances or heal injured tissues. These are three primary mechanisms i) physical and chemical barriers, ii) blood proteins such as complement and iii) phagocytic cells. These aspects are collectively known as the **INNATE IMMUNE SYSTEM** and are directed towards microbes: They respond in essentially the same way to repeated infections [1]. In contrast to innate immunity, there are other immune responses which are more intricate and increase in magnitude and defensive capacities upon each successive exposure to the offending microbe. This form of **ADAPTIVE IMMUNITY** is characterized by specificity to distinct molecules, memory and increased responses upon repeated exposure of the same entity. These two immune responses function cooperatively and the innate mechanisms not only provide early defense against microbes but also play an important role in the induction of adaptive actions.

1.3 CELLS AND TISSUES INVOLVED IN ADAPTIVE IMMUNITY

Lymphocytes are the only cells that can specifically recognize foreign antigens and upon recognition of a particular antigen for the second time they show an enhanced response and memory. The adaptive immune response can be separated into two types: **HUMORAL IMMUNITY** is mediated by B lymphocytes, these cells can recognize extracellular antigens and differentiate into antibody-secreting cells. T cells on the other hand recognize and destroy intracellular microbes or the infected cells and thus control **CELLULAR IMMUNITY**.

Although lymphocytes can specifically recognize foreign antigens, they are unable to capture antigens themselves. Antigen-presenting cells (APC) are specialized cells that can capture, transport, process and finally display antigens to specific lymphocytes. Macrophages [2], B cells [3;4] and in particular dendritic cells (DCs) [5;6] can act as professional APC. Once and antigen is presented to lymphocytes,

it will be eliminated by different mechanisms. In order to effectively perform host defensive functions, the cells responsible for immune responses are localized in special tissues or lymphoid organs. Lymphoid organs include the bone marrow, in which newly generated immature lymphocytes can mature to B cells; the thymus, which is the site of T cell maturation; the lymph nodes, where B and T cells respond to antigens that are collected by the lymph draining from peripheral tissues and the spleen, which responds to blood-borne antigens. Other specialized areas include the cutaneous and mucosal immune systems which consist of distinct populations of APC and lymphocytes that primarily focus on environmental antigens encountered in the skin or introduced through the respiratory and gastrointestinal tracts [7-9].

1.4 ANTIGEN PROCESSING AND PRESENTING TO T CELLS

In contrast to B cells, which can recognize intact protein molecules, classic αβT cells only recognize antigens in the form of peptides displayed by the major histocompatibility complex (MHC) on the surface of APC [10;11]. $CD8^+$ T cells recognize peptide antigens presented by MHC class I molecules, whereas $CD4^+$ T cells recognize antigens displayed by MHC class II molecules [11]. MHC class I molecules are expressed in all nucleated cells [12;13] but only those on the surface of APC can present antigens to $CD8^+$ T cells [14;15]. The antigens presented by MHC class I molecules are normally endogenously synthesized [15] and thus include the products resulting from virus infections or other microbes. MHC class II molecules are constitutively expressed on DCs, macrophages, B cells and to an extent on endothelial and thymic epithelial cells. However, a wide variety of cell types can be induced to express these molecules after cytokine expression [16]. These molecules present antigens from extracellular sources to $CD4^+$ T cells [15]. Functionally, most $CD4^+$ T cells are cytokine producing helper cells (therefore also called Th cells) whereas $CD8^+$ T cells are cytotoxic cells (therefore, called cytotoxic T lymphocytes, CTLs) [17]. According to the patterns of cytokines they produced, $CD4^+$ Th cells can be further differentiated into Th1 and Th2 cells. Th1 cells activate the microbicidal properties of macropages, and induce B cells to make IgG antibodies that are very effective at opsonizing extracellular pathogens for uptake by phagocytic cells. Thus, Th1-dominated responses are potentially effective in eradicating infectious agents. Th2 cells initiate the humoral immune response by activating naïve antigen-specific B cells to produce IgM and other type of antibodies [18].

1.5 T CELL ACTIVATION

When peptides are presented in the MHC complexes, these antigens are recognized by specific T cell receptors (TCR) on the T cells. With the participation of either the CD4 or CD8 co-receptors [19;20], and other accessory molecules, the peptide:TCR recognition activates a series of signaling pathways through the other parts of the TCR complex, such as CD3 and the TCR ζ-chain leading to T cell activation [10]. However, to achieve full T cell activation the APC need to present the specific antigen (signal I) and costimulatory molecules (signal II) [21;22]. Activation of the TCR in the presence of costimulatory signals results in T cell clonal expansion and the induction of effect or functions. In contrast, interaction of the TCR with antigens in the absence of costimulatory molecules induces T cell unresponsiveness or apoptosis [23;24]. The B7/CD28 costimulatory pathway is widely recognized as

the major costimulatory pathway in T cell activation. In brief, engagement APC residing B7-1(CD80) and/or B7-2 (CD86) molecules to CD28 on T cells induces T cell proliferation and IL-2 production [25]. The process of T cell activation is completed in the lymphoid tissues or organs.

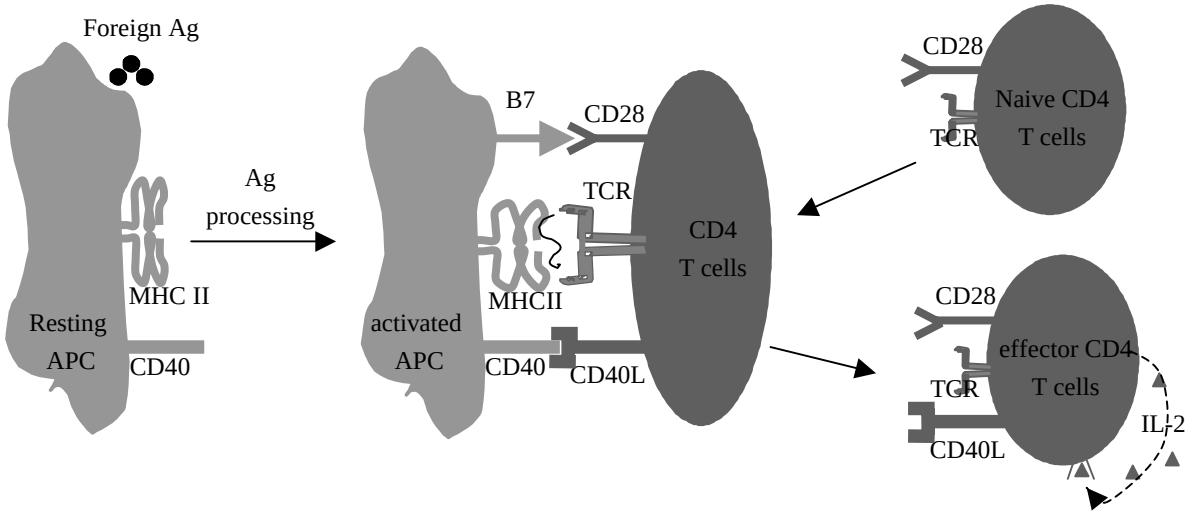


FIGURE 1.5 T cell activation. MHC-class II and costimulatory molecules CD40, B7.2 are consistently expressed on resting APC at low levels. Early in the immune response, peptides that are derived from the endocytototic pathways within APC are presented on MHC-class II molecules. The binding and processing Ag lead to the activation of APC by upregulating MHC-class II, CD40, B7-2, inducing the expressing of costimulatory molecule B7-1. Later, the peptides are presented to and recognized by antigen specific $CD4^+$ T cells. Antigen recognition by $CD4^+$ T cells induces the expression of CD40 ligand (CD40L). Interaction of CD40:CD40L stimulates the expression of more B7 molecules and secretion of cytokines. Engagement of B7 molecules on activated APC and costimulatory molecule CD28 on T cells lead to full activation of the $CD4^+$ T cells. CD8 T cells are activated by the presentation of antigens on MHC-class I molecules.

DCs are considered the most effective activators of naïve T cells. Immature dendritic cells (iDCs) are located in the peripheral tissues and cannot only detect and ingest microbes through specific recognition but also have a potent capacity to take up antigens. At the onset of infection they engulf antigens and migrate to the local lymphoid tissues. Upon maturation these DCs upregulate MHC molecules, adhesion molecules and costimulatory molecules such as B7 molecules, they also gradually loose their capacity to take up antigens. When they arrive at the local lymphoid tissues, these DCs are able to present antigens and simultaneously activate naive T cells [5;6].

Besides DCs, which can efficiently present a wide variety of antigens, macrophages and B cells can also present antigens to T cells and are regarded as the main APC during the effector phase of the immune response. The effector phase is the elimination of antigen after specific activation of lymphocytes [26]. Macrophages mainly present particulate antigens [2] due to their strong phagocytic function. B cells, which travel through the peripheral tissues to reside in the secondary lymphoid organs, need to be activated by DCs and cognate T cells before they obtain their antigen-presenting capacity. They take up soluble protein antigens by binding these antigens to their cell-surface through immunoglobulin (i.e. B cell receptors, BCR), and then internalize them. The peptide fragments of antigens are then displayed in form of peptide-MHC class complexes [3;4]. Once naïve T cells are

activated, they synthesize IL-2 and its receptors, proliferate and finally differentiate into effector T cells. Effector T cells can perform their function in the presence of antigens without costimulatory signals [27].

1.6 OVERVIEW OF HYPERSENSITIVITY

When foreign antigens invade an individual, the immunological mechanisms are stimulated. Once this antigen is eliminated, all normal immune responses decline and the immune system returns to its basal state. However, sometimes an overreaction of the immune responses can occur and this is termed hypersensitivity. Disorders caused by overactive immune reactions are called hypersensitivity diseases and there are four classes. Type I hypersensitivity is a reaction caused by IgE antibodies that are produced in response to an antigen binding to Fc receptors on mast cells. The tissue damage is caused by inflammatory mediators that are released by mast cells. This reaction begins rapidly after the second contact with a particular antigen (**CHALLENGE**), so it is also called **IMMEDIATE HYPERSENSITIVITY** [1]. Type II hypersensitivity results from IgG antibodies binding to cell surface or extracellular matrix antigens. These antibodies may then cause the destruction of cells or tissues that express those antigens. Type III hypersensitivity is caused by immune complexes of IgG antibodies against soluble antigens which may lead to the damage of the tissues at the site of the immune complex deposits. Finally type IV hypersensitivity reactions are elicited by antigen specific Th1 or $CD8^+$ cytotoxic T cells. Both of these effector cell types secrete cytokines that activate macrophages and induce inflammation. In some cases, $CD8^+$ cytotoxic T cells can directly kill target cells. Since the activation of antigen specific T cells requires several days, these types of reaction also take several days to develop. Therefore, type IV hypersensitivity is more commonly known as **DELAYED-TYPE HYPERSENSITIVITY** (DTH).

1.7 IMMUNOLOGICAL TOLERANCE

When encountering an antigen, lymphocytes can be either activated and then induce immune responses or inactivated and then induce tolerance. Immunological tolerance is a state of unresponsiveness to an antigen and is induced by previous exposure to that antigen. The unresponsiveness can arise from different mechanisms.

1.7.1 Overview of tolerance mechanisms

The most critical aspect of an immune system is the non-reactivity to self. This is initiated in the thymus during T cell maturation by clonal deletion or **NEGATIVE SELECTION** in which the lymphocytes whose antigen receptors bind strongly to self-antigens are eliminated by apoptosis [28]. Consequently, mature T cells released into the periphery remain unresponsive to self-antigens. Since the thymus is a central lymphoid organ, tolerance established in this manner is **CENTRAL TOLERANCE**. In addition to clonal deletion, clonal anergy also plays a role in central tolerance[29-32].

However, after entering the periphery, mature T cells can still encounter self-antigens that were not expressed in the thymus, especially tissue specific antigens. These T cells are then either activated and

induce autoimmune responses, or remain silence and become tolerant to these self-antigens. Here, since tolerance occurred in the peripheral lymphoid organs, it is called **PERIPHERAL TOLERANCE**.

The mechanisms of peripheral tolerance include clonal deletion, ignorance, anergy, and suppression. In peripheral tolerance, a high dosage of foreign antigens can induce clonal deletion in the periphery[33]. The term ignorance refers to T cells which can recognize certain antigens but remain inactivated due to the too weak signals their receive. This is because their TCR bind to the specific antigens at very low affinity [34], or alternatively, the level of antigen is too low to deliver any signal to the T cells [35]. Anergy is the state of unresponsiveness to antigenic stimulation. Anergic T cells can arise from four signaling errors; i) they are not fully activated due to the delivery of a low-affinity TCR ligand by the APC [36;37], ii) the presence of a strong TCR signal in the absence of costimulatory signals [37;38], iii) a strong TCR signal in the presence of costimulatory molecules but also cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) or other costimulation inhibitory molecules [39;40] or iv) T-T presentation of antigens. A population of antigen-presenting rat [41] and human [42] T cells express high levels of MHCII and costimulatory molecules such as CD80 and CD86. Therefore, these activated T cells can act as APC to present antigens to T cell clones with the same reactivity and induce the latter into an anergic state [43;44]. Although mouse T cells fail to express high levels of MHCII molecules, they can take up MHCII and B7 molecules from APC and thus can also function as APC [45]. Finally, suppression is another tolerance mechanism and here regulatory T (Treg) cells can suppress the activation and inhibit the functions of effector T cells. This tolerant mechanism is most intensified study in recent years and different populations of regulatory T cells have been found. The regulatory T cell populations will be overviewed in the next section.

1.7.2 Overview of regulatory T cells

Regulatory T cells can be found in $CD4^+$ T cells, $CD8^+$ T cells, double negative T cells [46], [47] and NKT cells [48]. $CD4^+$ Treg cells are widely studied and at least three major populations have been described. $CD4^+CD25^+$ Treg constitutively express the IL-2 receptor α-chain and consist of approximately 5-10% of peripheral $CD4^+$ T cells. They can be generated in the thymus [49], the periphery [50;51] and *ex vivo* [52]. Although they can expand in the presence of high doses of IL-2, CD28 [53;54], or even TGF-ß [55], $CD4^+CD25^+$ Treg are essentially anergic (i.e. fail to proliferate after TCR stimulation) and suppressive (prevent IL-2 production and proliferation of $CD4^+CD25^-$ or $CD8^+$ T cells) [54;56]. Their suppression depends on cell-cell contact between responder and themselves and requires the activation of the Treg via the TCR [54]. Once activated, their suppressive function is completely antigen unspecific [57]. $CD4^+CD25^+$ Treg cells are also characterized by the constitutive expression of CTLA-4 [58;59], glucocorticoid-induced tumor necrosis factor receptor (GITR) [60] and the transcription factor forkhead box P3 (FoxP3) also known as Scurfins [61-63].

The mechanisms underlying the suppressive function of $CD4^+CD25^+$ Treg cells are still not fully understood, but the suppressive activities of this population at least relate to the expression of the above molecules. FoxP3 plays an important role in the development and function of $CD4^+CD25^+$ Treg and forced expression of FoxP3 in conventional naive $CD4^+$ T cells (including $CD4^+CD25^-$ T cells) renders them as suppressive as naturally occurred $CD4^+CD25^+$ Treg [61;64]. CTLA-4 is a negatively

regulating molecule [58;59], whereas stimulation of $CD4^+CD25^+$ Treg through GITR abrogates the suppression [60;65]. Hence, one of the suppressive pathways used by these cells could be the down-regulation of costimulatory molecules on APC in a cell-cell contact-dependent manner [66]. In addition, the involvement of TGF-ß in the mediation of suppression of $CD4^+CD25^+$ Treg remains controversial. Piccirillo, C.A. *et al.*. reported that neither anti-TGF-ß1 nor soluble TGF-betaRII-Fc could block the suppression of $CD4^+CD25^+$ Treg [67]. However, other studies found that the suppression of $CD4^+CD25^+$ Treg is mediated by the membrane but not soluble form of TGF-ß, and anti-TGF-ß completely or partially abrogates the suppression [68-70].

The specificity of $CD4^+CD25^+$ Treg also remains unresolved since the repertoire of antigen specificity in the naturally occurring $CD4^+CD25^+$ Treg population is as broad as that of naive T cells [71;72]. Induced $CD4^+CD25^+$ Treg are antigen specific in systems [72] indicating that the $CD4^+CD25^+$ Treg are a heterogeneous sunpopulation. Researchers have been able to further characterize cell subsets within this population, for example, Lehmann *et al.* found that the combination of surface intergrin $\alpha_E\beta_7$ with CD25 could further divide this population into $CD4^+CD25^+\alpha_E{}^+$, $CD4^+CD25^+\alpha_E{}^-$ and $CD4^+CD25^-\alpha_E{}^+$ subpopulations, with $CD4^+CD25^+\alpha_E{}^+$ T cells having the most potent suppressive capacity [73]. Furthermore, Bruder, D. *et al..* found that neuropilin-1(Nrp1), a receptor involved in axon guidance, angiogenesis, and the activation of T cells, was constitutively expressed on the surface of $CD4^+CD25^+$ Treg, regardless of their activation status. This receptor is down-regulated in naive $CD4^+CD25^-$ T cells after TCR stimulation. $CD4^+Nrp1^{high}$ T cells express high levels of FoxP3 and suppress $CD4^+CD25^-$ T cells. Thus, Nrp1 can be used as a marker to distinguish $CD4^+CD25^+$ Treg and recently activated $CD4^+CD25^+$ non-regulatory T cells [74]. Fu, S. *et al.* separated $CD4^+CD25^+$ Treg into $CD62L^+$ and $CD62L^-$ subpopulations and found that while both subsets were anergic and expressed FoxP3, the $CD62L^+$ subset was more potent in suppression on a per cell basis, and proliferated and maintained suppressive functions far better than the $CD62L^-$ and non-separated $CD4^+CD25^+$ Treg. This $CD62L^+$ subpopulation was also more responsive to chemokines such as CCL19, MCP-1 and FTY720, chemokines responsible for successful migration to secondary lymphoid organs [75]. $CD4^+CD25^+$ Treg have also been separated into subsets according to their expression of $\alpha_4ß_1$ or $\alpha_4ß_7$ intergrins. Upon activation, both subsets of T cells expressed FoxP3 and were able to suppress conventional $CD4^+$ T cells. However, the characters of these Treg subsets were rather distinct: $\alpha_4ß_1$-expressing $CD4^+CD25^+$ Treg induced TGF-ß-producing Treg (Th3-like), whereas $\alpha_4ß_7$-expressing $CD4^+CD25^+$ Treg induced IL-10 producing Treg (Tr1-like) [76]

Th3 cells are $CD4^+$ Treg cells that produce high amount of TGF-ß, little or no IFN-γ [77], and various amounts of IL-4 and IL-10 [78]. Th3 cells can be induced in TGF-ß rich environments, either *in vivo* [79], especially after oral antigen administration [80] or *in vitro* [81]. Weiner, H.L. and co-workers have demonstrated that anti-IL-12 treatment facilitates the induction of Th3 cells[82]. Th3 cells can suppress immune response both *in vivo* and *in vitro*.

Tr1 cells are CD4 Treg cells that have low proliferation capacity, produce high levels of IL-10, low levels or no IL-2 or IL-4[83], variable amounts of IL-5, and some IFN-γ [84]. The low proliferative response of Tr1 cells can be overcome by IL-15, and to lesser extent IL-2 [85]. Tr1 cells can be induced *in vitro* with high level of IL-10[83], and *in vivo* [86] in both human and murine. However, In

human, some study found *in vitro* induction of Tr1 cells need both IL-10 and INF-α [87]. Tr1 cells suppress naive and memory Th1 or Th2 responses by means of production of IL-10 [88]. Th3 and Tr1 cells share some common aspects. First, both Th3 [82] and Tr1 cells [83;89] can trigger suppression in an antigen-specific manner but suppress in an antigen-non-specific manner. Second, both Treg types can be infectiously generated from conventional $CD4^+$ T cells by $CD4^+CD25^+$ Treg. Jonuleit, H. *et al.* demonstrated that using human cells, $CD4^+CD25^+$ Treg can infectiously render conventional $CD4^+$ T cells suppressive and this is partially mediated by soluble TGF-ß [90]. This implies that $CD4^+CD25^+$ Treg may induce Th3 cells. Tr1 cells can also be generated from $CD4^+CD25^-$ by $CD4^+CD25^+$ Treg [91].

Many studies have shown that $CD8^+$ T cells can also act as regulatory T cells [92]. Similar to $CD4^+$ Treg, $CD8^+$ Treg are also heterogeneous. Amongst them, a subset of $CD8^+$ Treg with a phenotype of $CD8^+CD28^-$ was extensively studied. However, $CD8^+CD28^-$ Treg are probably also heterogeneous because they can perform suppressive actions with different mechanisms in different systems. Therefore, Filaci, G. *et al*. classified human $CD8^+$ Treg into three types: type 1 CD8 Treg possess a $CD8^+CD28^-$ phenotype and mediate suppression by alternating the expression of costimulatory molecules on DCs in a cell-cell contact manner [93]; type 2 CD8 Treg are also $CD8^+CD28^-$ but they mediate suppression via cytokines (IFN-γ, IL-6) secretion; type 3 CD8 Treg mediate suppression through the secretion of IL-10 but their phenotype remains unclear. However, the above classification is not complete since the authors concentrated on human CD8 Treg and these regulatory T cells in general coordinate suppression by other mechanisms including: 1) cytokines with inhibitory functions such as TGF-ß [94]; 2) the induction of $CD4^+$ T cell apoptosis via ligation of Fas [95]; 3) suppression of CD40L upregulation on Th cells [96] and therefore blockade of the costimulatory signals required for the activation of T cells; 4) direct elimination of $CD4^+Vß8^+$ T cells that express Qa-1 MHC class Ib molecules [97]; 5) suppression via CTLA-4 [98] and 6) human $CD8^+CD25^+$ thymocytes, which express FoxP3, GITR, TNFR2, CTLA-4, suppress in the same manner as $CD4^+CD25^+$ Treg [99]. Therefore, the mechanisms of $CD8^+$ Treg can be divided into a cytokine dependent, a cell-cell contact dependent, and an apoptosis dependent pathway. These regulatory T cells can be generated via different methods which include oral tolerization [94], exposure of $CD8^+$ T cells to IL-10 [100-102] or TGF-ß [103] given exogenously or produced by other cell types and by intravenous injection of TGF-ß-treated Ag-pulsed APC into naïve mice [104].

1.7.3 The role of APC on immune tolerance

In section 1.5, it was briefly mentioned that when APC present antigen without costimulatory molecules the responding T cells remain unresponsiveness or enter into apoptosis. However, the role of DCs, B cells and macrophages on the induction and maintenance of tolerance is more complicated [105-108]).

Two distinct subsets of DCs with different origins have been intensely studied in humans and mice. In humans, the myeloid DCs (also called DC1) have a $CD11c^+CD33^{+/-}CD1a^+MHCII^+CD80^+CD86^+$ phenotype whilst the lymphoid DCs (DC2, or plasmocytoid DC) are characterized by the phenotype $CD11c^-CD4^+CD3^-IL3Ra^{++}HLA\text{-}DR^+$. Mouse myeloid and lymphoid DCs display the phenotypes

$CD8\alpha^{-}DEC\text{-}205^{-/lo}$ and $CD8\alpha^{+}DEC\text{-}205^{hi}$, respectively [108]. In general, Ag presentation by mature myeloid DCs leads to immunity whereas immature myeloid DCs induce immune tolerance. Ag presentation by lymphoid DCs elicits immune tolerance. The mechanisms in which immature myeloid DCs produce immune tolerance include the induction of T cell anergy due to lack of costimulatory molecules provided by iDCs [108] and the induction of peripheral T cell deletion or regulatory T cells [108;109]. Lymphoid DCs also elicit immune tolerance in their steady state, they can take up Ag from dying Ag-loading myeloid DCs and present that Ag to T cells in a tolerogenic fashion [108]. In an inflammatory setting, the regulation of tolerance induction is more complex. One possible mechanism is that IFN-γ, generated during inflammation, could signal $CD8\alpha^{+}$ DCs to induce the rate-limiting enzyme indoleamine 2,3-dioxygenase (IDO) in the tryptophan degrading pathway and thus halt T cell proliferation and responsiveness [108].

Fuchs *et al.* found that whereas activated T cells responded to antigens presented by either resting B cells or LPS-activated B cells, naive T cells were rendered tolerant [106]. These findings were fully confirmed in some systems [110] but only partially supported in other studies [111-113]. The latter researchers found that naïve T cells could only be rendered tolerant when antigens were presented by resting B cells. One of the key reasons that resting B cells but not preactivated ones can render naïve T cells tolerant is the low expression of CD40 on resting B cells which in turn fails to upregulate CD40L expression on naive T cells [111;113]. CD40L expression is critical for the upregulation of B7 molecules on resting B cells which subsequently deliver the costimulatiory signals necessary for T cell proliferation [113;114]. Two further concepts of tolerance induction via B cell mechanisms are i) the finding that CD1d expressing marginal zone B cells in the spleen can present antigens to NKT cells, which in turn induce antigen-specific Treg cells [115] and ii) histocompatibility at the TL/Qa region allows B cells to induce antigen-specific Treg in the ACAID (anterior chamber associated immune deviation) model [116].

Finally antigen presentation by macrophages has been shown to elicit both B cell [117] and T cell [107;118] tolerance. Even though T cell deletion has been shown to play a role in macrophage-induced T cell mediated tolerance [119], the more important mechanism appears to be T cell anergy [107;120]. In correlation to resting B cells, macrophage-induced T cell anergy stems from low levels of expressed costimulatory molecules. The application of antigen to UV (ultraviolet) irradiation skin has been demonstrated to induce T cell mediated tolerance. This tolerant state is positively correlated with the number of infiltrated macrophages and conversely correlated with the number of Langerhans cells (LC) in the UV-irradiated skin [121]. These infiltrated macrophages displayed lower levels of CD40 and B7 molecules when compared with the LC [122]. The $CD4^{+}$ T cells activated by these macrophages are IL-2R alpha deficient [122;123]. Interestingly, both the low levels of CD40 and B7 on these macrophages and the IL-2R alpha expression on activated T cells could be restored by the presence of IFN-γ [122]. Furthermore, research on the ACAID model found that macrophage-derived signals, such as the chemokine macrophage-inflammatory protein-2 (MIP-2), could selectively recruit NKT cells and bias their cytokine synthesis to generate $CD8^{+}$ Treg [124;125]. Therefore, macrophages are indirectly involved in the induction of regulatory T cells.

1.7.4 Oral tolerance

There are different levels of tolerance to certain antigens. The antigen dose used in tolerance induction affects the suppressive capacity of the regulatory T cells induced during this process. Usually the higher the antigen dose the higher the anergic state and suppressive activity of the Treg [126;127]. Oral tolerance refers to the oral administration of an antigen lend the eventual unresponsiveness to that antigen. This form of peripheral immune tolerance is dependent on the dose of the antigen and can be mediated by different mechanisms such as T cell deletion, anergy, and suppression [128]. High doses of oral antigens usually activate T cell deletion [33;129] and/or anergic mechanisms [130-133]; whereas low doses induce active suppression [130;134]. Active suppression has been extensively studied and it appears that the oral administration of antigen preferentially generates a Th2(IL-4/IL-10) [82;134] response or regulatory T cells such as Th3 (TGF-ß) [82;135], $CD4^+CD25^+$ [136;137] and $CD8^+$ [138;139].

The reason why oral application of antigen develops into tolerance remains unsolved. The following points however, are critical aspects to the immune mechanism: i) oral administration of Ag induces tolerance but only in the steady state; during inflammation [140;141] or in the presence of adjuvant [142], the oral administration of Ag produces immunity; ii) DCs from Peyer's patches express high levels of IL-10 upon the stimulation with CD40L [143] or the receptor of NFκB (RANK) [144], whilst splenic DC favor IL-12 production [144]. These Peyer's patches DCs are particularly capable of priming naive T cells to secrete high levels of IL-4 and IL-10, whereas DCs from non-mucosal sites prime naive T cells to produce IFN-γ [143]. iii) "gut processing" of an antigen is a critical factor since the binding of antigens to enterocytes and the ensuing passage through the epithelium can convert antigens into a tolerogenic form and thus induce tolerance. Any defect in "gut processing" would fail to induce oral tolerance [145]; iv) the induction of oral tolerance is enhanced by oral administration of IL-4 and IL-10 [82], and abrogated by systemic administration of anti-TGF-ß or recombinant IL-12 [146]. v) finally the mesenteric lymph nodes (MLN) are also critical because no oral tolerance can be induced in the mice without MLN [147;148].

1.8 NICKEL IS A SPECIAL ANTIGEN

Nickel is one of most common metals in the environment. As a ubiquitous component of metal alloys, nickel can be found not only in catheters, needles, dental braces and many other medical devices but also in everyday items such as watches, coins, jewelry, and even in some foods. Even though we enjoy the widespread benefits that this metal brings us, we also need to realize its potential side effects. The most prominent side effect that nickel has is that it is an allergen and can therefore cause T cell-mediated DTH responses [149]. The sensitization rate to nickel in the general population is about 15% [150].

1.8.1 Induction of nickel allergy

Although quite a large population of people are allergic to nickel, clinical investigations have shown that in humans, allergic contact hypersensitivity to nickel develops much more readily in inflamed

than normal skin. Consistent with this, ear piercing in order to wear nickel-releasing costume jewelry results in a high rate of nickel allergy [151;152]. In animal models, which were reared in conventional cages with nickel-containing stainless steel lids and water drinking bottles with nickel-containing stainless steel outlets (Ni^{low} environment), were difficult to be *de novo* sensitized with nickel alone [153-155]. For sensitization, these animals were given a high concentration of Ni (20%) on cotton gaze which was fixed with a bandage to clipped flanks for 7 days [156]. Conceivable, these priming conditions elicited skin irritation. Alternatively, these naïve Ni^{low} animals (reared in a Ni^{low} environment) could be sensitized using nickel in combination with adjuvants such as H_2O_2, SDS or PMA [153]. The use of complete Freund's adjuvant (CFA) in the immunization of Ni^{low} animals remains controversial since although Artik *et al.* could sensitize Ni^{low} mice CFA and nickel ions [153], van Hoogstraten *et al.* could not [157]. However, this latter group did find that nickel ions and CFA could sensitize animals that were bred and reared in a special non-metal environment, these animals and environment are termed $Ni^{very\ low}$ [157]. Thus, due to the difficulty in immunizing Ni^{low} animals studies of nickel allergy *in vivo* have remained relatively few.

1.8.2 Nickel specific effector T cells and their activation

In human peripheral blood mononuclear cells (PBMC), Ni-reactive T cells are primarily found within $CD4^+$ T cell subset [158-160]. At the clonal level, whereas only murine-derived nickel specific $CD4^+$ T cell hybridomas have been described [153], both Ni-reactive $CD4^+$ and $CD8^+$ human T cell clones have been reported [161-165]. Although some Ni-reactive T cell clones proliferated to nickel in the absence of APC [166;167], T cell reactions towards nickel normally required the presence of APC [160;164;166-169]. In contrast to typical conventional antigens, the role of APC in Ni-specific T cell proliferation are heterogeneous because the proliferation of Ni-specific T cell clones are either processing independent or processing dependent [170]. Some studies have shown that the uptake and processing mechanisms might not play a major role [171] and Lu *et al.* [172] have even demonstrated that immobilized MHCII was sufficient to present Ni to the Ni-specific cell line, Ani2.3. Lisby *et al.*, [160] found that preincubation of monocytes/macrophages with nickel resulted in the proliferation of not only memory but also naive T cells. Therefore, unlike the conventional antigen, nickel can activate nickel specific T cells (mainly $CD4^+$ T) through either processing dependent or processing independent pathway .

1.8.3 Tolerance induction to nickel

In section 1.8.1, it was commented that nickel alone is unable to immunize Ni^{low} animals. However, nickel alone is sufficient to elicit recall responses both *in vivo* and *in vitro*, therefore nickel alone is able to provide an effective signal I for T cell activation, but is unable to provide enough signal II for priming [153]. It is well known that repeated giving signal I (here, Ni-neoantigen) without signal II (costimulatory molecules) leads to the development of tolerance (see also section 1.5). Therefore, nickel should be a good tolerogen and indeed it is. For example, adolescent humans that have worn nickel-releasing orthodontic braces before ear piercing show a lower incidence of nickel allergy than those who wear no brace at all or braces after ear piercing [173;174]. In animal models, continual oral administration of nickel allowed this mice (Ni^{high} mice) to be unresponsiveness to nickel in the DTH reaction [156;157;175;176]. This phenomena was regardless to the rearing condition of the mice, that

is, Ni^{low} or $Ni^{verylow}$. Furthermore this unresponsiveness could be transferred to syngeneic naive recipient animals [156;157;175]. In addition, repeated intraperitoneal (i.p.) injections of Ni^{low} mice with nickel alone also develops tolerance [177].

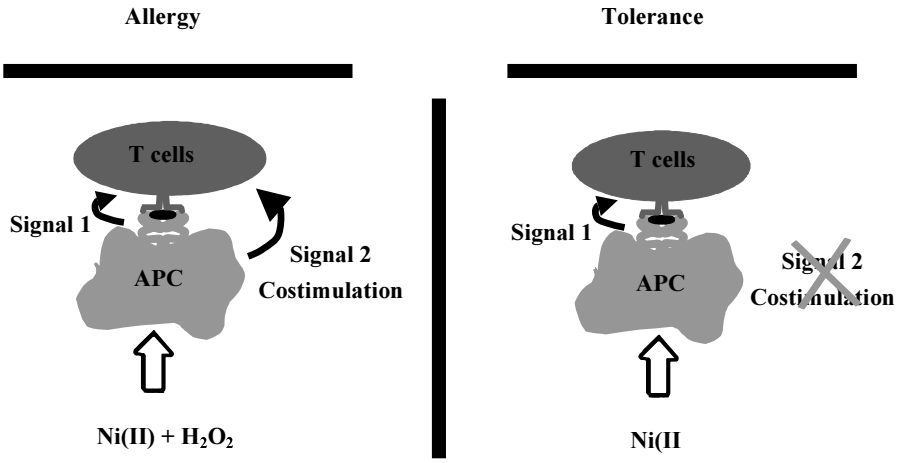


FIGURE 1.8.3 Artik *et al*'s mouse model for Ni-allergy. Artik, S. *et al* developed a mouse model for Ni-allergy. In this model, they could prime the Ni^{low} mice with $NiCl_2$ plus H_2O_2, but not with $NiCl_2$ alone. Although $NiCl_2$ alone was ineffective in T cell priming, it sufficed for eliciting recall response *in vivo* and *in vitro*. Therefore, they hypothesized that Ni^{2+} alone was able to provide an effective signal I for T cell activation, but was unable to provide an adequate signal II for priming. The successful sensitization of Ni^{low} mice with $NiCl_2$ plus H_2O_2 was due to in additional to the signal I provide by $NiCl_2$, H_2O_2 was able to induce costimulatory signals.

1.8.4 Nickel specific Treg cells

In animal models, the DTH unresponsiveness to nickel can be transferred by T cells from Ni^{high} animals to naïve recipients indicating that the oral administration of nickel can induce Treg. van Hoogstraten *et al.* [157] and Ishii *et al.* [156] reported that it was the $CD8^+$ T cells in Ni^{high} mice that can transfer tolerance to syngeneic naive recipient mice. In contrast, Cavani *et al.* [178] have isolated nickel specific $CD4^+$ T cell clones from skin lesions of nickel-allergic humans, and from the peripheral blood of nickel-allergic and non-allergic individuals. These T cell clones displayed a cytokine profile of Tr1, i.e. they produce high levels of IL-10, IL-5, variable amounts of TGF-ß and low or undetectable levels of IFN-γ and IL-4. When activated with nickel *in vitro*, these Tr1 cells blocked the maturation and function of Ni-presenting DCs in a cell-cell contact independent, IL-10-dependent fashion. Hence, these DCs displayed an impaired capacity to activate specific Tc1 and Th1 effector cells as well as T cells with a different specificity. Cavani *et al*. also identified $CD4^+CD25^+$ Treg in the peripheral blood of healthy, non-allergic individuals [179]. In a dose dependent manner, these isolated $CD4^+CD25^+$ T cells were able to strongly suppress nickel-specific responses of $CD4^+CD25^-$ T cells via a cell-cell contact-dependent, cytokine independent pathway. Surprisingly, $CD4^+CD25^+$ T cells from nickel allergic individuals have either a limited or absent capacity to suppress nickel-specific $CD4^+$ and $CD8^+$ T cell responses. Hence, in persons non-allergic to nickel both Tr1 cells [178;180], and $CD4^+CD25^+$ Treg [179] control the magnitude and duration of Ni-induced allergic contact dermatitis (ACD) and more importantly, they can prevent sensitization towards nickel. Interestingly, whereas

nickel-specific Tr1 cells can regulate the ACD by releasing cytokines, nickel-specific $CD4^+CD25^+$ Treg suppress or prevent ACD in a cell-cell contact dependent manner.

1.9 THE AIM OF THIS THESIS

From section 1.8, the following conclusions can be drawn:

1) $Ni^{very\ low}$ animals are easier to sensitize with nickel than Ni^{low} ones;
2) Nickel ions alone can provide effective signal I but not signal II, therefore Ni is a tolerogen;
3) When combined with an adjuvant, such as H_2O_2, Ni^{low} animals can be sensitized with nickel alone allowing both the study of Nickel-allergy and tolerance.

The aim of this thesis were to clarify the following points concerning nickel-allergy and tolerance.

1) Direct evidence of a relationship between oral nickel uptake and the susceptibility to become sensitized to nickel: section 3.1;
2) Direct evidence that lymph node cells (LNC) from DNFB sensitized Ni^{low} mice respond to nickel *ex vivo*. Further investigations demonstrate that this response was not due to the cross-reactivity between Ni and DNBS, but was because APC from "non-nickel" antigen sensitized Ni^{low} mice were able to activate nickel-specific T cells *in vitro*: section 3.2;
3) To investigate whether T cells from Ni^{high} and Ni^{low} mice are suppressive: sections 3.3.1-3.3.3;
4) The characterization of these suppressive T cells from Ni^{high} and Ni^{low} mice: section 3.3.4;
5) The tolerogenicity of APC from Ni^{high} mice: section 3.4

2. MATERIALS AND METHODS

This chapter describes the materials and methods used throughout this work. The materials are summarized in section 2.1 whereas equipment, reagents and protocols necessary to prepare the various buffers and solutions are listed in the appendices A-D. After section 2.1, the methods are described in the following order, cell preparation procedure (2.2), immunization of mice (2.3), challenge and mouse ear-swelling test (2.4), cell enrichment and sorting (2.5), adoptive transfer (2.6), *in vitro* restimulation (2.7), ELISA protocol for the detection of IL-2 (2.8), mixed lymphocyte reaction (MLR) (2.9), generation and testing of T cell hybridomas (2.10) and statistical analysis (2.11).

2.1 MATERIALS

This section describes the central materials used in this study including cell lines, antibodies, medium as well as plastic and glass ware. The different environmental breeding protocols for the mice are also described.

2.1.1 mice

Specific pathogen-free female C57Bl/6J (H-2^b), which express Ly5.2 (CD45.2), and BALB/c (H-2^d) mice were purchased from Janvier (Le Genest St. Isle, France). Congenic Ly5.1^+ (CD45.1^+) C57Bl/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were 7 to 10 weeks of age at the onset of experiments. They had free access to drinking water and standard rodent laboratory food (No. 1324, Altromin, Lage/Lippe, Germany).

Ly5.2 C57Bl/6J (H-2^b) mice, however, were bred and reared in Ni^{low}, $Ni^{very\ low}$ or Ni^{high} environments. Following is the detail description of Ni^{low}, $Ni^{very\ low}$ or Ni^{high} mice

Ni^{low} mice: BALB/c mice, Ly5.1 and Ly5.2 C57Bl/6J were reared only in a Ni^{low} environment, therefore these mice are referred to as BALB/c Ni^{low} and C57Bl/6J Ni^{low} mice respectively. No measures were taken to protect these animals from exposure to nickel since both the cage lids and drinking water bottle nozzles were manufactured from nickel containing stainless steel.

$Ni^{very\ low}$ mice were generated by breading and rearing Ly5.2 C57Bl/6J Ni^{low} mice in a metal free environment. In brief, mice were housed in plastic cages with plastic lids and received drinking water (tap water) from plastic bottles with glass outlets. Animals for experimentation were taken from at least the second generation.

Ni^{high} mice were generated by treating Ly5.1 or Ly5.2 C57Bl/6J Ni^{low} mice with 10 mM $NiCl_2$ in the drinking water for at least 4 weeks.

Unless otherwise stated, from now on, Ni^{low}, $Ni^{very\ low}$ or Ni^{high} mice refer to Ni^{low}, $Ni^{very\ low}$ or Ni^{high} Ly5.2 C57Bl/6J mice.

2.1.2 Cell line

BW5147(TCRα^-ß$^-$) murine thymoma line, the fusion partner for $CD4^+$ T cell hybridomas was kindly provided by Prof. Dr. H. U. Weltzien (MPI, Freiburg, Germany).
EXC-5 supernatant that rich in IL-2, IL-3, IL, and IL-5 was generated from the culture of EXC-5 cells, kindly donated from Dr. Weisner (Cologne, Germany)

2.1.3 Antibodies, microbeads and recombinant IL-2

All anti-mouse antibodies necessary for flow cytometry and ELISA were purchased from BD Biosciences (Heidelberg, Germany), and included PE-labeled anti-I-A/I-E (clone M5/114.15.2), FITC-labeled anti-$I\text{-}A^b$ (clone: AF6-120.1), FITC- and PE-labeled anti-CD8β.2 (clone: 53-5.8), PerCP-labeled CD4 (clone: RM4-5), APC-labeled anti-CD3ε (clone: 145-2C11), FITC-labeled anti-TCRβ chain (clone: H57-597), PE-labeled anti-CD45.1 (clone A20), FITC-labeled anti-CD45.2 (clone 104), FITC- and biotin-labeled anti-CD11c (clone HL3), and FITC- labelled anti-CD19 (clone 1D3) antibodies. For ELISA purified anti-mouse IL-2 (JES6-1A12) and biotin-labeled anti-mouse IL-2 (JES6-5H4) were used as capture and detection antibodies. Recombinant mouse IL-2 were also from BD Biosciences. Magnetically labelled anti-mouse CD4, anti-mouse CD90, anti-mouse MHC-II, anti-mouse CD19, anti-mouse CD11c, and anti-PE microbeads were obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

2.1.4 Medium

In the MLR, cells were cultured in DMEM medium (PAA, Linz, Austria) that was supplemented with 10% FCS, 10 U/ml penicillin/streptomycin and 50 µM 2-ME. In all *in vitro* lymphocyte transformation tests (LTT), complete RMPI 1640 medium (PAA), was used and was supplemented with 10% FCS, SC (Appendix C) and 10 U/ml penicillin/streptomycin. For the culture and *in vitro* restimulation of T cell hybridomas, RMPI 1640 medium was supplemented with 5% FCS, TC (Appendix C) and 10 U/ml penicillin/streptomycin. For the first weeks after generation, hybridomas were cultured in first HAT and then HT medium, protocols for these supplements are described in Appendix C.

2.1.5 Plastic and glass

All plastic and glassware equipment was from one of the following sources unless otherwise stated: Eppendorf, Hamburg, Germany; Flacon, Becton Dickinson, Heidelberg, Germany; Greiner, Frickenhausen, Germany or Schott, Düsseldorf, Germany.

2.2 CELL PREPARATION PROCEDURE

All plastic and glassware equipment was from one of the following sources unless otherwise stated: Eppendorf, Hamburg, Germany; Flacon, Becton Dickinson, Heidelberg, Germany; Greiner, Frickenhausen, Germany or Schott, Düsseldorf, Germany.

2.2.1 Cell centrifugation

Unless otherwise stated, all cell suspensions were centrifuged at 1200 rpm for 8 minutes at 4^{o}C using a Megafuge, from Heraeus Instruments, Germany.

2.2.2 Cell counting

Unless otherwise stated, cell suspensions were diluted 1:10 with tryptan blue solution (Appendix D) and the cell concentrations were counted using a Neubauser-Kammer haemocytometer and a light microscope (Diaplan, Lietz). Dead cells can be identified by the blue staining of tryptan blue. If more than 20% of the cells were dead the cell suspension was discarded.

2.2.3 Cell preparation

Mice were sacrificed by asphyxiation with CO_2, and the areas of incision disinfected with 70% ethanol. For the isolation of spleen or popliteal lymph nodes (PLN) the mice were handled freely; for isolation of inguinal lymph nodes (ILN) mice were first fixed upon an operation board.

2.2.3.1 Preparation of spleen cells

Mouse spleens were removed and placed into a sterile 15 ml or 50 ml tube containing sterile PBS (Appendix D). Under sterile conditions, spleens were homogenized in a small petri dish and the cell suspension transferred to a 15 ml or 50 ml tube and centrifuged. The cell pellet was resuspended by gentle tapping and 3 ml of ACT solution (Appendix D) was added at room temperature for 5 minutes to lyse the erythrocytes. Thereafter, the tube was filled up with PBS and centrifuged. After centrifugation, the cell pellet was resuspended either in PBS or in medium depending on the next experimental step, the cell suspension was then passed through a gauze filter into a fresh 15 ml or 50 ml tube.

2.2.3.2 Preparation of lymph node cells

Mouse lymph nodes (LN) were removed and placed into a 24-well plate containing 1ml of sterile complete RMPI 1640 medium (Appendix C). LN were then individually homogenized using tweezers and passed through gauze filters into 15 ml or 50 ml tubes.

2.2.4 Cell culture

All cell cultures, either primary cells or hybridomas, were incubated at 37°C with 6.5% CO_2 in a water saturated atmosphere.

2.2.5 Cell freezing and thawing

After establishment, batches of T cell hybridomas were frozen. To freeze, these cells were centrifuged, resuspended in freezing solution (Appendix D) at a concentration of 1-5 x 10^6/ml, and then aliquotted into sterile cryotubes (1 ml/tube). After 30 mins at 4°C, the cells were frozen at –80°C overnight and then moved to liquid nitrogen (–196°C) for long term preservation. When required for experimentation, frozen aliquots of cells were quickly heated in a glass of warm water (37°C) and placed into a 50 ml tube. After washed twice with TC medium, cells were placed into culture flasks and incubated as described in section 2.2.4.

2.3 SENSITIZATION OF MICE

2.3.1 Sensitization of mice for the mouse ear-swelling test (MEST)

In the case of Ni, Ni^{low} mice were injected intradermally (i.d.) into both flanks (50 µl each) with either sterile, pyrogen-free saline, 10 mM $NiCl_2$ in saline, or 10 mM $NiCl_2$ in saline containing 1% H_2O_2. When sensitizing $Ni^{very\ low}$ mice, however, instead of using 10 mM $NiCl_2$ in saline or in saline containing 1% H_2O_2, mice were injected with 1 mM $NiCl_2$ in saline or in saline containing 1% H_2O_2. For sensitization with 2,4-dinitrofluorobenzene (DNFB) both Ni^{low} or $Ni^{very\ low}$ mice were primed by painting 0.5% (w/v) DNFB on shaved flanks (25 µl each). DNFB was dissolved in a 4:1 (v/v) mixture of acetone and olive oil.

2.3.2 Sensitization of mice for lymphocyte transformation test (LTT)

In the case of Ni, both Ni^{low} or $Ni^{very\ low}$ mice were injected subcatenously (s.c.) into both hind footpads (50 µl each) with either sterile, pyrogen-free saline, 100 µM $NiCl_2$ in saline, or 100 µM $NiCl_2$ in saline containing 1% H_2O_2. For DNFB both Ni^{low} or $Ni^{very\ low}$ mice were primed by painting 0.5% (w/v) DNFB on both hind footpads (25 µl each). In experiments using fluorescein-isothiocyanate (FITC), Ni^{low} mice were primed by painting 0.5% (w/v) FITC on both hind footpads (80 µl each): FITC was dissolved in a 1:1 (v/v) mixture of acetone and dibutylpthalate.

2.4 CHALLENGE FOR RECALL AND EAR-SWELLING TEST

Ten days after priming, Ni^{low} or $Ni^{very\ low}$ mice were challenged for recall by injecting 50 µl of 10 mM $NiCl_2$ in sterile, pyrogen-free saline into the pinna of each ear, or by applying 50 µl of 0.2% DNFB. 48 hours after challenge with $NiCl_2$ and 24 hours after challenge with DNFB, DTH reactions were determined by measuring the increment in ear thickness compared to pre-challenge values. For determination of pre-challenge values, mice were anaesthesized with ether. For measurement after challenge the mice were killed by asphyxiation with CO_2 and then immediately measured.

Measurements were performed using a micrometer (Oditest D 1000 gauge, The Dyer Co., Lancaster, PA, USA) and in a blind fashion.

2.5 CELL ENRICHMENT AND SORTING

Single-cell suspensions of erythrocyte-depleted spleen cells or LNC were prepared as described in section 2.2.3. For T cell enrichment or purification, the first step was to enrich the T cell population using nylon wool columns.

2.5.1 T cell enrichment by nylon wool column

Sterile nylon wool filled columns were clamped onto stands and attached with sterile valves. Pre-warmed (37^{o}C) "SC medium" was then pipetted onto the columns and allowed to travel slowly through the tube so that the nylon wool was saturated with medium. Air bubbles were removed using a sterile pasteur pipette. After rinsing the columns with approximately 15-20 ml medium, the valves were tightened and the columns filled up with medium. Columns were then incubated at 37^{o}C for at least 30 minutes.

After incubation, the columns were washed again with warmed medium. After retightening the valves, 1.5-2 ml of spleen cell suspension (maximum 1.5x10^{8} cells) was pipetted on top of the nylon wool. The valves were then shortly opened so that the cells were allowed to penetrate into the nylon wool. After tightening the valves again, columns were filled up with medium and then placed at 37^{o}C for 45-60 minutes. Thereafter, enriched T cells are collected by eluting the nylon wool columns with warmed medium. During the elution the medium needs to pass through the nylon wool very slowly, at a rate of about 1 ml/min, and columns are not allowed to run dry. Elution can be stopped after a volume of 15-20 ml has been reached. Normally, the purity of nylon wool enriched T cells is about 80%. In some *in vivo* transfer experiments, only nylon wool enriched T cells were used.

2.5.2 Cell sorting by magnetic cell sorting equipments

To obtain highly purified cells, magnetic cell sorting (MACS; Miltenyi Biotec) equipment was used. The principle of this technique is to incubate and therefore label a particular cell population with magnetic beads coupled to specific antibodies. When these cells are then passed through a separation column, placed in a magnetic field, the cells labeled with magnetic particles will be retained while the unlabeled cells run through. So, one can obtained their desired population either directly (positive selection) or indirectly (depletion of unwanted cells). In this study only depletion was used. The magnetic cell sorting equipment used in this study includes the AutoMACS separator, the MiniMACS and the MidiMACS; they use autoMACS columns, MS columns and LS columns, respectively. In each separation, an autoMACS column can process up to 4 x 10^{9} cells and can hold up to 2 x 10^{8} magnetically labeled cells. AutoMACS columns can be repeatedly used. An MS column can process up to 2x10^{8} cells and can hold up to 10^{7} magnetically labeled cells whereas an LS column can process up to 2x10^{9} cells and can hold up to 10^{8} magnetically labeled cells. So, one needs to select the type of

separator according to the cell type and number required. Both MS and LS columns can not be repeatedly used.

After red blood cells are depleted, cells are washed with running buffer (Appendix D) and centrifgued. Thereafter, unless otherwise stated, the cell pellet is resuspended in 90 µl of running buffer and 10 µl microbeads per 10^7 cells and then incubated at 4^oC for 15 minutes. Cells are then washed, centrifuged, and resuspended in 500 µl running buffer (for MS column) or 3 ml (for LS column) or in a volume between 100 µl to 50 ml (for the autoMACS). Separation was then performed according to the manufactures instructions. In case of autoMACS, the program "DEPLETES" was used through out this study.

2.5.3 Cell enrichment and sorting

In some *in vivo* transfer experiments and *in vitro* MLR experiments, nylon wool enriched splenic T cells were further purified by depletion of MHC-II$^+$ cells with magnetic cells sorting. In some *in vivo* transfer experiment, T cells were further purified by depletion of CD11c$^+$, CD19$^+$ and MHCII$^+$ cells using sorting unit of FACSalibur (BD Biosciences, San Jose, CA) or autoMACS. The sorted T cell fractions were contaminated with <0.5% of CD11c$^+$ MHCII$^+$, CD19$^+$MHCII$^+$ cells, respectively. In "criss-cross" restimulation experiments, the APC from LNC were depleted by using MHC-II microbeads and after depletion contamination with MHC-II$^+$ cells was less than 1%. In some *in vivo* transfer experiments and *in vitro* characterization assays of Ni- or DNBS- reactive T cells, bulk T cells or unseparated LNC were depleted of CD4$^+$ or CD8$^+$ T cells. CD4$^+$ T cells were depleted using anti-CD4 microbeads. To deplete CD8$^+$ T cells, the cell populations were incubated in running buffer with PE- labeled anti-CD8β.2 antibody (diluted 1:100/ 10^7 cells) and incubated at 4^oC for 10 minutes. After washing several times, the cells were further incubated with anti-PE microbeads and cells sorted as described in section 2.5.2. The contamination of CD4$^+$ or CD8$^+$ T cells were less than 2% and 1%, respectively. In *in vitro* MLR experiments, APCs were sorted by depleting CD90$^+$ T cells from spleen cells. The contaminated T cells in sorted APC fractions were less than 1%. In *in vivo* transfer experiments, APC were sorted by depleting CD4$^+$, CD8$^+$, and CD90$^+$ T cells from spleen cells using autoMACS. The contaminated T cells in sorted APC fractions were < 0.5% CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ cells. In the serial transfer assay using T cells and APC, an additional depletion of Ly5.1$^+$ (CD45.1$^+$) cells was performed between the first set of recipients (i.e. second donors) and the second set of recipients. After this depletion, the fraction of cells required for transfer contained <0.1% Ly5.1$^+$ cells.

2.5.4 Cell purity check

After enrichment or sorting, cell purities were checked using flow cytometry, a technique that measures and analyzes the optical properties of single cells passing through a focused laser beam. When cells pass through the laser beam, they disrupt and scatter the laser light, which is detected as forward and sidewared scattered light. Forward scattered (FSC) light is related to cell size; sideward scattered (SSC) light is related to a cell's internal complexity. Besides FSC and SSC, the cytometer can also measure the relative amount of different dyes because the dyes can absorb the laser light and emit a portion of this absorbed light in different regions of the spectrum. So, when cells are stained

with fluorescent dyes or fluorochromes which are coupled to antibodies directed against cell surface or intracellular antigens, the cytometer can measure the properties of each cell. Typical antibody combinations used in this study can be found in table 2.5.4.

Table 2.5.4 Typical antibody combinations used for staining cells

FITC-labeled Ab	PE-labeled Ab	PerCP-labeled Ab	APC-labeled Ab
TCRß chain	I-A/I-E		
CD19	I-A/I-E		CD3ε
CD11c	I-A/I-E		CD3ε
CD8β.2	I-A/I-E	CD4	CD3ε
$I\text{-}A^b$	CD45.1		CD3ε

In order to obtained an optimal staining effect (i.e. brightest staining/lowest background), the dye-labeled antibodies need to be titrated and this work had been done in previous studies in this laboratory for all the antibodies used in this study. To stain, cells (upto 10^6) are washed and resuspended in 100 µl of anti-CD16/CD32 mAb (Fc block) diluted 1:100 in Facs buffer (Appendix D). Cells are incubated at 4^oC for 10 minutes and then washed. Cells are then stained in 50 µl of desired antibody cocktail for 10 mins at 4°C in the dark. Cells are then washed again, resusupended in buffer and acquired using the FACSCalibur. Results were analyzed with CellQuest software (Becton Dickinson).

2.6 ADOPTIVE TRANSFERS

After enrichment and/or sorting, cell suspensions were extensively washed with sterile, pyrogen-free PBS. Following cell counting, cell suspensions were diluted to the desired concentration and intravenously (i.v) injected into the tail vein of recipient mice (150 µl/mouse). To investigate primary immune responses, mice were sensitized intradermally (i.d.), as described in section 2.3.1 one day after transfer. Ten days thereafter, mice were challenged for recall at the ears, and 48 hours after rechallenge with nickel or 24 hours after rechallenge with DNFB, their ear-swelling response were measured. To determine secondary immune responses mice were first sensitized and 10 days later received an adoptive transfer of cells. On the next day, mice were challenged at the ears, and 48 hours after rechallenge with nickel or 24 hours after rechallenge with DNFB, their ear-swelling responses were measured. The detailed description of rechallenge and ear-swelling measurement can be found in section 2.4.

2.7 LYMPHOCYTE TRANSFORMATION TEST (LTT)

Ten days after sensitization (see section 2.3.2), mice were sacrificed and the draining popliteal and inguinal lymph nodes from each group were isolated and pooled. Single-cell suspensions of these pooled cells (LNC) were prepared as described in section 2.2.3.2. Cells were then plated (200 µl/well)

onto 96-well round-bottom plates in triplicates or quadruplicates and cultured at 37° C either alone or with the following antigens: 75 µM $NiCl_2$, 100 µM DNBS or 200 µM FITC. The experimental assays performed were summarized in table 2.7.

After 3 days of culture, cells were pulsed with 0.5 µCi/well [^{3}H] thymidine for 16 hours. In the majority of the experiments, cells were harvested onto filters using the Inotech Sample Harvesting System (Inotech AG, Dottikon, Switzerland), which were then dried before MiltiLex A scintillator sheets were added (Wallac Oy, Turku, Finland). After the melt-on scintillator sheets were cooled, [^{3}H] thymidine incorporation was measured with a 1450 MicroBeta TriLux Liquid Scintillation and Luminescence counter (Wallac Oy, Turku, Finland).

Table 2.7 the different LTT assays performed in this study

	Assay	Cell types used
1	Standard	10^5 LNC from saline, Ni, Ni + H_2O_2, DNFB or FITC sensitized Ni^{low} mice
2	Ni- or DNBS-reactive cell	10^5 LNC from Ni + H_2O_2 or DNFB sensitized Ni^{low} mice that were depleted of either $CD4^+$ or $CD8^+$ T cells
3	Modified limiting dilution	Different numbers of either bulk LNC or CD8β.2 depleted LNC from either Ni + H_2O_2 or DNFB treated Ni^{low} mice
4	Criss-cross restimulation	7 x 10^4 sorted LN T cells from naive, Ni + H_2O_2, DNFB primed Ni^{low} mice were cocultured with 10^5 irradiated LNC (1000 rad) from these differently treated mice.
5	"suppressor" T cell	10^5 LNC from Ni, Ni + H_2O_2 sensitized $Ni^{very\ low}$ mice or Ni + H_2O_2 sensitized Ni^{low} mice were cocultured with 3 x 10^5 splenic T cells from Ni^{low} mice Ni^{high} mice

2.8 DETECTING IL-2 CONCENTRATION BY ELISA

IL-2 production in the supernatants from cell cultures was measured by a sandwich ELISA using two monoclonal antibodies (capture antibody: clone JES-1A12, detecting antibody: clone JES6-5H4) according to following protocol.

96-well Nunc Maxisorb ELISA plates (Nunc GmbH, Germany) were coated overnight at 4°C with 50 µl of 1 µg/ml capture antibody dissolved in Binding Solution (Appendix D). Plates were then washed (Washing Buffer Appendix D) and blocked for 2 hours at room temperature with 200 µl/well Blocking Buffer (Appendix D). After a further washing standards (500pg/ml to 7.81 pg/ml) and cell culture supernatants were plated in triplicate (100 µl/well) in the Blocking Buffer/Tween and incubated at 4°C overnight. Plates were then washed 5 times before 100 µl/well of biotinylated anti-IL-2 detecting antibody (0.5 µg/ml) in Blocking Buffer was added. After 1 hours incubation at room temperature, plates were washed a further 4 times and then incubated with 100µl/well, 1:2000 Streptavidin-Horseradish Peroxidase (AV-HRP) (Amersham Pharmacia Biotech) diluted in PBS/0.1% milk powder

for 30 minutes. Following 5 times washing, color reaction was developed by adding 100 µl/well TMB substrate (Kem-EN-Tec A/S, Denmark) and incubating at room temperature in dark for 30-60 minutes. After the color reaction was stopped with 100 µl/well 0.2 M H_2SO_4 for 10 minutes (also in dark), the optical density (OD) of each well was read using a 96-well-plate ELISA reader (Dynex Technologies) at a wavelength of 450 nm. The levels of IL-2 in the tested samples were determined by linear regression from the standard curve constructed using standard recombinant IL-2. Cell cultures assays used in these experiments were performed as described in section 2.7, the only difference was that, $5x10^5$ cells/well were seeded instead of 1 x 10^5 cells/well. After 24 hours later the supernatant from each well was transferred to a fresh 96-well plate and froze at –80^{o}C before use.

The optimal concentrations of capture and detecting antibodies (i.e. 1 µg/ml and 0.5 µg/ml, respectively) used in the above measurement were determined in pilot experiments by generating standard curve with recombinant IL-2 following above protocol. This protocol was based on the one provided by BD PharMingen (Heidelberg, Germany).

2.9 MIXED LYMPHOCYTE REACTION (MLR)

APC from C57Bl/6 Ni^{low} and Ni^{high} mice and BALB/c Ni^{low} mice were obtained by depleting $CD90^+$ cells from their spleen cell populations. T cells from Ni^{low} BALB/c mice were enriched by nylon wool columns and then depletion of MHC-II^+ cells using microbeads, as described in section 2.5.1 and 2.5.2. The purity of these sorted populations was determined by flow cytometry. After sorting, cells were extensively washed and then cultured in DMEM medium.

After irradiation with 2000 rad (Gammacell 2000, Copenhagen Science Park Symbion, Copenhagen, Danmark), APC from both Ni^{low} and Ni^{high} C57Bl/6 mice were pipetted into 96-well round-bottom plates (10^5 cells/100µl/well) in quadruplicates either alone or with 75 µM $NiCl_2$. As a control, APC from BALB/c mice were cultured in the same manner. On the second day, 10^5/well T cells from BALB/c mice were added and $NiCl_2$ concentration was adjusted to perform mixed lymphocyte reaction (final volume was 200 µl/well). Four days later, 0.5 µCi/well [^{3}H] thymidine was added for 16 hours. The cells were harvested as described in section 2.7. The results are expressed as stimulation index (SI) + SD (SI = mean cpm of C57Bl/6 APC stimulated BALB/c T cells/ mean cpm of BALB/c APC stimulated BALB/c T cells).

2.10 GENERATION AND TESTING OF T CELL HYBRIDOMAS

The best way to study the specificity of T cells to a particular antigen is propagating by a population of T cells specific to the particular antigen from a single cell. This can be done by generating T cell clones or T cell hybridomas. Since the T cell hybridomas are easier to grow in larger numbers, the generation of T cell hybridomas was used.

To generate T cell hybridomas, antigen activated T cells are immortalized by fusing them with a T cell lymphoma or thymoma (here we use BW5147$\alpha^-\beta^-$TCR thymoma cells). The BW5147 thymoma cells are resistant to the purine analogue 6-thioguane because of their deficiency in hypoxanthine-guanine

phosphoribosyl transferase (HGPRT). This deficiency results in lethal sensitivity to aminopterin that blocks *de novo* synthesis of purines. The normal T cells that are efficient in HGPRT are not sensitive to aminopterin when hypoxanthine and thymidine are supplied because they can use a salvage pathway in which purine is synthesized from exogenously supplied hypoxanthine by HGPRT. So, after fusion with antigen-activated T cells, cells are cultured in "HAT medium" (Appendix C) which contains hypoxanthine and thymidine. Only hyridomas that are a complete fusion of thymoma cells and antigen-activated T cells can survive since the thymoma cells obtain the necessary genes from the normal T cells [181]. After screening and subcloning, antigen specific T cell hybridomas should be obtained.

2.10.1 Preparation of antigen specific T cells

Pooled popliteal and inguinal lymph node cells from DNFB sensitized C57Bl/6 Ni^{low} mice (see section 2.3.2) were restimulated with either Ni or DNBS, as described in section 2.7 for 3 days. Thereafter, the activated T cells were propagated with 5 µg/ml Con A (Sigma) overnight.

2.10.2 Preparation for cell fusion

After centrifugation, expanded T cells (from section 2.10.1) were resuspended in 3 ml PBS and dead cells were removed using a Ficoll Gradient (Ficoll-Plaque, Phamocia, Freiburg, Germany). In brief, the 3 ml T cell suspension is slowly pipetted onto 5 ml of Ficoll solution in a 15 ml tube. After centrifugation at 2200 rpm for 20 minutes and stopped without brake, the living cells were carefully collected from the top of Ficoll solution and placed into a fresh 50 ml tube. The collected T cells were washed once with RPMI 1640 medium containing 10% FCS. After washing, the T cell suspension was mixed with the BW5147$\alpha^-ß^-$TCR fusion partner at the ratio of 1:1 in naked RMPI 1640 medium and then washed twice. In the second washing, the cells were centrifuged at 800 rpm for 5 minutes.

2.10.3 Cell fusion

After washing, cell pellets were resuspended and the tube incubated in a beaker of warm water (37^{o}C). 1 ml of pre-warmed polyethylene glycol 1500 (PEG 1500) was then added 1 drop at a time over a period of 45 seconds with continuously stirring of the tube. Stirring continues for another 45 seconds after PEG addition. Thereafter, 1 ml of pre-warmed (37^{o}C) naked medium was added in the same manner as PEG over a period of 30 seconds, followed by another 2 ml, 3 ml and 4 ml of naked medium. After adding another 20 ml pre-warmed naked medium, the cells were centrifuged at 800 rpm for 5 minutes. Following centrifugation, the cells were resuspended in RMPI medium (10% FCS) and pipetted (2 drops/well) into flat bottomed 96-well plates using a 2 ml pipette. On the next day, 2 drops of 2 times "HAT medium" was put on each well. Afterwards, the fused cells need to be monitored, and fed at certain time points. Resulting colonies are transferred to 24-well plates when appropriate and grown to 1-2 x 10^6 cells which is sufficient for screening. At different time points after fusion, different culture mediums are used: during the first 2 weeks of culture, "HAT medium" is applied; in the third week, the "HAT medium" was replaced by "HT medium" (Appendix C) and 3 weeks after cell fusion, "TC medium" (Appendix C) is used for continuous culture.

2.10.3 Screening and detection of T cell hybridomas

It is well known that activated T cells proliferate to IL-2. Here, this principle was used in the screening and detecting T cell hybridomas. Briefly, in the presence of APC and the specific antigen, T cell hybridomas will secrete IL-2 into the supernatants. When Con A activated T cells are incubated with IL-2 containing supernatant they will proliferate, this is called an IL-2 bioassay (see section 2.10.3.3). So, in order to perform this assay, one first needs to prepare the supernatants that may contain IL-2 by using T cell hybridomas stimulation assay (section 2.10.3.1) and Con A blasts (section 2.10.3.2).

2.10.3.1 T cell hybridoma stimulation assay

In the absence or presence of 75 µM Ni or 100 µM DNBS, 1 x 10^5 T cell hybridomas and 5 x 10^5 irradiated (2000 rad) spleen cells from naive C57Bl/6 Ni^{low} mice were cocultured in triplicates in "TC medium" (200 µl/well). 24 hours after culture, 50 µl of the culture supernatants were transferred to a new 96 well plate and frozen at -80^{o}C at least overnight ready for the IL-2 bioassay (see section 2.10.3.3).

2.10.3.2 Preparation of Con A blasts

C57Bl/6 spleen cells were prepared as described in section 2.2.3.1. After adjusting to a concentration of 1 x 10^6 cells/ml, they were cultured in "TC medium" with 1.25 µg/ml Con A for 24 hours. After washing and culture in "TC medium" without Con A for another 24 hours, the cells were ready for the IL-2 bioassay.

2.10.3.3 IL-2 bioassay

After thawing, the presence of IL-2 in the hybridoma-derived culture supernatants (section 2.10.3.1) was tested by adding IL-2 dependent Con A blasts (2 x 10^4/50 µl). For a positive control, Con A blasts were incubated in "TC medium" with 10% EXC-5 supernatant. After 18 hours of culture, 0.5 µCi/well thymidine was added. Six hours later, the cells were harvested as described in section 2.7. The results are expressed as the stimulation index (SI) + SD. SI is calculated as the mean cpm of Con A blasts cultured in the presence of supernatants of antigen-stimulated hybridoma/ mean cpm of Con A blasts cultured in the presence of supernatants of the same hybridoma cultured in medium alone.

2.10.4 Subcloning

To guarantee a single colony T cell hybridoma response, hybridomas were subcloned after the original screening test. Those hybridomas which showed positive responses to either Ni or DNBS were further subcloned using a limiting dilution technique. Briefly, 9, 3, 1 or 0.3 cells from each hybridoma were plated onto 96-well-plates in 200 µl of TC medium. After several days of culture, the grown cells were

transferred from 96-well-plates to 24-well-plate. When the cells grew to a concentration of about 1-2 x 10^6 cells, they were retested. In this study, hybridomas were subcloned twice. These established T cell hybridomas were then tested for their specificity with Ni or DNBS as described in last section.

2.11 Statistical analysis

Statistical significance of results was determined by ANOVA followed by Newman-Keuls test using GraphPad Prism Softward, (GraphPad Prism, California, USA).

3. RESULTS

In this chapter, nickel specific reactivity and suppressive activity will be described by using Ni^{high}, Ni^{low} and $Ni^{very\ low}$ mice. Section 3.1 provides the evidences of different susceptibilities of these three types of mouse to the sensitization to nickel. Section 3.3 deals with tolerance and suppression, describes the different suppressive activity of the T cell from different type of mice. Section 3.4 provides the evidences of tolerogenicity of APCs from Ni^{high} mice.

3.1 DIFFERENT SUSCEPTIBILITIES OF NI^{HIGH}, NI^{LOW} AND $NI^{VERY\ LOW}$ MICE TO THE SENSITISATION TO NICKEL

In order to deal with this issue, *in vivo* mouse ear-swelling test (MEST) and/or *in vitro* proliferation and/or IL-2 secretion from the cultured supernatants were measured. Due to the different focus at the different experimental stages, only Ni^{high} mice versus Ni^{low} mice, and Ni^{low} mice versus $Ni^{very\ low}$ mice were compared, but had no direct comparison of all these three types of mouse at one time.

3.1.1 Comparison of Ni^{low} and Ni^{high} mice: Ni^{low} mice can be immunized of with $NiCl_2/H_2O_2$ but not with Ni alone; Ni^{high} mice can not be immunized even with $NiCl_2/H_2O_2$

Artik *et al* established a mouse model to induce DTH reaction to nickel in mice. In this model, they found that Ni^{low} mice could be immunized with $NiCl_2$ combining with adjuvant such as H_2O_2, but not with $NiCl_2$ alone. The reason was $NiCl_2$ alone could only provide signal 1 (antigen) without signal 2 (co-stimulation), therefore, although it could elicit recall immune response, $NiCl_2$ alone was ineffective to *de novo* activate nickel-specific T cells[153]. As it has been described in section 1.5, repeated giving only signal 1 without signal 2 induces tolerance. Combining this principle and Artik *et al*'s finding [153], one can conclude that $NiCl_2$ alone should be a tolerogen. Here, I employ this Ni-allergic mouse model to study oral tolerance to nickel.

Oral administration of $NiCl_2$ to naive Ni^{low} mice decreased the ear-swelling response to $NiCl_2$ in a dose-dependent manner. If the naive Ni^{low} mice were treated with 10 mM $NiCl_2$ in the drinking water for periods of 10, 5, and 4 weeks, respectively, complete tolerance was induced (table I of ref [177]). Based on this results, from now on, a 4- to 8- week course of 10 mM $NiCl_2$ in the drinking water was chosen for induction of oral tolerance. When mice thus treated were sensitized 1 week after the termination of oral treatment and challenged 10 days later, they only showed a background ear-swelling response to nickel (bar 3, **Figure 3.1.1A**), but a completely normal response to DNFB (bar 6, **Figure 3.1.1A**). Virtually identical results were obtained when the mice were sensitized and

challenged after a treatment-free interval of 20 weeks (**Figure 3.1.1B**), indicating that long term tolerance had been induced. ***These results were obtained by in cooperation with Drs. S. Artik and K. Roelofs-Haarhuis.***

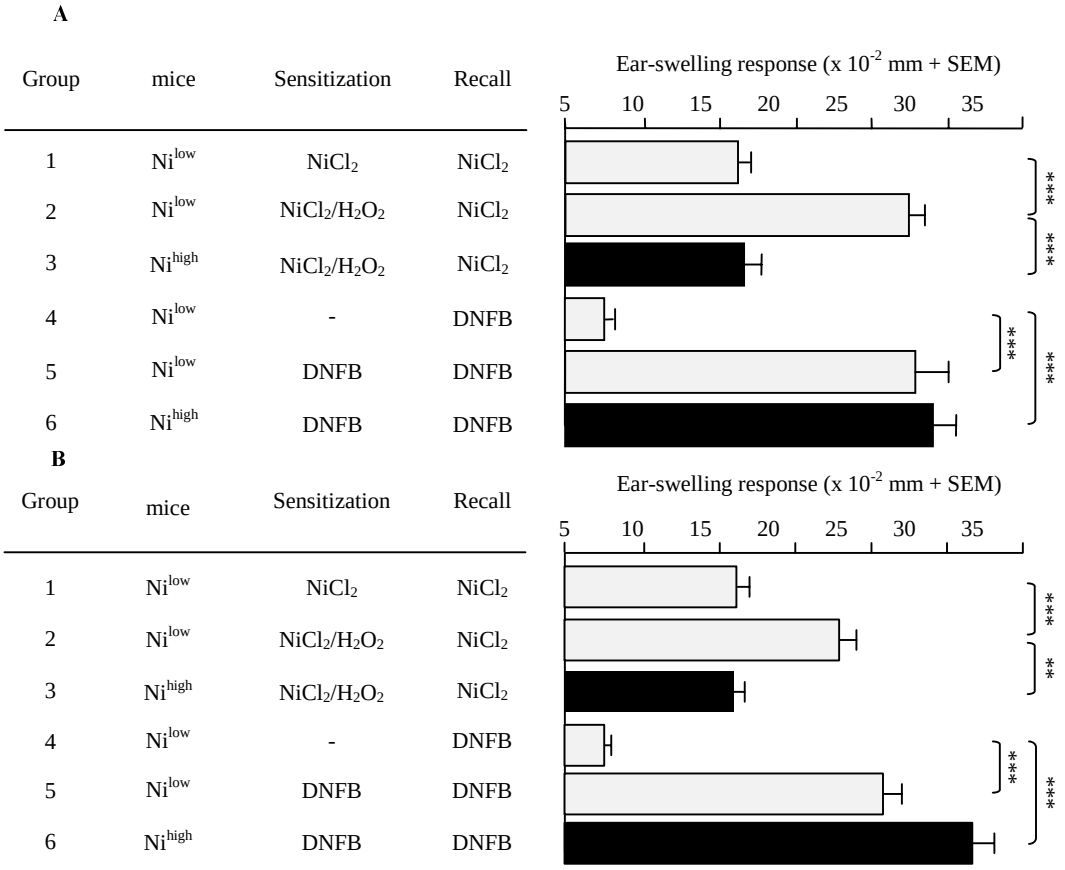


FIGURE 3.1.1. Oral tolerance to Ni induced in naive Ni^{low} mice is specific and lasts for at least 20 wks. Non-sensitized Ni^{low} mice were treated with 10 mM $NiCl_2$ in the drinking water for a period of 4 wks ($\rightarrow Ni^{high}$) or were left untreated, as indicated. After a treatment-free interval of 1 wk (*A)* or 20 wks *(B)*, mice were injected with either $NiCl_2$ alone (negative control), $NiCl_2$ in H_2O_2, or DNFB and challenged for recall as indicated, and their ear-swelling response was determined. Asterisks indicate a significant difference (**, $p \leq 0.01$, and ***, $p \leq 0.001$) between the groups compared by brackets. Experiment *(A)* was performed five times and experiment *(B)* two times, and each time comparable results were obtained. In this and the following Figs., black bars indicate the decisive experimental groups.

3.1.2 T cells from Ni^{high} mice were anergic: show in vitro proliferation and IL-2 production results

The *in vivo* MEST (**Figure 3.1.1**) demonstrated that oral nickel treatment did induce tolerance to nickel. We then asked whether the T cells of tolerized mice were anergic in the presence of $NiCl_2$. To

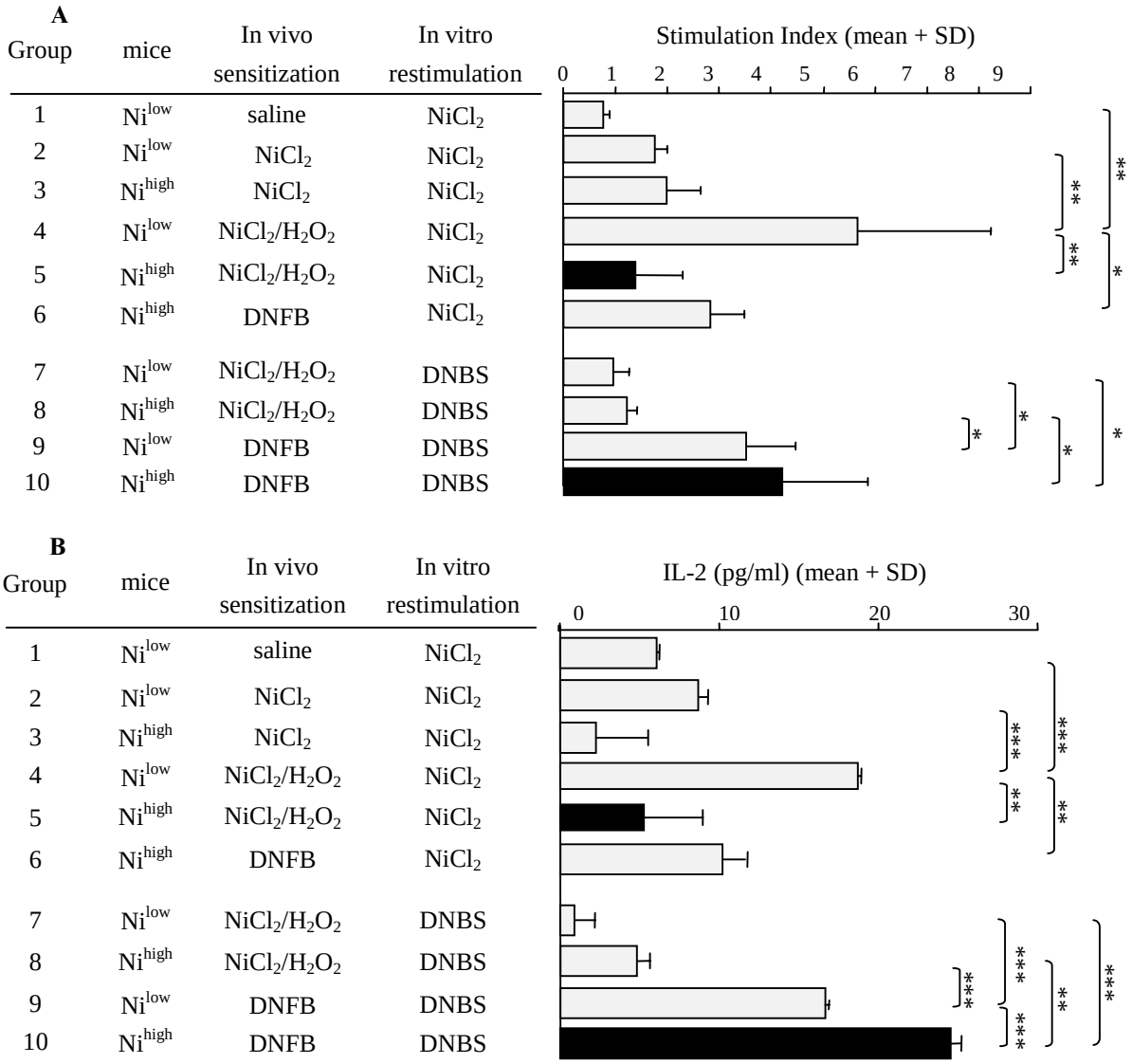


FIGURE 3.1.2. LNC of Ni^{high} mice fail to proliferate and produce IL-2 in response to Ni ions, but respond normally to DNFB. Non-sensitized Ni^{low} mice were treated with 10 mM $NiCl_2$ in the drinking water for a period of 4 wks ($\rightarrow Ni^{high}$) or left untreated, as indicated. Six wks after the termination of tolerance treatment, they were injected with either saline, $NiCl_2$ alone, $NiCl_2$ in H_2O_2, or DNFB. Ten days later, pooled cells from the draining lymph nodes of these mice were restimulated in vitro with Ni ions or DNBS, and cell proliferation and IL-2 secretion, respectively, were determined. In the proliferation assay *(A),* cultures were pulsed with [^{3}H] thymidine three days after restimulation and the isotope incorporation was determined. Background values obtained from cells cultured in medium only varied between 310 ± 16.3 and 825 ± 106 cpm. In the IL-2 secretion experiments *(B)*, after 24 hours of culture, the supernatants were transferred to ELISA plates to measure their IL-2 levels. Asterisks indicate a significant difference (*, $p \leq 0.05$, **, $p \leq 0.01$, and *** $p \leq 0.001$) between the groups compared by brackets. A representative result of four independent experiments is shown.

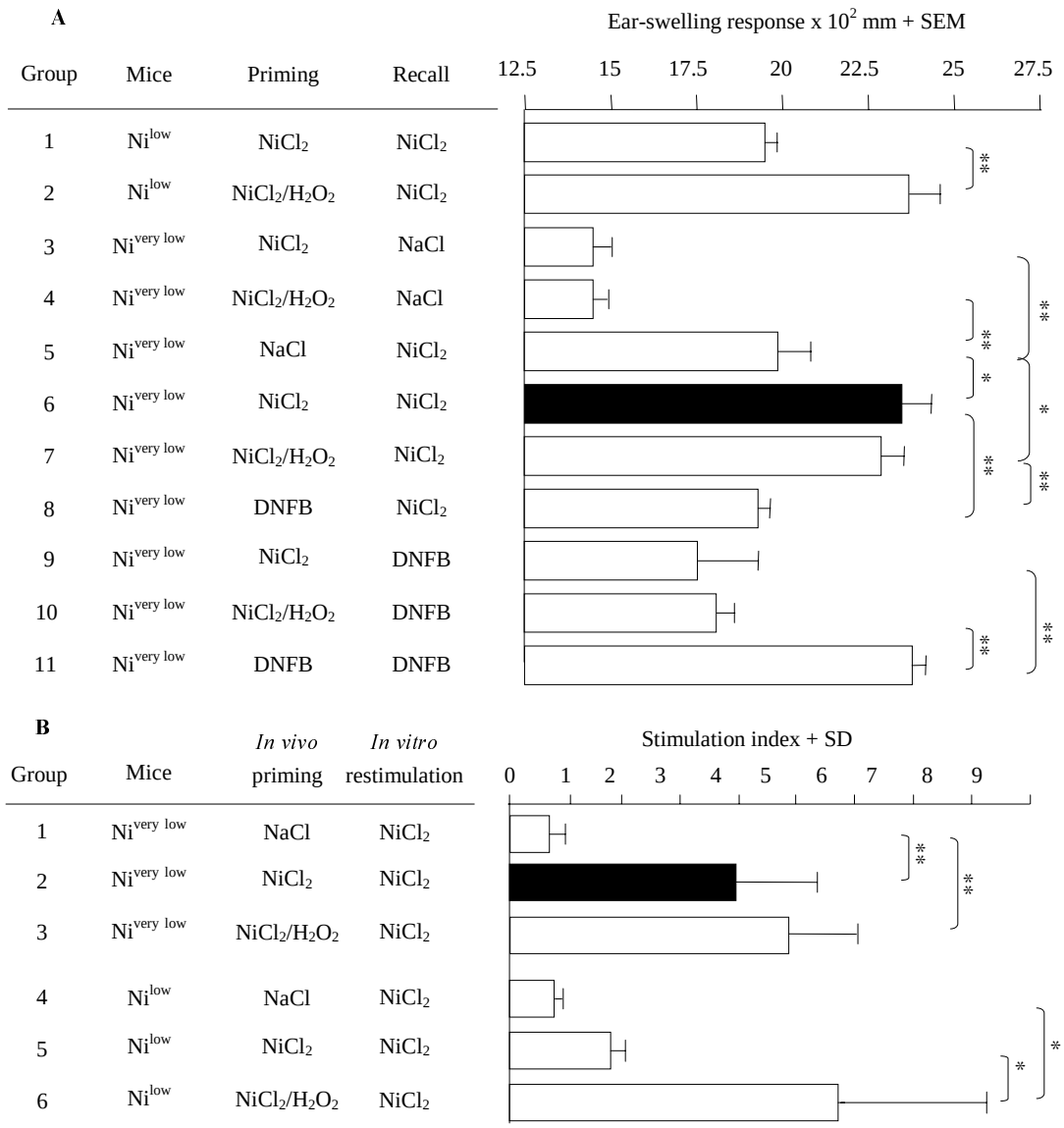


FIGURE 3.1.3. $Ni^{very\ low}$ mice, but not Ni^{low} mice, can be immunized by exposure to Ni ions without adjuvant. $Ni^{very\ low}$ and Ni^{low} mice were injected with either NaCl (negative control), $NiCl_2$ alone, $NiCl_2$ in H_2O_2, or painting with DNFB as described in Materials and Methods. (A) after being challenged for recall as indicated, and their ear-swelling response was determined. (B) Ten days later, pooled cells from the draining lymph nodes of these mice were restimulated *in vitro* with Ni ions. Three days after restimulation, cultures were pulsed with [^{3}H] thymidine and the isotope incorporation was determined. Asterisks indicate a significant difference (*, $p \leq 0.05$; **, $p \leq 0.01$) between the groups compared by brackets. Experiment A repeated three times and B at least four times, and each times got comparable results.

address this issue, we tested their capacity for proliferation and IL-2 production following *in vivo* sensitisation with $NiCl_2$ plus H_2O_2 and restimulation with Ni ions *in vitro* (**Figure 3.1.2**). Groups of Ni^{high} and Ni^{low} groups of mice were sensitised (see section 2.3.2) with either saline, $NiCl_2$ alone, $NiCl_2$ in H_2O_2, or DNFB. Ten days later, LNC (see section 2.7) were restimulated *in vitro* with $NiCl_2$ and DNBS (the water soluble analogue of DNFB), respectively. As expected, LNC of Ni^{low} mice sensitised with $NiCl_2$ in H_2O_2 showed an enhanced cell proliferation and IL-2 production (group 4 in both **Figure 3.1.2 A and B**). In contrast, LNC of Ni^{high} animals completely failed to do so (group 5 in both **Figure 3.1.2 A and B**). Their anergic state was specific for $NiCl_2$, because they did respond to DNFB

sensitisation and restimulation with DNBS (group 10 in both **Fig. 3.1.2 A and B**). These results obtained *in vitro* parallel those obtained *in vivo*, demonstrating Ni-specific unresponsiveness (cf. groups 3 and 6 in both **Fig. 3.1.1A and B**). These data indicate that the LNC from Ni^{high} mice were anergic.

3.1.3 Comparison of Ni^{low} and $Ni^{very\ low}$ mice: in contrast with Ni^{low} mice, $Ni^{very\ low}$ mice can also be immunized with $NiCl_2$ alone

Although Ni^{low} animals were difficult to be *de novo* sensitized with nickel alone [153-155;157] due to nickel alone fail to provide sufficient signal II [153]. However, nickel ions do can upregulate costimulatory molecules or other accessory molecules on DC [182] or other "non-professional" APC such as keratinocytes [183-186] when these "APC" were cultured *in vitro*. These findings hint that $NiCl_2$ may be able to provide certain level of costimulation. More recently, we found an upregulation of CD80 expression on DC of draining auxiliary lymph nodes upon injection $Ni^{very\ low}$ mice with $NiCl_2$ alone (M. Fang, unpublished data). Interestingly, van Hoogstraten *et al* [157] found that the $Ni^{very\ low}$ mice were easier to be sensitized than Ni^{low} mice. From these findings, we concluded that: 1) $NiCl_2$ alone is able to provide certain but not high level of costimulation; 2) $Ni^{very\ low}$ mice may require lower costimulatory signal to be sensitized than the Ni^{low} mice. Therefore, by using *in vivo* MEST and *in vitro* proliferation assay, I compared the susceptibilities of Ni^{low} and $Ni^{very\ low}$ mice to the sensitization to nickel.

From *in vivo* MEST, I found that unlike Ni^{low} mice, in which the high ear-swelling could only be observed when they were immunized with $NiCl_2/H_2O_2$ (bar 2, **Figure 3.1.3A**), but not with $NiCl_2$ alone (bar 1, **Figure 3.1.3A**), the $Ni^{very\ low}$ mice showed high ear-swelling response when they were immunized with either $NiCl_2/H_2O_2$ (bar 7, **Figure 3.1.3A**) or $NiCl_2$ alone (bar 6, **Figure 3.1.3A**). The immune response to nickel in the $Ni^{very\ low}$ mice are nickel specific. Because $NiCl_2$ or $NiCl_2/H_2O_2$ sensitized mice only response to Ni (bars 6 and 7, **Figure 3.1.3A**) but not to DNFB (bars 9 and 10, **Figure 3.1.3A**); vice versa, DNFB immunized mice showed high ear-swelling upon re-challenging with DNFB (bar 11, **Figure 3.1.3A**) but not with Ni (bar 8, **Figure 3.1.3A**).

Parallel to *in vivo* MEST, the *in vitro* proliferation assay also confirmed these findings (**Figure 3.1.3B**). After *in vitro* restimulation with $NiCl_2$, an enhanced proliferation was observed in LNC of $Ni^{very\ low}$ mice when they were immunized with either $NiCl_2$ alone (bar 2, **Figure 3.1.3B**) or $NiCl_2/H_2O_2$ (bar 3, **Figure 3.1.3B**); whereas the enhanced proliferation could only be found in LNC of Ni^{low} mice which was injected with $NiCl_2/H_2O_2$ (bar 6, **Figure 3.1.3B**), but not with $NiCl_2$ alone (bar 5, **Figure 3.1.3B**).

3.1.4 Summary and conclusion concerning the susceptibilities of Ni^{high}, Ni^{low} and $Ni^{very\ low}$ mice to the sensitisation to nickel

The results from section 3.1.1 to 3.1.3 clearly show that the higher the mice oral uptake of nickel, the more difficult they can be immunized: Ni^{high} mice could not be immunized at all; Ni^{low} mice could be

immunized with Ni ions combining with adjuvant such as H_2O_2; $Ni^{very\ low}$ could be immunized with either Ni ions alone or Ni ions combining with adjuvant.

3.2 IN VITRO PRIMING OF NI-REACTIVE T CELLS FROM LNC OF NI^{LOW} MICE

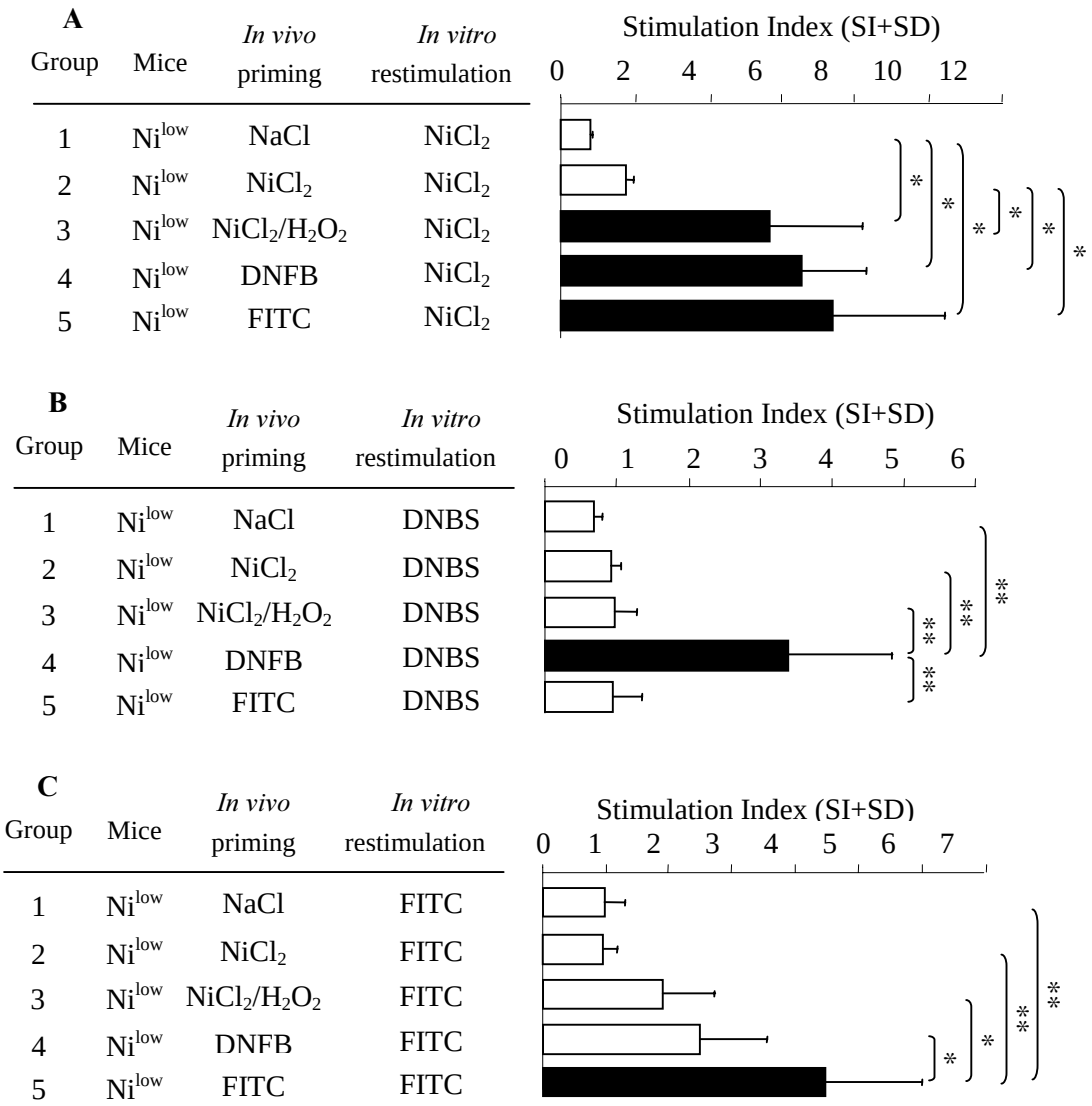


FIGURE 3.2.1. LNC of Ni^{low} mice immunized with DNFB or FITC also proliferate in response to Ni ions. Ni^{low} mice were primed with either saline, $NiCl_2$ alone, $NiCl_2$ in H_2O_2, DNFB, or FITC. Ten days later, pooled cells from the draining lymph nodes of these mice were restimulated *in vitro* with Ni ions (A), DNBS (B), or FITC (C). Cultures were pulsed with [^{3}H] thymidine three days after restimulation and the isotope incorporation was determined. Asterisks indicate a significant difference (*, $p \leq 0.05$, **, $p \leq 0.01$, and *** $p \leq 0.001$) between the groups compared by brackets. A representative result of six independent experiments is shown.

Artik *et al*. [153] found that the DTH response induced by $NiCl_2/H_2O_2$ in Ni^{low} mice was nickel-specific, because the mice thus immunized failed to mount a secondary response (in the MEST) to the control $K_2Cr_2O_7$. By using DNFB as control antigen, the data obtained in this thesis confirmed this finding in both Ni^{low} and $Ni^{very\ low}$ mice (**Figures 3.1.1 and 3.1.3**). Hence, mice that were

immunized with $NiCl_2$ alone, or $NiCl_2/H_2O_2$, showed a high ear-swelling response when they were re-challenged at the ears with Ni, but not with control antigens. Vice versa, that were immunized with control antigen showed high ear-swelling upon re-challenge with the particular control antigen, but not with Ni. However, as will be shown below this specificity detected *in vivo* (in the MEST) could not be found, when the response of the Ni^{low} mice was studied *in vitro* by using the lymphocyte proliferation assay.

3.2.1 Upon restimulation in vitro, LNC of Ni^{low} mice immunized with DNFB and FITC also reacted to Ni

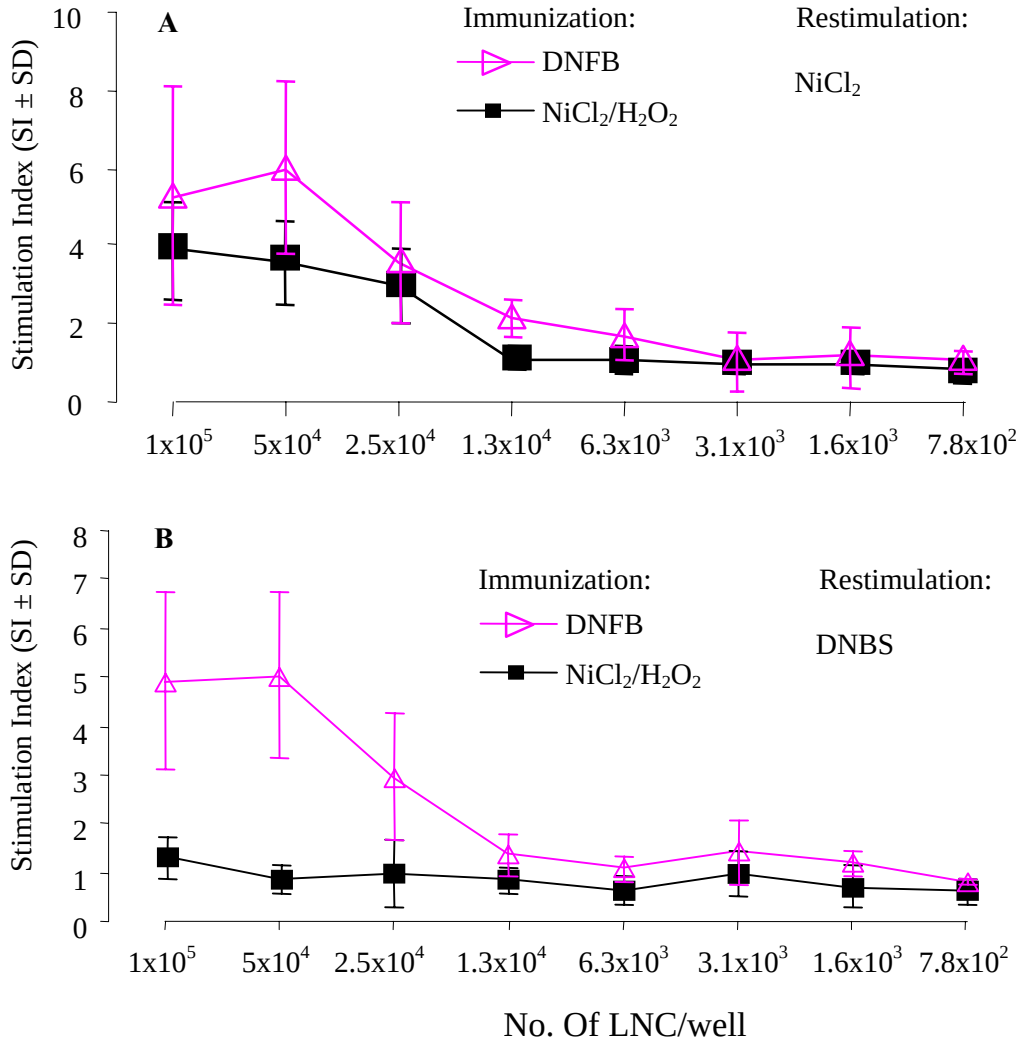


FIGURE 3.2.2. LNC of Ni^{low} mice that were immunized with either DNFB or $NiCl_2/H_2O_2$ show comparable frequencies of Ni-reactive lymph-node cells. Ni^{low} mice were primed with either $NiCl_2$ in H_2O_2 (—■—) or DNFB (—▷—). Ten days later, pooled cells from the draining lymph nodes of these mice were serially diluted, as indicated, and restimulated *in vitro* with Ni ions (A) or DNBS (B) Cultures were pulsed with [3H] thymidine three days after restimulation and the isotope incorporation was determined. A representative result of three independent experiments is shown.

LNC of Ni^{low} mice which had been immunized with either $NiCl_2$ alone, $NiCl_2/H_2O_2$, DNFB, FITC, or saline were restimulated *in vitro*, as described in section 3.1.2. As expected, the LNC from mice immunized with DNFB and FITC could only be restimulated with DNBS (bar 4, **Figure 3.2.1B**) and FITC (bar 5, **Figure 3.2.1C**), respectively. In contrast, Ni ions not only restimulated the LNC from mice immunized with $NiCl_2/H_2O_2$ (bar 3, **Figure 3.2.1A**), but also those from DNFB- and FITC-sensitized mice (bars 4 and 5, **Figure 3.2.1A**).

3.2.2 LNC of Ni^{low} mice immunized with $NiCl_2/H_2O_2$ had comparable frequencies of Ni-reactive T cells as those immunized with DNFB

In order to find out whether the Ni-reactive cells from $NiCl_2/H_2O_2$-sensitized mice are different from those of mice that were immunized with antigen other than nickel, I compared the frequencies of Ni-reactive T cells in LNC from $NiCl_2/H_2O_2$-immunized mice with those from DNFB-immunized mice. For this purpose, I set up dilution series of the LNC obtained from $NiCl_2/H_2O_2$- and DNFB-immunized mice, respectively, and added either $NiCl_2$ or DNBS to the cultures. As expected, the LNC of DNFB-immunized mice responded to DNBS, but not to $NiCl_2$ (**Figure 3.2.2B**) indicating a specific secondary response. In contrast, when the LNC obtained from the same mice were (re)stimulated with $NiCl_2$ comparable frequencies of Ni-reactive cells of $NiCl_2/H_2O_2$ and DNFB sensitized mice were observed (**Fig 3.2.2A**).

3.2.3 Ni- and DNBS- reactive T cells in LNC of DNFB sensitized Ni^{low} mice are different cells

Since LNC from Ni^{low} mice primed with DNFB also reacted to Ni (**Figure. 3.2.1**) and had a comparable frequency of Ni-reactive T cells as $NiCl_2/H_2O_2$-primed mice, a new question arose: are the Ni-reactive T cells found in the DNFB sensitized mice identical with the ones reacting to DNBS, the soluble analogue of DNFB used *in vitro*? In other words, do the DNBS-reactive T cells in DNFB-sensitized mice show true cross-reactivity to nickel? To answer this question, the experimental approaches described below (sections 3.2.3.1 and 3.2.3.2) were used.

3.2.3.1 Whereas Ni-reactive T cells in the LNC of DNFB-primed Ni^{low} mice belong to the $CD4^+$ subset, the DNBS-reactive ones belong to the $CD8^+$ subset

In human PBMC, Ni-reactive T cells belong mainly to the $CD4^+$ subset [160], albeit some Ni-reactive $CD8^+$ T cells also were detected [165]. In contrast, T cells reacting to TNCB (trinitrochlorobenzene) [187] and even more pertinent here DNBS [188], were mainly found within the $CD8^+$ subset. If the same were true in mice, one would expect Ni- and DNBS-reactive T cells within the $CD4^+$ and $CD8^+$ T cell subset, respectively.

In order to test this, the LNC obtained from $NiCl_2/H_2O_2$- and DNFB-primed mice, respectively, were first depleted of either $CD4^+$ or $CD8^+$ T cells and then restimulated *in vitro* with either Ni or DNBS. In the presence of Ni **(Fig. 3.2.3.1A**), the bulk LNC and $CD4^+$ LNC (i.e., the $CD8^+$ T cell-depleted LNC), but not the $CD8^+$ LNC (i.e., the $CD4^+$ T cell-depleted LNC), strongly proliferated irrespective of

whether the donors of these responder cells had been primed with $NiCl_2/H_2O_2$ (black bars) or DNFB (open bars). The reverse was seen, however, when the LNC of the same donors were restimulated with DNBS **(Fig. 3.2.3.1B)**. In that case, the bulk LNC and $CD8^+$ LNC (i.e., the $CD4^+$ T cell-depleted LNC) from DNFB-primed mice (open bars) proliferated, whereas all the cells from $NiCl_2/H_2O_2$-primed mice (black bars) failed to do so. I concluded from these results that the Ni-reactive T cells were $CD4^+$ T cells while the DNBS-reactive T cells were $CD8^+$ T cells. Therefore, cross-reactivity between Ni-reactive and DNBS-reactive T cells was excluded as an explanation for the non-specific stimulation of DNFB-primed T cells/ in the presence of Ni seen *in vitro*.

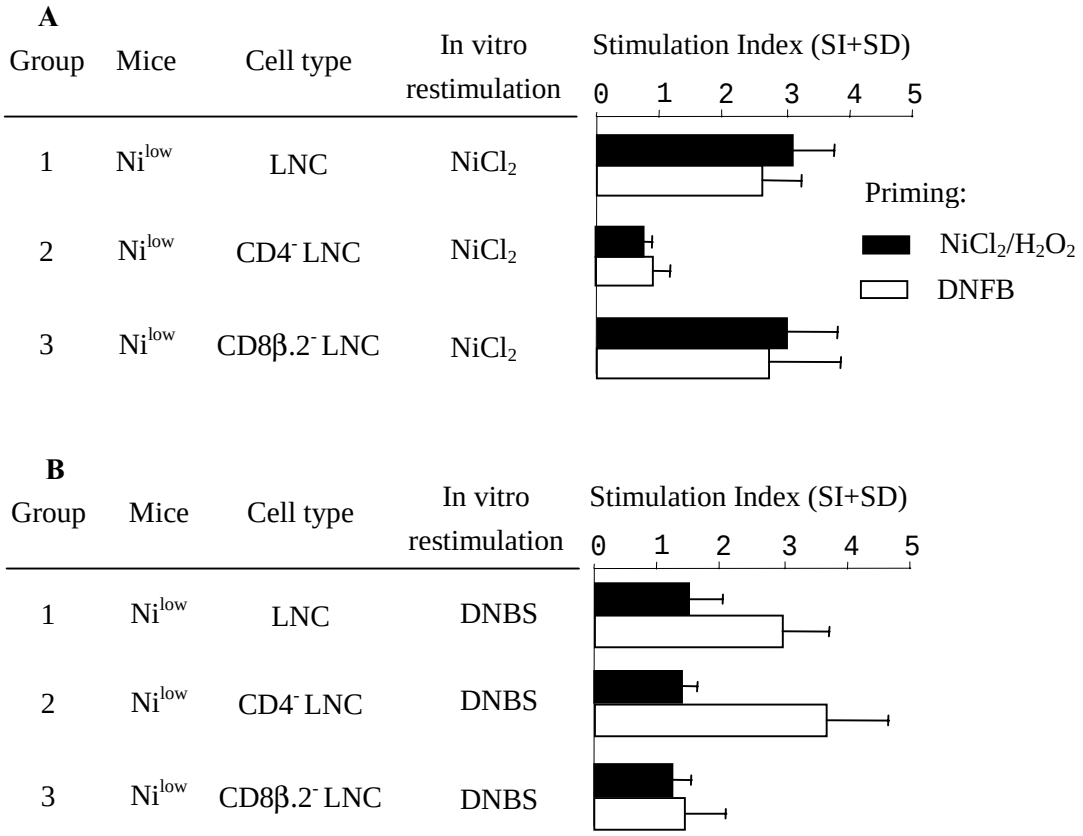


FIGURE 3.2.3.1. Ni-reactive cells in the LNC of DNFB-primed Ni^{low} mice belong to the $CD4^+$ subset, whereas DNBS-reactive LNC belong to the $CD8^+$ subset. Groups of Ni^{low} mice were primed with either $NiCl_2$ in H_2O_2 (■) or DNFB (□). Ten days later, cells from the draining lymph nodes of these mice were pooled, $CD4^+$ or $CD8^+$ T cells depleted, as indicated, and restimulated *in vitro* with Ni ions (*A*), or DNBS (*B*). Cultures were pulsed with [^{3}H] thymidine three days after restimulation and the isotope incorporation was determined. A representative result of three independent experiments is shown.

3.2.3.2 Lack of cross-reactivity between Ni-reactive and DNBS-reactive T cell hybridomas developed from the LNC of DNFB-primed Ni^{low} mice.

To further clarify whether or not there is a classical cross-reactivity between the DNBS-reactive and Ni-reactive T cells found in DNFB-primed mice, LNC from DNFB sensitized mice were restimulated

No.	Name of hybridoma	Before cell fusion, LNC Restimulated with	Stimulation Index (SI) ± SD to Ni	SI ± SD to DNBS
1	Ni0.3A9-3B4	Ni	**5.04 ±0.43**	1.09 ± 0.21
2	Ni0.3A9-3D2	Ni	**4.12 ±0.69**	0.97 ± 0.16
3	Ni0.3A9-3G2	Ni	**4.79 ±0.59**	1.07 ± 0.13
4	Ni0.3B8-0.3G8	Ni	**4.22 ±1.11**	0.80 ± 0.21
5	Ni0.3B8-3B3	Ni	**5.53 ±0.49**	0.99 ± 0.09
6	Ni0.3B8-3E3	Ni	**4.24 ±0.56**	1.09 ± 0.22
7	Ni3G11-0.3C5	Ni	**9.57 ±0.88**	1.42 ± 0.18
8	Ni3G11-0.3H2	Ni	**6.55 ±0.21**	0.93 ± 0.16
9	Ni3G11-0.3H9	Ni	**14.5 ±1.26**	1.66 ± 0.14
10	Ni3H7-0.3G11	Ni	**3.43 ±0.27**	0.89 ± 0.10
11	Ni3H7-3D4	Ni	**2.17 ±0.16**	0.97± 0.10
12	Ni3H7-3E5	Ni	**4.86 ±0.43**	1.08 ± 0.07
13	Ni9F2-0.3G8	Ni	**4.17 ±0.63**	1.07 ± 0.16
14	Ni9F2-0.3H1	Ni	**8.28 ±0.32**	1.48 ± 0.20
15	Ni9F2-3B1	Ni	**5.35 ±0.95**	0.97 ± 0.17
16	DNBS0.3H4-0.3C4	DNBS	1.12 ±0.28	**7.04 ± 1.78**
17	DNBS0.3H4-3B3	DNBS	1.07 ±0.09	**6.14 ± 0.49**
18	DNBS0.3H4-3G3	DNBS	1.05 ±0.15	**8.27 ± 1.11**
19	DNBS0.3H4-9D9	DNBS	0.82 ±0.24	**3.18 ± 0.94**
20	DNBS0.3H4-9G9	DNBS	0.80 ±0.24	**2.89 ± 0.88**
21	DNBS3B4-0.3B12	DNBS	1.44 ±0.27	**9.56 ± 1.76**
22	DNBS3B4-0.3C10	DNBS	1.80 ±0.24	**12.5 ± 1.57**
23	DNBS3B4-0.3D4	DNBS	1.57 ±0.38	**13.03 ± 0.85**
24	DNBS3B4-3B5	DNBS	1.44 ±0.34	**12.35 ± 2.89**
25	DNBS3B4-3G6	DNBS	1.19 ±0.11	**10.88 ± 1.00**
26	DNBS9G6-0.3E6	DNBS	1.81 ±0.28	**15.41± 1.22**
27	DNBS9G6-0.3E10	DNBS	1.25 ±0.29	**10.14 ± 2.35**
28	DNBS9G6-3B4	DNBS	1.83 ±0.33	**11.79 ± 2.14**
29	DNBS9G6-3E3	DNBS	1.66 ±0.24	**15.83 ± 1.35**
30	DNBS9G6-3H6	DNBS	1.39 ±0.31	**25.86 ± 5.09**

TABLE 3.2.3.2. Both Ni-reactive and DNBS-reactive T cell hybridomas can be developed from LNC of DNFB-primed Ni^{low} mice. Ni^{low} mice were primed with DNFB. Ten days later, the pooled cells from the draining lymph nodes of these mice were restimulated *in vitro* with Ni ions or DNBS. The resulting T cells were then fused with fusion partner cell line BW5147 and T cell hybridomas were generated, as described under *Materials and Methods*. The reactivities of representative hybridomas are shown. A representative result of three independent experiments is shown.

with either Ni or DNBS, and T cell hybridomas were generated from them, as described in section 2.10. As shown in **Table 3.2.3.2**, the hybridomas generated from the LNC restimulated with Ni proliferated in response to Ni, but not to DNBS. Vice versa, the hybridomas generated from LNC restimulated with DNBS reacted to DNBS, but not to Ni. These results confirmed that at a clonal level there was no cross-reaction between Ni-reactive and DNB-reactive T cells.

3.3 T CELL SUPPRESSIVE ACTIVITY IN Ni^{HIGH}, Ni^{LOW} AND $Ni^{VERY\ LOW}$ MICE

Two conclusions can be drawn from the results described in section 3.1. First, the higher the oral uptake of Ni; the more difficult it is to sensitize the mice to this metal, and second, the complete tolerance of the Ni^{high} mice was associated with T cell anergy to nickel. This suggested the involvement of Ni-specific Treg cells in the tolerance of the Ni^{high} mice, since such cells can be anergic to the tolerogen. Therefore, I asked whether there are differences in nickel-specific Treg cells beween Ni^{high}, Ni^{low} and $Ni^{very\ low}$ mice that could account for their different susceptibility to nickel sensitization.

In chapter 1, I summarized the mechanisms of immunological tolerance (section 1.7), in particular the mechanisms of oral tolerance (section 1.7.4). Oral tolerance is based on the activity of specific Treg cells and this mechanism should also operate in the case of oral tolerance to Ni (sections 1.8.3 and 1.8.4). Conceivably, the existence of Ni-specific Treg cells in Ni^{high} and Ni^{low} mice could be the reason why these animals cannot be sensitized with $NiCl_2$ alone. If so, their might be differences among their Treg cells which could account for their different susceptibility to sensitization with $NiCl_2/H_2O_2$.

3.3.1 Profoundly suppressive activity of the T cells obtained from Ni^{high} mice

From section 3.1.2, we already knew that the T cells in the Ni^{high} mice were anergic in the presence of Ni. Now, I want to know if their anergic T cells also are suppressive. To address this issue, adoptive cell transfers from from Ni^{high} donor mice to Ni^{low} and $Ni^{very\ low}$ recipients were performed, and the recipients' susceptibility or resistance to Ni sensitization was determined by MEST.

3.3.1.1 T cells of Ni^{high} mice can prevent the establishment of Ni specific effector cells (i.e. suppress the 1^o immune response) shown by adoptive transfer: using Ni^{low} and $Ni^{very\ low}$ recipients

From the data shown in **Figure 3.3.1.1**, we can see that as few as 10^2 splenic T cells from Ni^{high} donors the sufficed to render the Ni^{low} recipients completely resistant to subsequent sensitization with $NiCl_2/H_2O_2$ and challenge with $NiCl_2$ (bar 2, **Figure 3.3.1.1**); in contrast, such recipients showed normal ear-swelling responses upon sensitization and challenge with DNFB (bar 6, **Figure 3.3.1.1**). ***These results were obtained in cooperation with Drs. S. Artik and K. Roelofs-Haarhuis.*** In other words, nickel-specific Treg cells, which are present in Ni^{high} mice, are able to suppress the primary response to nickel of Ni^{low} mice which normally is inducible by their immunization with $NiCl_2/H_2O_2$.

Based on the above observation and using the same method, the study of suppressing the primary immune response to nickel was extended to $Ni^{very\ low}$ recipients. Here, 10^7 nylon wool enriched splenic T cells were transferred into $Ni^{very\ low}$ recipients. One day later, the recipients were sensitized, re-challenged and ear-swelling measured as described in section 2.3-2.4. The data in **Figure 3.3.2.1A** clearly showed that only background ear-swelling responses were observed in the $Ni^{very\ low}$ recipients

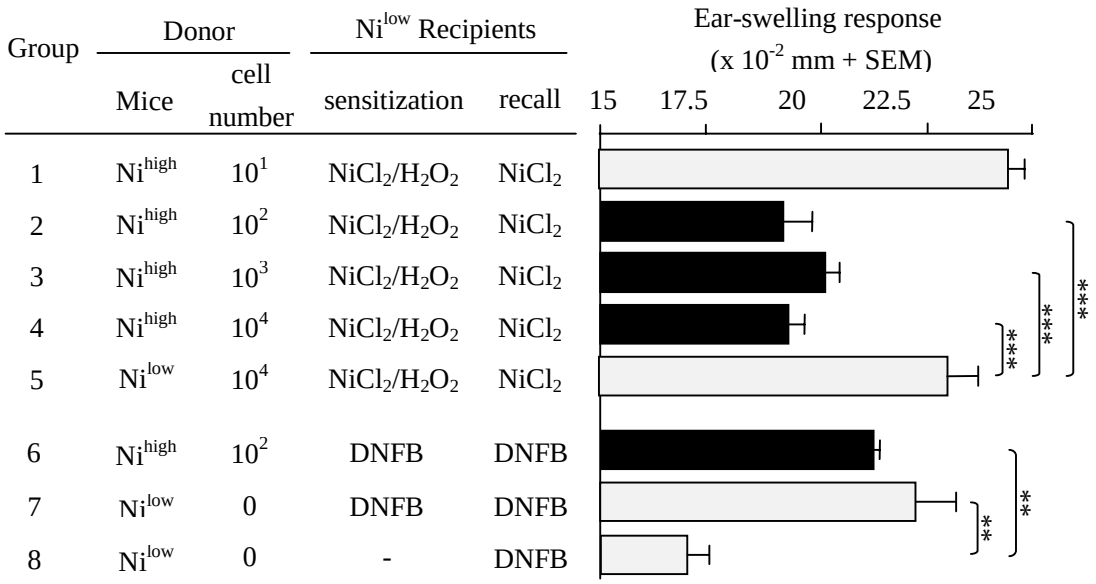


FIGURE 3.3.1.1 As few as 10^2 live T cells from Ni^{high} donors are sufficient for adoptive transfer of tolerance to Ni^{low} recipients. Prospective donor mice were treated with 10 mM $NiCl_2$ in the drinking water for a period of 4 wks ($\rightarrow Ni^{high}$) or left untreated($\rightarrow Ni^{low}$), as indicated. Two (groups 1-5) or four (groups 6-8) wks after termination of tolerance treatment, the enriched splenic T cells of Ni^{high} and Ni^{low} donors were further purified by depletion of $CD11b^+$, $CD11c^+$, $CD19^+$, and $MHCII^+$ cells using the FACScalibur, and the indicated numbers of T cells were transferred to syngeneic Ni^{low} recipients. One day after transfer, the recipients were sensitized and challenged for recall, as indicated. After challenge their ear-swelling response was determined. Asterisks indicate a significant difference (**, $p \leq 0.01$ and ***, $p \leq 0.001$) between the groups compared by brackets. The transfer of 10^2 enriched splenic T cells from Ni^{high} donors and Ni^{low} donors, respectively, was performed five times and the experiments shown in the other groups were performed at least twice, yielding comparable results each time.

that received T cells from Ni^{high} donors (bar 3, **Figure 3.3.2.1A**). **To see Figure 3.3.2.1, please go to section 3.3.2.1.**

The above results demonstrated that T cells from Ni^{high} donors can suppressive primary immune responses to nickel in both Ni^{low} and $Ni^{very\ low}$ recipients.

3.3.1.2 The suppressive activity of T cells from Ni^{high} mice is long lasting

In section 3.1.1, I reported that the Ni^{high} mice were still tolerant after a treatment-free interval of 20 weeks. This raised the question if the T cells of such animals can still transfer the tolerance. In order to answer this question, the T cells from Ni^{high} mice, which had a treatment interval of 1 week and 20 weeks, respectively, were purified and transferred to Ni^{low} recipients. As the data shown in **Figure 3.3.1.2**, after a treatment-free interval of 20 weeks, the T cells from Ni^{high} mice still could transfer tolerance to Ni^{low} recipients (**Figure 3.3.1.2**). Therefore, the suppressive capacity of T cells from Ni^{high} mice were long lasting. It lasted at least 20 weeks. But, unlike after 1 or 2 weeks treatment interval that only 10^2 T cells were enough to transfer tolerance (see section 3.3.1.1), after a treatment-free interval of 20 weeks, 10^4 T cells were needed to transfer tolerance.

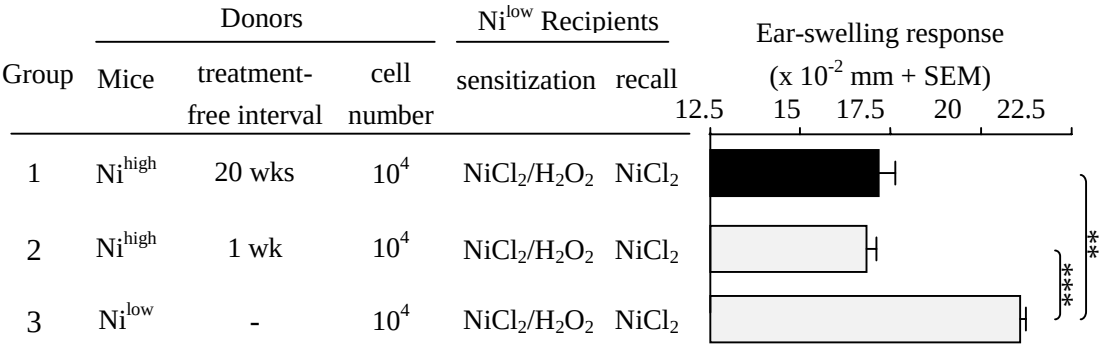


FIGURE 3.3.1.2 Persistence of the suppressive capacity of T cells obtained from orally tolerized donors. Prospective donor mice were treated with 10 mM $NiCl_2$ in the drinking water for a period of 4 wks ($\rightarrow Ni^{high}$) or were left untreated (Ni^{low}), as indicated. After a treatment-free interval of 20 wks (group 1) or 1 wk (group 2), 10^4 sorted splenic T cells of Ni^{high} and Ni^{low} donors, respectively, were transferred to syngeneic Ni^{low} recipients. One day after transfer, the recipients were sensitized with $NiCl_2$ in H_2O_2, 10 days later they were challenged for recall with $NiCl_2$, and after 2 days the ear-swelling response was determined. Asterisks indicate a significant difference (***, $p \leq 0.001$) between the groups compared by brackets. The figure is a representative of two independent experiments.

3.3.1.3 T cells of Ni^{high} mice can suppress the 2^o immune response to nickel

Data shown in the last two sections clearly demonstrated that Treg cells in Ni^{high} mice are able to suppress the primary immune response to nickel. In other words, the Treg cells of Ni^{high} mice prevent the induction of nickel-specific effector T cells and thus prevent the *de novo* induction of nickel allergy. In this context, it is important to know whether or not these Treg cells can also inhibit the functions of already established nickel-specific effector T cells. If this were the case, these Treg cells could be used not only to prevent nickel allergy, but also to cure an already existing nickel allergy. In order to this, I performed experiments designed to suppress the secondary immune response to nickel. For this purpose, T cells of Ni^{high} mice were adoptively transferred to $Ni^{very\ low}$ recipients which had been sensitized sensitized to Ni before transfer.

10^7 nylon wool-enriched splenic T cells from different donors were transferred to $Ni^{very\ low}$ recipients that had been sensitized to nickel 10 days prior to transfer. The T cells from $Ni^{very\ low}$ donors

completely failed to suppress the nickel hypersensitivity at all (*bars 2* and *3*, **Figure 3.3.2.2A**), whereas the T cells of Ni^{high} donors profoundly suppressed the elicitation of nickel hypersensitivity in the sensitized $Ni^{very\ low}$ recipients, irrespective of whether the recipients had been sensitized by the injection of $NiCl_2$ alone or $NiCl_2/H_2O_2$ (*bars 4* and *5*, **Figure 3.3.2.2A**).

The capacity of Treg cells to suppress secondary immune responses to nickel was also demonstrated *in vitro* **(Figure 3.3.2.2B)**. Here, the suppressive capacity of splenic T cells from Ni^{high} and Ni^{low} mice was assessed by co-culturing them in the presence of $NiCl_2$ with a variety of LNC used as indicator cells. As specified in **Figure 3.3.2.2B,** the indicator cells were obtained from $Ni^{very\ low}$ mice (b*ars 1-6)* immunized with either $NiCl_2$ alone or $NiCl_2/H_2O_2$ and from Ni^{low} mice immunized with $NiCl_2/H_2O_2$.(*bars 7-9*), respectively. ***To see Figure 3.3.2.2, please go to section 3.3.2.2.***

3.3.1.4 The suppression by Ni^{high} T cells of the secondary immune response in vitro is nickel-specific

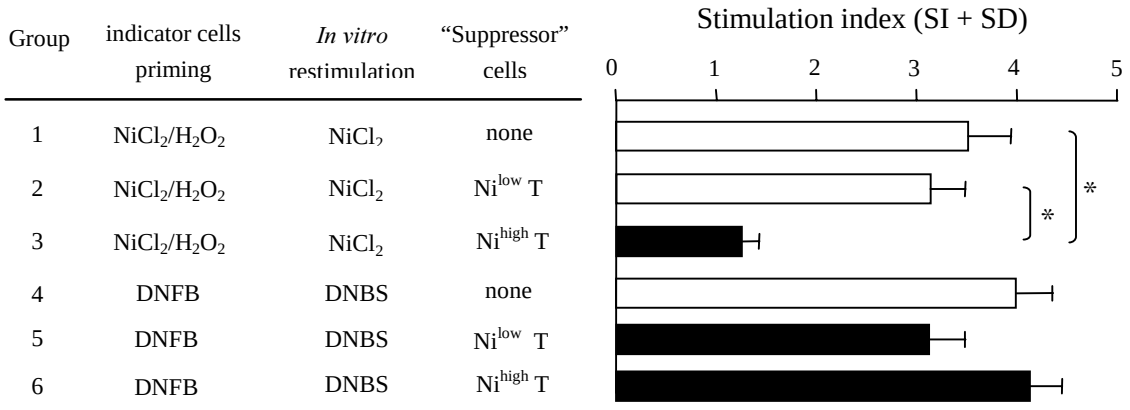


FIGURE 3.3.1.4. The suppression by Ni^{high} T cells of the secondary immune response *in vitro* is nickel-specific. Ni^{low} mice were immunized with either $NiCl_2$ in H_2O_2 or DNFB. Ten days later, pooled cells from the draining lymph nodes of these mice were cocultured with splenic T cells (ratio 1:3) from either Ni^{low} or Ni^{high} mice, and restimulated *in vitro* with Ni ions or DNBS, as indicated. Three days after restimulation, cultures were pulsed with [^{3}H] thymidine and the isotope incorporation was determined. Asterisks indicate a significant difference (*, $p \leq 0.05$) between the groups compared by brackets. A representative result of four independent experiments is shown.

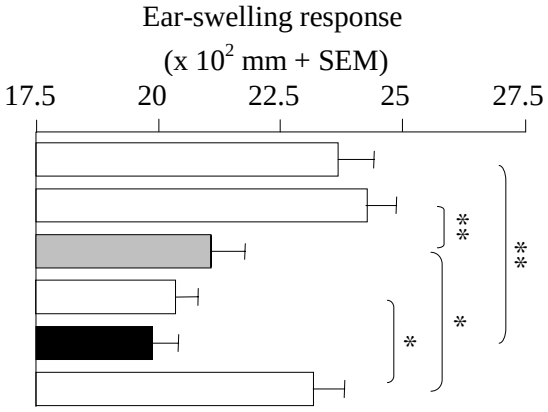
To test whether the suppression by Ni^{high} T cells of the secondary immune response is nickel-specific, T cells of Ni^{high} and Ni^{low} mice were co-cultured with LNC of Ni^{low} mice primed with either $NiCl_2/H_2O_2$ or DNFB. In the presence of $NiCl_2$, the T cells of Ni^{high} mice completely suppressed the proliferation of LNC from $NiCl_2/H_2O_2$-sensitized mice (*bar 3,* **Figure 3.3.1.4**), but not that of the LNC from Ni^{low} mice (*bar 2*), confirming the observation in the previous section that T cells of Ni^{high} mice suppress the secondary immune response to nickel. However, in the absence of $NiCl_2$ and the presence of DNBS, the proliferation of LNC from-DNFB primed mice was not influenced by adding T cells from either Ni^{high} mice (*bar 6,* **Figure 3.3.1.4**) or Ni^{low} (*bar 5*) mice. Therefore, the suppression by Ni^{high} T cells of secondary immune response is nickel-specific.

3.3.2 The suppressive activity of T cells from Ni^{low} mice depends on the immunization mode of $Ni^{very\ low}$ recipients

The above data demonstrated that Ni^{high} mice were resistant to sensitization with $NiCl_2/H_2O_2$ and that their T cells were anergic upon restimulation with $NiCl_2$ *in vitro*, were suppressive *in vitro*, and were able to transfer the nickel tolerance to Ni^{low} or $Ni^{very\ low}$ recipients. Whereas the existence of the Treg cells in the Ni^{high} mice prevented their sensitization following the injection of $NiCl_2/H_2O_2$, untreated Ni^{low} mice could be sensitized by $NiCl_2/H_2O_2$, albeit not by $NiCl_2$ alone. In view of the resistance of the Ni^{low} mice to sensitization with $NiCl_2$ alone we wondered weather, perhaps, they possess certain nickel-specific Treg cells. In order to test this possibility, T cell transfer experiments were performed.

A

Group	Donor T cells (10^7)	$Ni^{very\ low}$ recipients	
		Priming	Recall
1	$Ni^{very\ low}$	$NiCl_2$	$NiCl_2$
2	$Ni^{very\ low}$	$NiCl_2/H_2O_2$	$NiCl_2$
3	Ni^{high}	$NiCl_2/H_2O_2$	$NiCl_2$
4	Ni^{low}	NaCl	$NiCl_2$
5	Ni^{low}	$NiCl_2$	$NiCl_2$
6	Ni^{low}	$NiCl_2/H_2O_2$	$NiCl_2$



B

Group	Donor T cells (10^7)	$Ni^{very\ low}$ recipients	
		Priming	Recall
1	$Ni^{very\ low}$	NaCl	$NiCl_2$
2	$Ni^{very\ low}$	$NiCl_2$	$NiCl_2$
3	Ni^{low}	$NiCl_2$	$NiCl_2$
4	$Ni^{very\ low}$	-	DNFB
5	$Ni^{very\ low}$	DNFB	DNFB
6	Ni^{low}	DNFB	DNFB

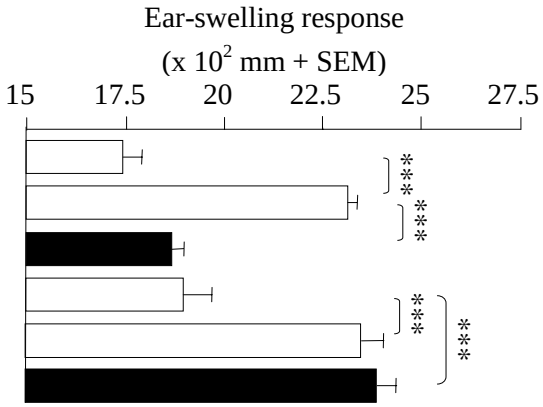


FIGURE 3.3.2.1 Adoptive transfer of T cells from different donors to untreated $Ni^{very\ low}$ recipients: unlike the T cells from Ni^{high} donors which contain potent Treg cells, those from Ni^{low} donors can suppress the recipients' susceptibility to being sensitized with Ni alone, but not that of being sensitized with $NiCl_2/H_2O_2$. 10^7 nylon-wool enriched splenic T cells from either Ni^{high}, Ni^{low} or $Ni^{very\ low}$ donors were transferred into $Ni^{very\ low}$ recipients. The $Ni^{very\ low}$ recipients were primed one day after transfer, and challenged ten days after priming, as indicated. Their ear-swelling response was determined, as described in Materials and Methods. Asterisks indicate a significant difference (*, $p \leq 0.05$; **, $p \leq 0.01$; **, $p \leq 0.01$) between the groups compared by brackets. Experiment repeated four times, and each times got comparable results**.**

3.3.2.1 T cells from Ni^{low} mice can suppress the primary immune responses to nickel when the $Ni^{very\ low}$ recipients were immunized with Ni alone, but not with $NiCl_2/H_2O_2$

Here, we adoptively transferred 10^7 nylon wool enriched splenic T cells of Ni^{low} donors to $Ni^{very\ low}$ recipients; the latter were immunized 1 day after the T cell transfer and rechallenged 10 days thereafter, as usual. We found that, indeed, the Ni^{low} donor mice provided nickel-specific Treg cells to $Ni^{very\ low}$ recipients that protected the latter from being sensitized by $NiCl_2$ alone; in contrast, T cells obtained from $Ni^{very\ low}$ donors were unable to do so (bars 1 and 2, **Figure 3.3.2.1A**). However, unlike the T cells of Ni^{high} mice that suppressed the sensitization of the $Ni^{very\ low}$ recipients regardless of whether these were immunized with $NiCl_2$ alone or $NiCl_2/H_2O_2$ (bar 3, **Figure 3.3.2.1A***)* the Treg cells from Ni^{low} donors only were able to prevent the $Ni^{very\ low}$ recipients' sensitization by $NiCl_2$ alone, but not that induced by immunization with $NiCl_2/H_2O_2$ (bar 5 vs 6, **Figure 3.3.2.1A**). The suppression exerted by the transferred Ni^{low} T cells was nickel--specific, because these cells only were able to suppress the ear-swelling response to nickel, but not to DNFB (bars 3 and 6, **Figure 3.3.2.1B**). In contrast to the T cells of Ni^{low} donors, those obtained from $Ni^{very\ low}$ donors failed to prevent the sensitization to either $NiCl_2$ alone or $NiCl_2/H_2O_2$ (bars 1 and 2, **Figure 3.3.2.1A**), indicating that the $Ni^{very\ low}$ mice are devoid of nickel-specific Treg cells.

3.3.2.2 T cells from Ni^{low} donor mice are able to inhibit the function of nickel-specific memory/effetor T cells in $Ni^{very\ low}$ recipients previously sensitized with $NiCl_2$ alone

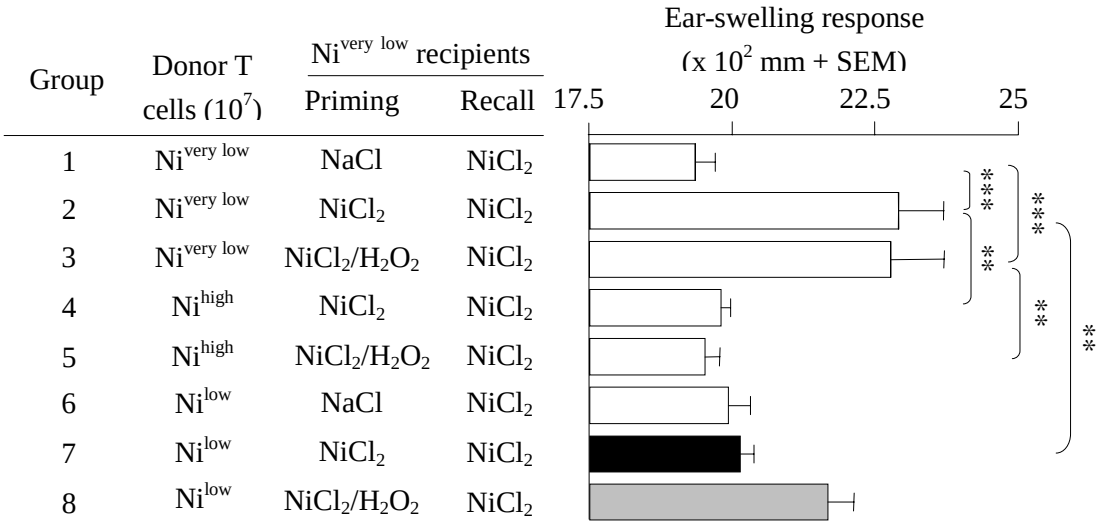


FIGURE 3.3.2.2A Adoptive transfer of T cells from different donors to $Ni^{very\ low}$ recipients which were primed prior to transfer: T cells from Ni^{high} donors completely suppress the hypersensitivity reaction (2^o reaction) irrespective of the recipients' priming, whereas the suppressive capacity from Ni^{low} donors depends on whether the recipients were primed with Ni alone or $NiCl_2/H_2O_2$. One day after the $Ni^{very\ low}$ recipients were primed, 10^7 nylon-wool enriched splenic T cells from either Ni^{high}, Ni^{low} or $Ni^{very\ low}$ donors were transferred into these sensitized recipients, as indicated. Ten days later, the mice were challenged, two days thereafter, the ear-swelling response was determined, as described in Materials and Methods. Asterisks indicate a significant difference (**, $p \leq 0.01$; ***, $p \leq 0.001$) between the groups compared by brackets. Experiment repeated two times, and each times got comparable results.

The results shown in **Figure 3.2.2.1** indicate that the Treg cells of Ni^{high} mice not only were able to prevent the *de novo* sensitization induced not only by $NiCl_2$ alone but also by $NiCl_2/H_2O_2$. In contrast, the Treg cells of Ni^{low} mice only were able to prevent the sensitization induced by $NiCl_2$ alone. Next, we asked whether the Treg cells of the two types of mouse also were able to inhibit the elicitation of nickel hypersensitivity in mice already sensitized, i.e., if they can suppress the secondary immune responses to $NiCl_2$. Therefore, T cells from three different types of donors (i.e., Ni^{high}, Ni^{low}, and $Ni^{very\ low}$ mice) were transferred into $Ni^{very\ low}$ recipients that had been immunized to nickel 10 days prior to transfer. As already mentioned in section 3.3.2.2, the T cells obtained from $Ni^{very\ low}$ mice completely failed to suppress the nickel hypersensitivity at all (*bars 2* and *3,* **Figure 3.3.2.2A**), whereas the T cells of Ni^{high} donors profoundly suppressed the elicitation of nickel hypersensitivity in sensitized $Ni^{very\ low}$ recipients, irrespective of whether the recipients had been sensitized by the injection of $NiCl_2$ alone or $NiCl_2/H_2O_2$ (*bars 4* and *5,* **Figure 3.3.2.2A**). Interestingly, the T cells of the Ni^{low} donors only partially suppressed the nickel hypersensitivity in the $Ni^{very\ low}$ recipients previously sensitized with $NiCl_2/H_2O_2$, but completely suppressed the hypersensitivity in the $Ni^{very\ low}$ recipients immunized with $NiCl_2$ alone (*bar 8* versus *bar 7,* **Figure 3.3.2.2A**).

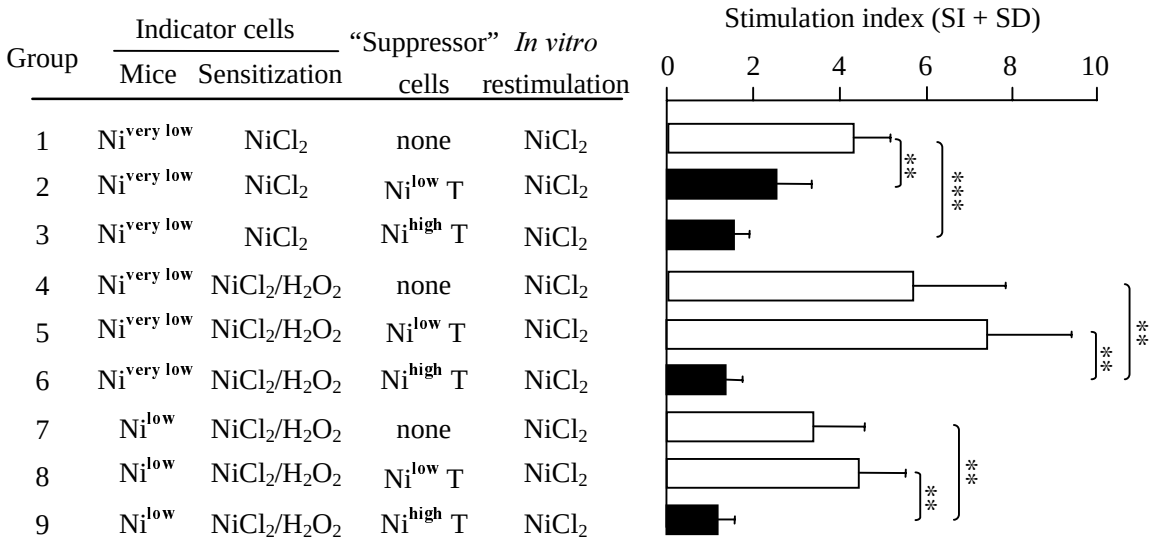
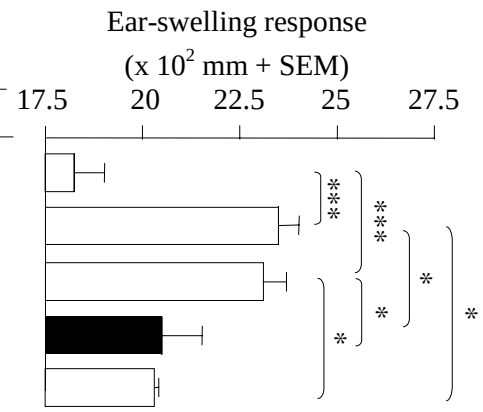


FIGURE 3.3.2.2B. The T cells of Ni^{high} and Ni^{low} donor mice suppress the secondary immune response to $NiCl_2$ *in vitro*. Ni^{low} mice were sensitized with $NiCl_2/H_2O_2$, whereas $Ni^{very\ low}$ mice were sensitized with either $NiCl_2/H_2O_2$ or $NiCl_2$ alone ;as indicated. Ten days later, pooled LNC of these mice served as indicators that were co-cultured with splenic T cells (ratio 1:3) from either Ni^{low} or Ni^{high} mice and were restimulated *in vitro* with Ni ions. After 3 days, the cultures were pulsed with [3H] thymidine and the isotope incorporation was determined. Asterisks indicate a significant difference (**, $p \leq 0.01$; ***, $p \leq 0.001$) between the groups compared by brackets.

Similar results were obtained from the *in vitro* co-culture experiments. As described in section 3.3.1.3, Ni^{high} T cells completely suppressed the $NiCl_2$-induced restimulation of LNC indicator cells, irrespective of the origin of the latter and the mode of nickel sensitization of their donors (*bars 3, 6, and 9,* **Figure 3.3.2.2B**). In contrast, Ni^{low} T cells could only suppress the restimulation of LNC from those $Ni^{very\ low}$ mice that had been immunized $NiCl_2$ alone (*bar 2,* **Figure 3.3.2.2B**), but not that of those mice which had been immunized with $NiCl_2/H_2O_2$ (*bar 5*), not to mention that of Ni^{low} mice immunized with $NiCl_2/H_2O_2$ (*bar 8*). Taken together, the results confirm the existence of a hierarchy

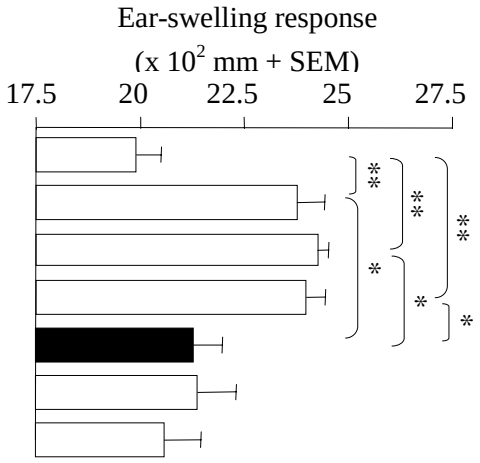
A

Group	T cell		$Ni^{very\ low}$ recipients	
	Donor	Number	Priming	Recall
1	-		NaCl	$NiCl_2$
2	-		$NiCl_2$	$NiCl_2$
3	Ni^{high}	10^2	$NiCl_2$	$NiCl_2$
4	Ni^{high}	10^3	$NiCl_2$	$NiCl_2$
5	Ni^{high}	10^4	$NiCl_2$	$NiCl_2$



B

Group	T cell		$Ni^{very\ low}$ recipients	
	Donor	Number	Priming	Recall
1	-		NaCl	$NiCl_2$
2	-		$NiCl_2/H_2O_2$	$NiCl_2$
3	Ni^{high}	10^2	$NiCl_2/H_2O_2$	$NiCl_2$
4	Ni^{high}	10^3	$NiCl_2/H_2O_2$	$NiCl_2$
5	Ni^{high}	10^4	$NiCl_2/H_2O_2$	$NiCl_2$
6	Ni^{high}	10^5	$NiCl_2/H_2O_2$	$NiCl_2$
7	Ni^{high}	10^6	$NiCl_2/H_2O_2$	$NiCl_2$



C

Group	T cell		$Ni^{very\ low}$ recipients	
	Donor	Number	Priming	Recall
1	-		NaCl	$NiCl_2$
2	-		$NiCl_2$	$NiCl_2$
3	Ni^{low}	10^2	$NiCl_2$	$NiCl_2$
4	Ni^{low}	10^3	$NiCl_2$	$NiCl_2$
5	Ni^{low}	10^4	$NiCl_2$	$NiCl_2$
6	Ni^{low}	10^5	$NiCl_2$	$NiCl_2$
7	Ni^{low}	10^6	$NiCl_2$	$NiCl_2$

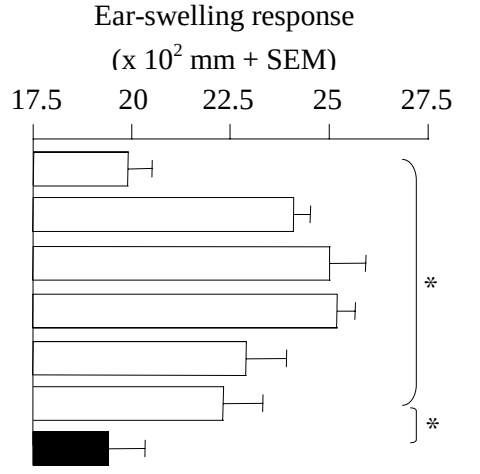


FIGURE 3.3.3 Both the type of the T cell donor and the mode of recipient sensitization determine the minimal cell number of splenic T cells required for transfer unresponsiveness to $Ni^{very\ low}$ recipients Indicated numbers of purified splenic T cells from Ni^{high} (A, B) and Ni^{low} (C) mice were transferred into $Ni^{very\ low}$ recipients. $Ni^{very\ low}$ recipients were primed one day after transfer with either $NiCl_2$ alone (A, C) or $NiCl_2/H_2O_2$ (B), and challenged ten days after priming, as indicated. NaCl injected $Ni^{very\ low}$ mice were used as negative control, and $NiCl_2$ alone and $NiCl_2/H_2O_2$ primed $Ni^{very\ low}$ mice were used as positive control in (A, C) and (B), respectively. Their ear-swelling response was determined, as described in *Materials and Methods*. Asterisks indicate a significant difference (*, $p \leq 0.05$) between the groups compared by brackets. Experiment repeated two times, and each times got comparable results.

of $Ni^{high} > Ni^{low} > Ni^{very\ low}$ in the effectiveness of nickel-specific Treg cells and, once again, indicate

that the sensitization induced by $NiCl_2$ alone can more easily be suppressed than that induced by $NiCl_2/H_2O_2$.

3.3.3 Both the type of the T cell donor and the mode of recipient sensitization determine the minimal cell number of splenic T cells required for transfer unresponsiveness to $Ni^{very\ low}$ recipients

From the results shown in **Figures 3.3.2.1** and **3.3.1.3** we may draw two conclusions: First, the immunization with $NiCl_2$ alone appears to activate a population of nickel-specific T effector cells which (by unknown criteria) differs from that activated by $NiCl_2/ H_2O_2$, since it was more difficult to suppress the nickel hypersensitivity induced by immunization with $NiCl_2/H_2O_2$ than that induced by immunization with $NiCl_2$ alone. Second, the suppressive capacity of the T cells transferred correlated with the oral nickel uptake of the donor mice. We observed a hierarchy of $Ni^{high} > Ni^{low} > Ni^{very\ low}$ with respect to the suppressive capacity of the transferred T cells. Whereas the T cells of the Ni^{high} mice almost completely suppressed the induction and elicitation of nickel hypersensitivity and those of Ni^{low} mice showed a moderate suppressive effect, those of the $Ni^{very\ low}$ mice failed to suppress at all.

In the experiments shown in **Figures 3.3.2.1** and **3.3.2.2A**, the number of enriched T cells that were adoptively transferred to the $Ni^{very\ low}$ recipients was 10^7 cells per recipient. However, when we used Ni^{low} instead of $Ni^{very\ low}$ mice as recipients, we found that as few as 10^2 Ni^{high} donor T cells sufficed to transfer the tolerance (**Figure 3.3.1.1**). Taken together, these experiments suggest that the minimal number of donor T cells required for the transfer of unresponsiveness to $Ni^{very\ low}$ recipients not only varies with the type of T cell donors used and the immunization regimen used for priming of the recipients, but also with the type of recipient used, i.e., Ni^{low} or $Ni^{very\ low}$ mice. To further test this concept, we established dose-response curves of donor T cells that were obtained from either Ni^{low} (**Figure 3.3.3C**) or Ni^{high} donors (**Figures 3.3.3A and B**) and were transferred to $Ni^{very\ low}$ recipients. When the latter were subsequently immunized with $NiCl_2$ alone, re-challenged, and tested for ear-swelling, it became evident that 10^3 purified T cells from Ni^{high} donors sufficed to tolerize them (bar 4, **Figure 3.3.3A**); in contrast, 10^4 T cells from the same donors were needed for tolerization when the $Ni^{very\ low}$ recipients were subsequently immunized with $NiCl_2/H_2O_2$ (bar 5, **Figure 3.3.3B**). Obviously, it is easier to prevent a sensitization induced by $NiCl_2$ alone than by $NiCl_2/H_2O_2$. Furthermore, whereas 10^3 T cells from Ni^{high} donors could transfer the tolerance to $Ni^{very\ low}$ recipients subsequently immunized with $NiCl_2$ alone (bar 4, **Figure 3.3.3A**), as many as 10^6 T cells from Ni^{low} donor mice were required to achieve the same effect (bar 7, **Figure 3.3.3C**). I conclude from this results that the number and/ or type of nickel-specific Treg cells of Ni^{high} mice is superior to that of Ni^{low} mice.

3.3.4 Characterization of Treg cells in Ni^{high} and Ni^{low} mice

Previously, the Treg cells of Ni^{high} mice were characterized by using the minimal number of splenic T cells required for the tolerance transfer from Ni^{high} donors to Ni^{low} recipient mice (**Figure 3.3.1.1**). It was found that the tolerance of Ni^{high} donors could only be transferred to the Ni^{low} recipients by a

combination of 5 x 10^1 $CD4^+$ and 5 x 10^1 $CD8^+$ T cells, but not by either 10^2 $CD4^+$ or 10^2 $CD8^+$ T cells alone (see Figure 6 of ref. [177]).

Here, I used basically the same experimental approach to compare the effects of the $CD4^+$ and $CD8^+$ T cell subsets obtained from the Ni^{high} donors with those obtained from the Ni^{low} donors. The only difference to the previous study was that here $Ni^{very\ low}$ mice, instead of Ni^{low} mice, were used as recipients and that, consequently, T cells from $Ni^{very\ low}$ donor mice were used as non-suppressor controls (**Figure 3.3.4.1**).

3.3.4.1 When Ni^{low} and $Ni^{very\ low}$ recipient mice were immunized with $NiCl_2/H_2O_2$, both CD4 and CD8 T cells from Ni^{high} donors were need to transfer tolerance

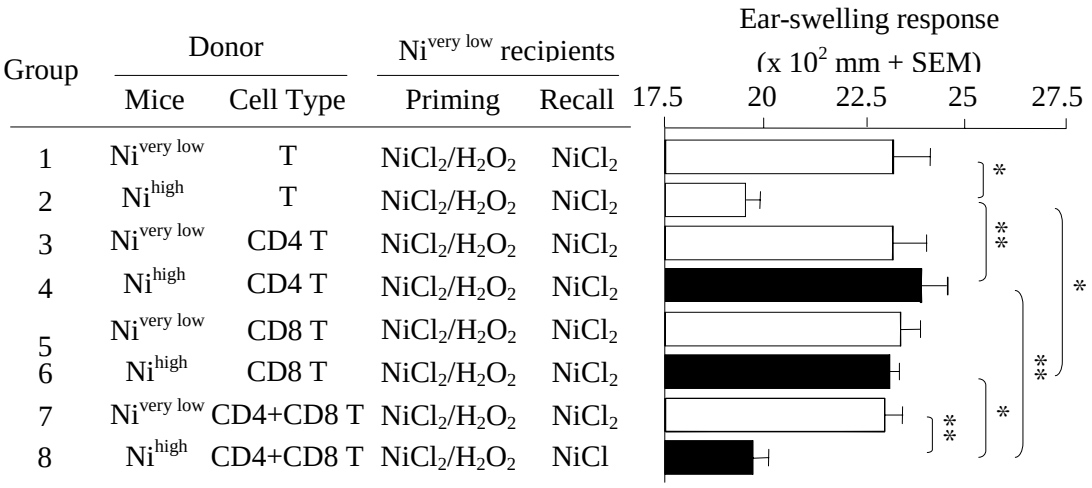


FIGURE 3.3.4.1 When $Ni^{very\ low}$ recipient mice were immunized with $NiCl_2/H_2O_2$, both CD4 and CD8 T cells from Ni^{high} donors were need to transfer tolerance

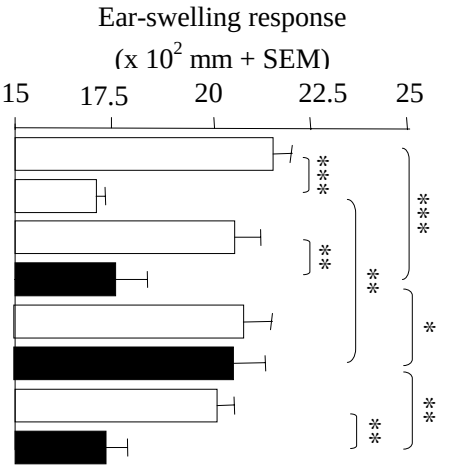
Ni^{low} mice were treated with 10 mM $NiCl_2$ in the drinking water for a period of 4 wks ($\rightarrow Ni^{high}$), as indicated. Untreated $Ni^{very\ low}$ mice were used as control donors. 10^4 purified splenic T cells from these donors were transferred to syngeneic $Ni^{very\ low}$ recipients. The purified T cells were sorted for either $CD8^+$ cells (groups 5 and 6) or $CD4^+$ cells (groups 3 and 4). In groups 7 and 8, 5x10^3 sorted $CD8^+$ and 5x10^3 sorted $CD4^+$ T cells of $Ni^{very\ low}$ and Ni^{high} donors, respectively, were recombined and 10^4 cells of this pool were transferred, as described above. One day after transfer, the recipients were sensitized with $NiCl_2$ in H_2O_2 and after challenge with $NiCl_2$ their ear-swelling response was determined. Asterisks indicate a significant difference (**, $p \leq 0.01$; ***, $p \leq 0.001$) between the groups compared by brackets. Similar results were obtained in two independent experiments.

As can be seen in **Figure 3.3.4.1**, when the $Ni^{very\ low}$ recipients were immunized with $NiCl_2/H_2O_2$ after the adoptive transfer, the tolerance of the Ni^{high} donors could only be transferred by a combination of $CD4^+$ (5 x 10^3) and $CD8^+$ (5 x 10^3) T cells (bar 8, **Figure 3.3.4.1**), but not by either 10^4 $CD4^+$ T cells or $CD8^+$ T cells alone (bars 4 and 6, **Figure 3.3.4.1**). Together with our previous finding (**Figure 6** of ref [177]), it can be concluded that both CD4 and CD8 T cells from Ni^{high} donors were needed to transfer tolerance to either Ni^{low} or $Ni^{very\ low}$ recipients, when the recipients were immunized with $NiCl_2/H_2O_2$. The only difference between using Ni^{low} and $Ni^{very\ low}$ mice as recipients was the Ni^{high} donor cell number required for transferring tolerance (10^2 versus 10^4).

3.3.4.2 Transfer of the CD4$^+$ T cells from Ni^{high} or Ni^{low} donors sufficed to prevent the subsequent sensitization of Ni$^{very\ low}$ recipients with $NiCl_2$ alone

A

Group	Donor		Ni$^{very\ low}$ recipients	
	Mice	Cell Type	Priming	Recall
1	Ni$^{very\ low}$	T	$NiCl_2$	$NiCl_2$
2	Ni^{high}	T	$NiCl_2$	$NiCl_2$
3	Ni$^{very\ low}$	CD4 T	$NiCl_2$	$NiCl_2$
4	Ni^{high}	CD4 T	$NiCl_2$	$NiCl_2$
5	Ni$^{very\ low}$	CD8 T	$NiCl_2$	$NiCl_2$
6	Ni^{high}	CD8 T	$NiCl_2$	$NiCl_2$
7	Ni$^{very\ low}$	CD4+CD8 T	$NiCl_2$	$NiCl_2$
8	Ni^{high}	CD4+CD8 T	$NiCl_2$	$NiCl_2$



B

Group	Donor		Ni$^{very\ low}$ recipients	
	Mice	Cell Type	Priming	Recall
1	Ni$^{very\ low}$	T	$NiCl_2$	$NiCl_2$
2	Ni^{low}	T	$NiCl_2$	$NiCl_2$
3	Ni$^{very\ low}$	CD4 T	$NiCl_2$	$NiCl_2$
4	Ni^{low}	CD4 T	$NiCl_2$	$NiCl_2$
5	Ni$^{very\ low}$	CD8 T	$NiCl_2$	$NiCl_2$
6	Ni^{low}	CD8 T	$NiCl_2$	$NiCl_2$
7	Ni$^{very\ low}$	CD4+CD8 T	$NiCl_2$	$NiCl_2$
8	Ni^{low}	CD4+CD8 T	$NiCl_2$	$NiCl_2$

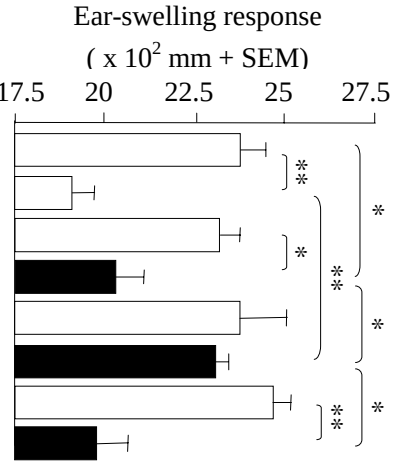


FIGURE 3.3.4.2 When Ni$^{very\ low}$ recipient mice were immunized with $NiCl_2$ alone, CD4 T cells from either Ni^{high} or Ni^{low} donors were able to transfer unresponsiveness Ni^{low} mice were treated with 10 mM $NiCl_2$ in the drinking water for a period of 4-6 wks ($\rightarrow$Ni^{high}) or were left untreated, as indicated. Untreated Ni$^{very\ low}$ mice were used as control donors. 10^3 and 10^6 purified splenic T cells from Ni^{high} (A) and Ni^{low} (B) donors, respectively, were transferred to syngeneic Ni$^{very\ low}$ recipients. The purified T cells were sorted for either CD8$^+$ cells (groups 5 and 6) or CD4$^+$ cells (groups 3 and 4). In groups 7 and 8, a combining of $5x10^2$ sorted CD8$^+$ and $5x10^2$ sorted CD4$^+$ T cells of Ni$^{very\ low}$ and Ni^{high} donors (A), respectively; or a combining of $5x10^5$ sorted CD8$^+$ and $5x10^5$ sorted CD4$^+$ T cells of Ni$^{very\ low}$ and Ni^{low} donors (B), respectively, were recombined and 10^3 (A) or 10^6 (B) cells of this pool were transferred, as described above. One day after transfer, the recipients were sensitized with $NiCl_2$ and after challenge with $NiCl_2$ their ear-swelling response was determined. Asterisks indicate a significant difference (*, $p \leq 0.05$; **, $p \leq 0.001$; ***, $p \leq 0.001$) between the groups compared by brackets. Similar results were obtained in two independent experiments.

The results shown in **Figure 3.3.4.1** indicated that both $CD4^+$ and $CD8^+$ Ni^{high} T cells were required in order to prevent the subsequent sensitization of the $Ni^{very\ low}$ recipients by $NiCl_2$/ H_2O_2. However, when the $Ni^{very\ low}$ recipients were immunized with $NiCl_2$ alone after the adoptive transfer, the tolerance of the Ni^{high} donors could be transferred by 10^4 $CD4^+$ T cells alone (*bar 4,* **Figure 3.3.4.2A**). Once again, $CD8^+$ T cells alone proved incapable of preventing the sensitization by $NiCl_2$/ H_2O_2 (*bar 6,* **Figure 3.3.4.2A**).

Similar results were found when Ni^{low} mice, instead of Ni^{high} mice, were used as donors: when the $Ni^{very\ low}$ recipients were immunized with $NiCl_2$ alone after the adoptive transfer, the unresponsiveness of the Ni^{low} donors could be transferred by 10^6 $CD4^+$ T cells alone (*bar 4,* **Figure 3.3.4.2B**). Here, the only difference between the use of Ni^{high} and Ni^{low} mice as donors was the cell numbers (10^6 versus 10^3) required for the successful transfer of unresponsiveness.

3.4 APC FROM Ni^{HIGH} MICE ARE TOLEROGENIC

In addition to Treg cells, particular types of APC also play a role in immune tolerance (see section 1.7). Therefore, in our mouse model we not only studied the role of T cells in oral tolerance to nickel, but also paid attention to the APC. Thus, our group reported that *(i)* the DCs of Ni^{high} mice showed higher DEC-205 and lower CD40 expression compared with those from Ni^{low} mice, and *(ii)* the B cells of Ni^{high} mice displayed lower CD40 and higher CD38 expression than their Ni^{low} counterparts [189]. Are these phenotypic alterations of the APCs of Ni^{high} mice associated with functional alterations of the APCs? This question can be answered in the affirmatory, as is evident from the results shown in sections 3.4.1 to 3.4.4.

3.4.1 APC from Ni^{high} mice show reduced stimulatory capacity in allo-MLR

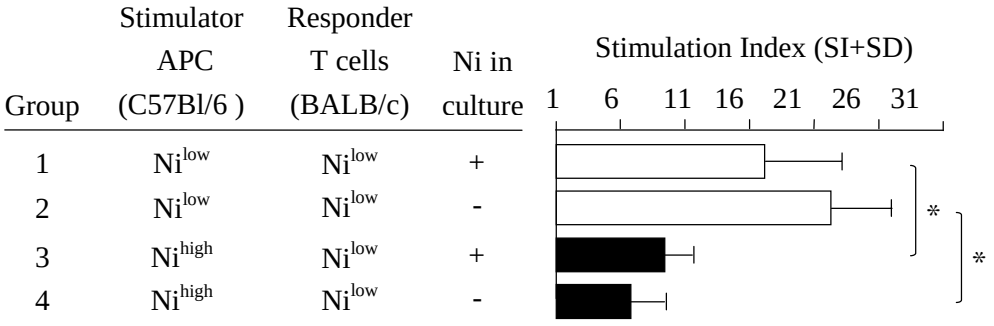


FIGURE 3.4.1. APC from Ni^{high} mice show decreased allostimulatory capacity in the MLR. Groups of C57BL/6 Ni^{low} mice were tolerized by oral $NiCl_2$ treatment($\rightarrow Ni^{high}$), or were left untreated, as indicated. Thereafter, the mice were sacrificed and their APC prepared and cultured over night with (+) or without (-) $NiCl_2$, before splenic T cells from untreated (Ni^{low}) BALB/c mice were added to start the MLR. Four days later, [^{3}H]-thymidine incorporation was measured. The asterisks indicate a significant difference (*, $p \leq 0.05$) between the groups compared by brackets. The results are taken from one of four experiments which yielded comparable results.

The tolerogenic phenotype displayed on the APC from Ni^{high} mice was mirrored by their reduced allo-

stimulatory capacity in the MLR *in vitro* (see section 2.9), where these cells were used as allo-stimulators. Irradiated APC (T cell-depleted spleen cells) from Ni^{high} C57Bl/6 mice (H-2^b) were cocultured with splenic T cells from untreated BALB/c mice (H-2^d). The proliferative responses of these T cells were then tested in the presence or absence of $NiCl_2$. In comparison with the APC from untreated C57Bl/6 Ni^{low} mice (bars 1 and 2, **Figure 3.4.1**), the APC from C57Bl/6 Ni^{high} mice (bars 3 and 4, **Figure 3.4.1**) showed a significantly reduced allostimulatory capacity, regardless of weather $NiCl_2$ was present.

3.4.2 APC from Ni^{high} mice can transfer tolerance to untreated Ni^{low} recipients

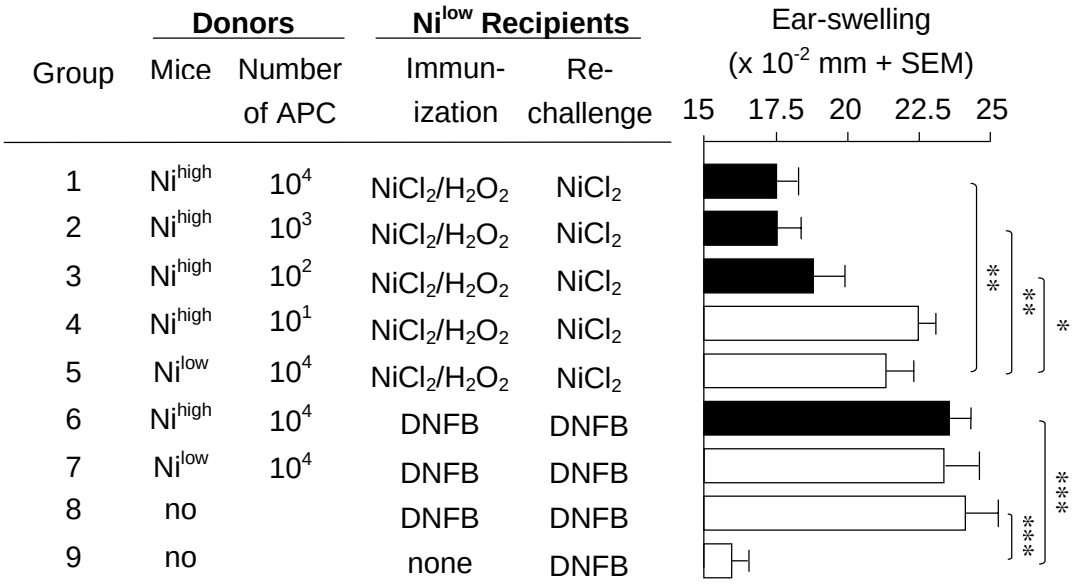


FIGURE 3.4.2. APC from Ni^{high} donors are able to adoptively transfer nickel tolerance. Prospective C57BL/6 Ni^{low} donor mice were orally tolerized ($\rightarrow Ni^{high}$) or left untreated, as indicated. Specified numbers of APC from Ni^{high} or untreated Ni^{low} donors were injected i.v. into untreated Ni^{low} syngeneic recipients. Twenty-four hours after the transfer, recipients were immunized by injection of $NiCl_2/H_2O_2$ into each flank, except for those which were painted with DNFB onto both flanks, as indicated. Ten days later, the mice were re-challenged at the ears with either $NiCl_2$ or DNFB, and their ear-swelling response determined 2 days thereafter. The data shown represents the mean ear-swelling response + SEM from groups of 5 mice each. The asterisks indicate a significant difference (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) between the groups compared by brackets. Results represent one of three experiments, which yielded comparable results.

.

The data in section 3.3.1.1 demonstrated that as few as 10^2 bulk T cells from the spleens of Ni^{high} donors were able to adoptively transfer the tolerance to untreated Ni^{low} recipients. Here, we can find that the APC also can transfer nickel tolerance. As shown in bars 1-3 of **Figure 3.4.2**, the transfer of 10^4, 10^3, even as few as 10^2 APC (T cell depleted spleen cells) from Ni^{high} donors succeeded in transferring nickel tolerance to naive syngeneic Ni^{low} recipients. The ear-swelling response in the recipients that received tolerogenic APC was as low as the negative ear-swelling previously observed

in Ni^{low} mice that did not received a cell transfer and were sensitized with $NiCl_2$ alone challenged with $NiCl_2$ (bar 1, **Figure 3.1.1A and B**). Moreover, these ear-swelling values correlated with those of Ni^{low} mice received between 10^2 and 10^4 splenic T cells from Ni^{high} donors, followed by sensitized with $NiCl_2/H_2O_2$ and challenged with $NiCl_2$ (bars 2-4, **Figure 3.3.1.1**). However, the transfer of only 10^1 APC from Ni^{high} donors or 10^4 APC from Ni^{low} donors (bars 4 and 5, **Figure.3.4.2**) failed to render the recipients resistant to subsequent sensitization with $NiCl_2/H_2O_2$. As a specificity control, a group of recipients that had received 10^4 APC from Ni^{high} donors were sensitized and challenged with DNFB. The anti-DNFB response of the recipients was normal (bar 6, **Figure 3.4.2**), indicating that the APC conferred unresponsiveness to nickel, but not to DNFB. Consistent with this, it was shown that the Ni^{high} mice failed to be generally immunosuppressed (see section 3.3.1.1.), even though this might be suggested by the tolerogenic phenotype (see section 3.4) and the reduced allostimulatory capacity of the APC (see section 3.4.1) of these animals.

By further characterization the APC from Ni^{high} donors, we found that 10^3 purified B cells could transfer tolerance (Figure 5 of ref. [189]). So, in the transfer of tolerance from Ni^{high} donors to Ni^{low} recipients, only need very few cells (10^2 bulk splenic T cells, 10^2 T depleted spleen cells, 10^3 purified B cells). Therefore, the purity is critical in the characterization of cell populations. To DCs, it was difficult for us to get enough purity, therefore, we were unable to direct test if they could transfer tolerance or not. However, the facts that only 10^2 T-depleted spleen cell could transfer tolerance whereas 10^3 purified B were needed to do this job strongly imply that DCs might transfer tolerance in an even more powerful way than B cells.

3.4.3 The tolerogenicity of APC from Ni^{high} mice are transient

Both APC (**Figure 3.4.2**) and T cells (**Figures 3.3.1.1, 3.3.2.1, 3.3.1.3**) from Ni^{high} mice were able to transfer nickel tolerance when the respective donor mice were sacrificed directly after oral nickel treatment, or after a treatment-free interval of a couple of weeks after oral nickel treatment. Both the tolerance of Ni^{high} mice (**Figure 3.1.1**) and the T cell suppressive activity (**Figure 3.3.1.2**) of these mice lasted at least 20 weeks. Next, we want to test if the tolerogenicity of the APCs was also long lasting.

Since 10^4, instead of 10^2, T cells were need to transfer tolerance from Ni^{high} mice that had stopped oral $NiCl_2$ treatment for 20 weeks to naive syngeneic Ni^{low} recipients (section 3.3.1.2), 10^4 APC or T cells were used in the next adoptive transfer experiment. After receiving 10^4 APC or T cells from either Ni^{high} donor mice, which had stopped oral $NiCl_2$ treatment for 1 week or 20 weeks, or Ni^{low} donor mice, the Ni^{low} recipients were sensitized with $NiCl_2/H_2O_2$ and challenged with $NiCl_2$, and ear-swelling was measured, as usual. Both APC and T cells from Ni^{high} mice were able to transfer nickel tolerance when the respective donors were sacrificed after a treatment-free interval of 1 week after oral tolerance induction (bars 1 and 4, **Figure. 3.4.3A**). Consistent with the results shown in 3.3.1.2, nickel tolerance could be transferred with bulk T cells as late as 20 weeks after the termination of oral $NiCl_2$ treatment (bar 5, **Figure 3.4.3A**). Obviously, nickel-specific T cells with a long-lasting suppressive capacity were formed during oral $NiCl_2$ treatment. In contrast with T cells, the APC from Ni^{high} mice had completely lost their capacity to transfer tolerance after a treatment-free interval of 20 weeks (bar

2, **Figure 3.4.3A**). The biological half-life of nickel ions is so short that, in men, 50% of the nickel ions in the body are eliminated within 2-3 days[190]. Hence, it is likely that after a treatment-free interval of 20 weeks, the concentration of nickel ions in the Ni^{high} mice was too low to induce new tolerogenic APC, and the original tolerogenic APC that had been induced during the four weeks of nickel-treatment were lost due to their short lifetime.

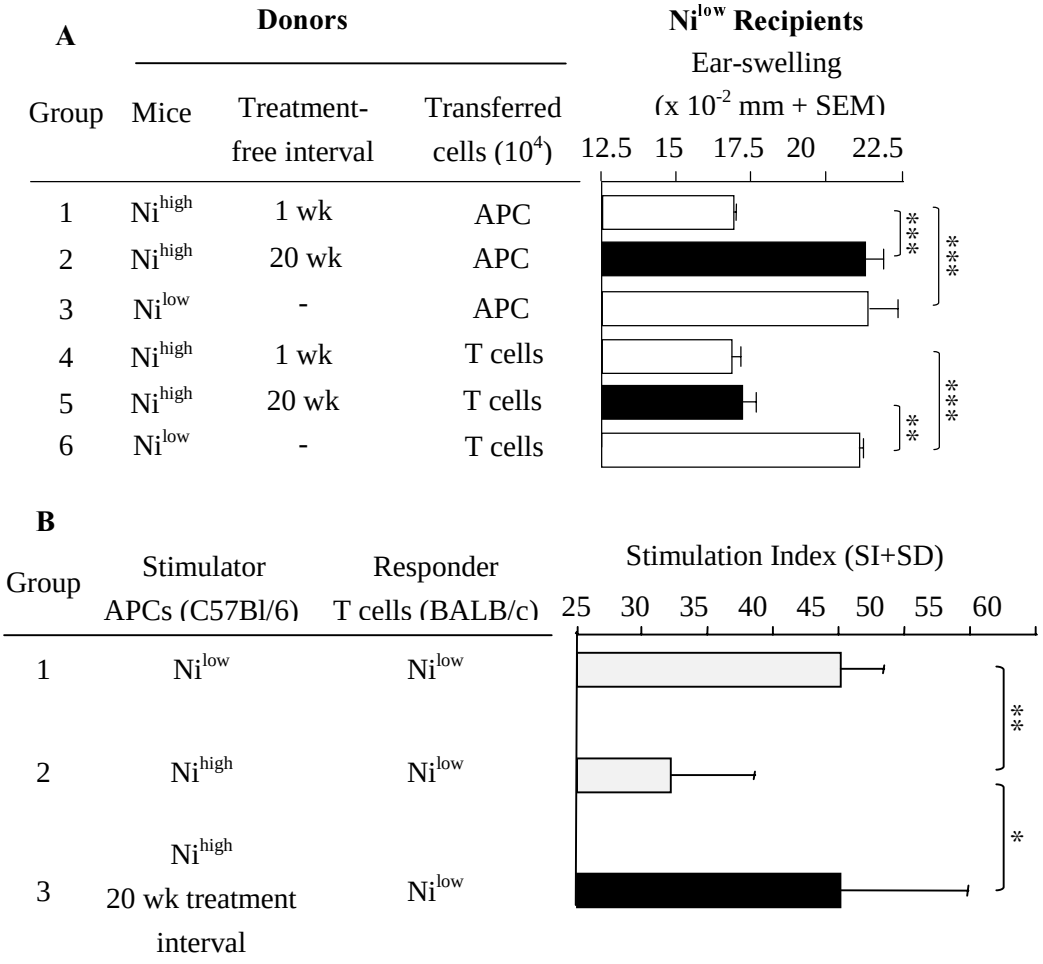


FIGURE 3.4.3. Unlike T cells, APC lose their tolerogenicity during a treatment-free interval of 20 wk after the termination of oral $NiCl_2$ treatment. Prospective C57BL/6 donor mice were orally tolerized ($\rightarrow Ni^{high}$) or left untreated, as indicated. (A) Following a treatment-free interval of 1 wk or 20 wk, as indicated, 10^4 APC or T cells from Ni^{high} or Ni^{low} donors were injected i.v. into Ni^{low} syngeneic recipients. Twenty-four hours after the transfer, all recipients were immunized by injection of $NiCl_2$ / H_2O_2 into each flank. Ten days later, the mice were re-challenged at the ears with $NiCl_2$ and their ear-swelling responses determined 2 days thereafter. Data depicted represents the mean ear-swelling response + SEM from groups of 5 mice each. (B) Direct after oral Ni treatment or following a treatment-free interval of 1 wk or 20 wk, as indicated, the mice were sacrificed and their APC prepared and cultured over night before splenic T cells from untreated (Ni^{low}) BALB/c mice were added to start the MLR. Four days later, [^{3}H]-thymidine incorporation was measured. The asterisks indicate a significant difference (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) between the groups compared by brackets. Results represent one of two (A) or three (B) experiments, which yielded comparable results.

The transient tolerogenicity of APC from Ni^{high} mice could also be found in allo-MLR experiment.

Because only the APC from Ni^{high} mice with a treatment-free interval of 1 week (bar 2, **Figure 3.4.3B** as well as bars 3 and 4, **Figure 3.4.1A**), but not with a treatment-free interval of 20 weeks (bar 3, **Figure 3.4.3B**), showed a reduced allo-stimulatory capacity compared with those from untreated Ni^{low} mice (bar 1, **Figure 3.4.3B**).

So, although the APC in the Ni^{high} mice were found to be an essential element in nickel tolerance, the T cells of these animals are the responsible factor for the long-term maintenance of tolerance.

3.4.4 Infectious tolerance: both APC and T cells are involved

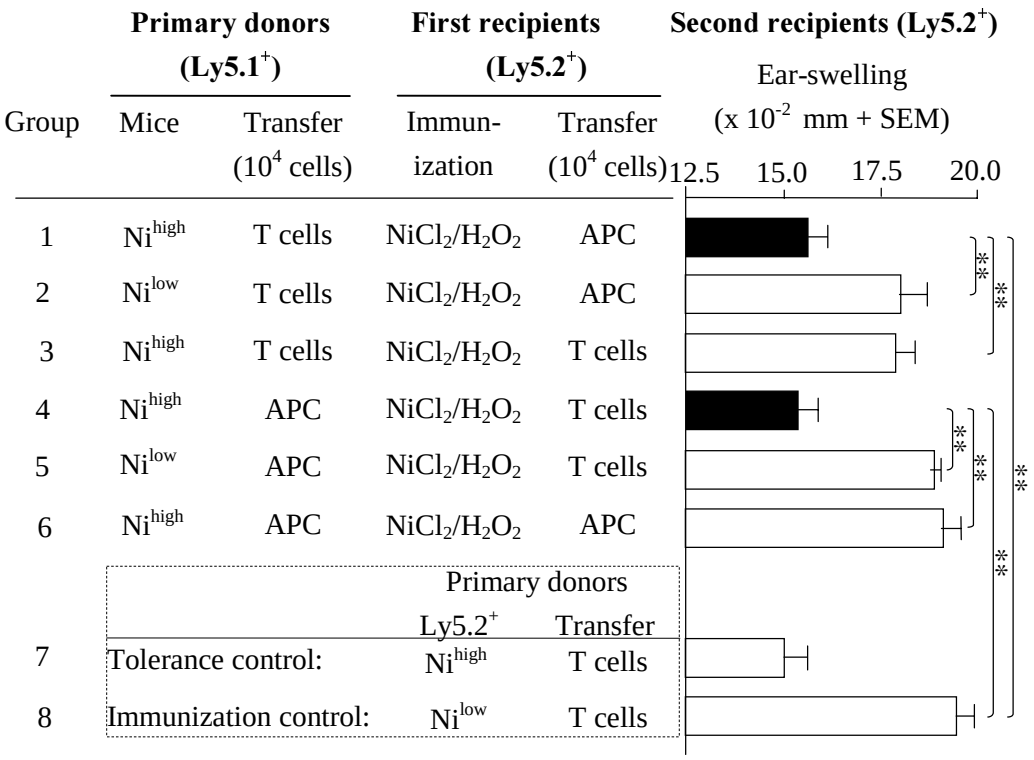


FIGURE 3.4.4. Infectious tolerance is the amplification mechanism accounting for the transfer of nickel tolerance in vivo; in order to be effective, T cells and APC must cooperate in this process. Prospective $Ly5.1^+$ donor mice were orally tolerized to nickel ($\rightarrow Ni^{high}$) or left untreated (Ni^{low}), as indicated. Thereafter, T cells or APC from the $Ly5.1^+$ primary donors were transferred (10^4 cells per recipient) to a first set of $Ly5.2^+$ recipients. The latter were injected with $NiCl_2$ / H_2O_2 within 24 h after transfer. On day 11, T cells and APC from these first recipients were isolated and depleted of donor $Ly5.1^+$ cells, and subsequently transferred (10^4 cells per recipient) into a second set of $Ly5.2^+$ recipients. As control groups for nickel tolerance and allergy (*bottom*), 10^4 nylon wool-enriched T cells from Ni^{high} or Ni^{low} $Ly5.2^+$ donors were transferred into groups of Ni^{low} $Ly5.2^+$ recipients, as indicated. All recipients were immunized, re-challenged, and the ear-swelling responses determined in accordance to the standard protocol. . The asterisks indicate a significant difference (**, $p \leq 0.01$; ***, $p \leq 0.001$) between the groups compared by brackets. The displayed data represents the mean ear-swelling response (+ SEM) from groups of 5 mice each. Results represent one of two experiments, which yielded comparable results.

Since only 10^2 T cells or 10^2 APC from Ni^{high} mice sufficed to transfer tolerance to Ni^{low} recipients, powerful amplification mechanisms must have been involved. Infectious tolerance was one of the mechanisms. In order to investigate the role of infectious tolerance in our model, serial adoptive cell

transfers were performed. These involved orally tolerized Ni^{high} mice as primary cell donors, a first set of (Ni^{low}) recipient mice which in turn became the secondary cell donors, and, finally, a second set of Ni^{low} recipient mice which were assayed for tolerance induction after immunization with $NiCl_2/H_2O_2$ and re-challenge at the ears. To be able to distinguish the cells from the primary donors from those of the recipients, congenic C57BL/6 mice, which express Ly5.1 on the surface of all lympho-hematopoietic cells were used as the primary donors, instead of the $Ly5.2^+$ wildtype mice. As shown in **Figure 3.4.4**, 10^4 T cells or APC from Ni^{high} or untreated Ni^{low} $Ly5.1^+$ primary donors were transferred to the first set of Ni^{low} $Ly5.2^+$ recipients, which were immunized with $NiCl_2/H_2O_2$ one day later. After a further 10 days, APC or T cells, were isolated from the first recipients and depleted of any contaminating $Ly5.1^+$ cells remaining from the primary donors. These cells (10^4 / mouse) were then transferred into the second set of Ni^{low} $Ly5.2^+$ recipients. If the latter received T cells or APC from those first recipients which themselves had been tolerized by injection of the opposite cell type (APC and T cells, respectively) originating from the Ni^{high} $Ly5.1^+$ donors, they were unresponsive to immunization (bars 1 and 4, **Figure 3.4.4**). Consequently, $Ly5.1^+$ cells derived from the primary donors possessed the ability to infectiously spread nickel tolerance to the first recipients (and prospective secondary donors), indicating that, indeed, infectious tolerance operates in orally induced nickel tolerance. Both T cells and APC from the $Ly5.1^+$ primary donors were able to initiate this cascade mechanism of tolerance in vivo. Interestingly however, although the tolerance was successfully transferred by T cells of the primary donors to APC of the first recipients and, vice versa, by APC of the primary donors to T cells of the first recipients, there was no direct tolerance transfer from either donor T cells to host T cells or from donor APC to host APC (bars 3 and 6, **Figure 3.4.4**).

The results shown in **Figure 3.4.4** demonstrate that the successful tolerance transfer from the primary $Ly5.1^+$ donors to the second set of $Ly5.2^+$ recipients involved infectious tolerance. We ruled out the possibility that the transfer or tolerance was due to a contamination through residual $Ly5.1^+$ cells derived from the tolerant primary donors, because immunofluorescent staining showed that amongst the cells isolated from the first set of $Ly5.2^+$ recipients there was a maximum of 0.1% contaminating $Ly5.1^+$ cells. Thus, along with the 10^4 cells obtained from the first recipients and transferred to each mouse in the second set of $Ly5.2^+$ recipients, a maximum of 10^1 cells derived from the $Ly5.1^+$ primary donors could have been co-transferred. This small contamination was negligible because neither 10^1 T cells (bar 1, **Figure 3.3.1.1**) , nor 10^1 APC (bar 4, **Figure 3.4.2**) from Ni^{high} donors were able to transfer the tolerance. Another reason why this small contamination was negligible came from the observation that the second set of recipients failed to be tolerized when they received 10^4 T cells from those first $Ly5.2^+$ recipients, which themselves were rendered tolerant by the injection of T cells from the primary $Ly5.1^+$ donors (bar 6, **Figure 3.4.4**). Although the first recipients (and thus the secondary donors) taken for this T cell →T cell transfer were identical with those used for the T cell →APC transfer, only the APC isolated from the first recipients were found to tolerize the second set of recipients (bar 3 vs. 4, **Figure 3.4.4**). These tolerogenic APC, however, contained the same percentage (≤0.1%) of contaminating $Ly5.1^+$ cells as the ineffective T cell fraction isolated from the same animals.

4. DISCUSSION

The oral administration of nickel to animals (Ni^{high}) rendered them unresponsiveness to nickel in the DTH reaction [156;157;175;176], and furthermore this unresponsiveness could be transferred to naive recipients [156;157;175]. The explanation for this finding resides in the fact that Ni ions alone can only provide signal I (i.e. Ni-neoantigen) but not signal II (costimulatory molecules) [153]. This implies that Ni ions act as tolerogens. In order to induce nickel-specific immune response in Ni^{low} animals (conventionally breed), a combination of Ni ions and adjuvant were needed for sensitization (see section 1.8.1). Moreover, we find that the nickel contained within stainless steel also influences the outcome of immune response to nickel. Mice bred in metal-free conditions, $Ni^{very\ low}$, were easier to be sensitized than their Ni^{low} counterparts [157]. All these findings imply that the oral contact of nickel released from metals is sufficient to induce certain levels of tolerance. Although it was shown Ni ions alone could only provide signal I but not sufficient signal II to activate naive T cells *in vivo*, at least in Ni^{low} mice, nickel did upregulate costimulatory molecules or other accessory molecules on DC [182] or other "non-professional" APC such as keratinocytes [183-186] when these "APC" were cultured *in vitro*. Together with the finding that $Ni^{very\ low}$ mice were easier sensitized than their Ni^{low} counterparts, it is reasonable to think that the T cells in the differently breed mice require different levels of costimulation in order to be activated and in turn, the T cells may also influence the expression of costimulatory molecules on APC. In order to comprehensively clarify this issue, I have studied the relationship of oral nickel uptake, the susceptibility of immunization to nickel and nickel-specific Treg development. In this chapter, the above points will be discussed in detail.

4.1 SUSCEPTIBILITY TO NICKEL SENSITIZATION IS INVERSELY CORRELATED TO ORAL EXPOSURE

The existence of an inverse dose-response relationship in mice between the amount of oral nickel uptake and the ease of sensitization to nickel was first realized by van Hoogstraten *et al.* [191] and Ishii et al. [156]. Later, using their newly developed model of co-administering $NiCl_2$ with H_2O_2 for the sensitization, Artik *et al.* [153] noted that the induction of oral nickel tolerance was both dose- and time-dependent [177]. Extending these observations, I find in this study that a distinct hierarchy exists after oral treatment between the $Ni^{very\ low} > Ni^{low} > Ni^{high}$ mice and their ease of sensitization: Ni^{high} mice proved completely resistant to sensitization, whereas Ni^{low} mice could be sensitized with $NiCl_2/H_2O_2$ but not $NiCl_2$ alone. An intriguing new observation was that the $Ni^{very\ low}$ mice, in contrast to the Ni^{low} mice, could be sensitized with $NiCl_2$ alone, that is, without additional adjuvant.

4.2 THE SUPPRESSIVE ACTIVITY OF T CELLS TO NICKEL POSITIVELY CORRELATES WITH ORAL EXPOSURE

Using *in vitro* suppression assays and *in vivo* transfer experiments, I have shown in section 3.3 that different breeding conditions can effect the development of nickel-specific Treg. The $Ni^{very\ low}$ mice were prove to be lack of nickel-specific Treg cells in the $Ni^{very\ low}$ mice. The protection provided by $CD4^+$ Treg in Ni^{low} mice was not absolute because their suppression could be broken by immunization with $NiCl_2/H_2O_2$. Only Treg from Ni^{high} mice could protect from sensitization with $NiCl_2/H_2O_2$ and here the cooperation between $CD4^+$ and $CD8^+$ T cells was necessary. Therefore, as far as the suppressive capacity of nickel-specific Treg cells are concerned there exists a reverse hierarchy, namely $Ni^{high} > Ni^{low} > Ni^{very\ low}$, between the different breeding groups of mice,

4.3 AN OVERALL RELATIONSHIP EXISTS BETWEEN ORAL UPTAKE OF NICKEL, THE SUSCEPTIBILITY TO NICKEL SENSITIZATION AND THE SUPPRESSIVE ACTIVITIES OF T CELLS

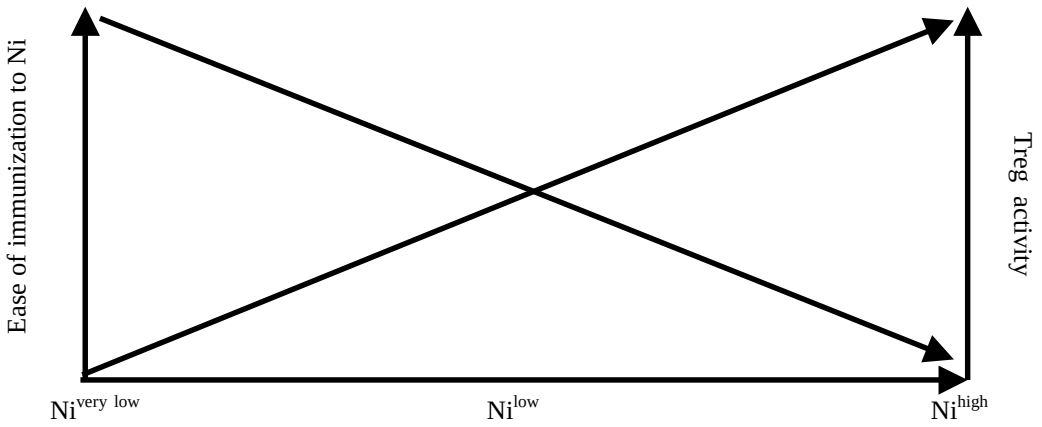


Figure 4.3 Schematic view of the inverse relationship between oral nickel exposure, Treg cell activity, and the ease of being sensitized to nickel. The higher the oral uptake of nickel, the higher the suppressive capacity of Treg and, hence the greater the difficulty to being sensitized

From the discussion in sections 4.1 and 4.2, it is apparent that there is a comprehensive relationship between the oral uptake of nickel, the susceptibility to nickel sensitization and the ensuing suppressive T cell activities depending of the level of nickel exposure. In figure 4.3, these relationships can be summarized in the following manner; the higher the oral uptake of nickel the higher the suppressive T cell activity, consequently, these mice are less susceptible to immunization to nickel.

4.4 UPREGULATION OF COSTIMULATION MOLECULES AFTER SUBCUTANEOUS OR INTRADERMAL INJECTION OF $NiCl_2$, WITH OR WITHOUT ADJUVANT, IS DETERMINED BY THE SUPPRESSIVE ACTIVITY OF NICKEL-SPECIFIC TREGS

Ni^{2+} ions alone are able to sensitize $Ni^{very\ low}$ mice, section 3.1.3. This evidence indirectly implies that Ni^{2+} ions themselves, at least when intradermally injected, can generate a 'danger' situation [22] and in turn upregulate the costimulatory molecules on DCs required for T cell priming. Recent data using our *in vivo* mouse model has shown that this indeed appears to be the case (M. Fang, unpublished data) and correlates with the upregulation of costimulatory molecules observed in human skin explants exposed to Ni^{2+} ions *in vitro* (Rustemeyer, T., personal communication). Moreover, human endothelial cells exposed to nickel *in vitro* were found to upregulate the expression of a variety of adhesion molecules [192], and in keratinocytes, nickel has been shown to upregulate MHC class II [183], CD80 [184], ICAM-1 [185;186], tumor necrosis factor-α, the very late antigen-3 [186], and the vascular endothelial growth factor [193]. In a broader sense, all of these effects presumably augment its adjuvant activity. Hence, nickel ions themselves are able to provide a certain level of costimulation that is sufficient to immunize the $Ni^{very\ low}$ mice which lack nickel-specific Treg. Therefore, our previous view that Ni^{2+} ions are devoid of intrinsic adjuvanticity [153] must be revised since it was based solely on studies performed in Ni^{low} mice.

In contrast to $Ni^{very\ low}$ mice, upregulation of CD80 and CD86 on DCs only occurred in naive Ni^{low} mice when they were injected with $NiCl_2/H_2O_2$ [189]. This CD80/CD86 upregulation however, was no longer detectable if the Ni^{low} mice received an adoptive transfer of Ni^{high} T cells prior to the injection (M. Fang, unpublished data). Therefore, these findings indicate that the combination of $NiCl_2/H_2O_2$ provides signals that are strong enough to overcome the suppression exerted by Treg in Ni^{low} but not in Ni^{high} mice.

4.5 THE DEVELOPMENT OF NICKEL SPECIFIC T CELLS AFTER SENSITIZATION WITH A "NON-NI" ANTIGEN

The data shown in section 3.2 shows that LNC prepared from DNFB- (or FITC-) primed mice react to Ni *in vitro.* This response was not due to possible cross-reactions between nickel and DNFB for the following reasons: 1) the cells responding to Ni are $CD4^+$ T cells whereas DNBS-reactive cells are $CD8^+$ T cells (Figure 3.2.3.1); 2) when T cell hybridomas were generated from DNFB-primed LNC, both Ni- and DNBS-reactive hybridomas were obtained and interestingly were individually specific for nickel or DNBS (table 3.2.3.2). In contrast, further experiments showed that the Ni-reactivity of LNC from either $NiCl_2/H_2O_2$- or DNFB-sensitized mice were similar: they had comparable frequencies (section 3.2.2) and the responding T cells were both within the $CD4^+$ subpopulation (section 3.2.3.1). These findings that sensitization with DNFB could generate Ni-specific T cells was a very unusual result and also contradicted the *in vivo* data that showed that $NiCl_2/H_2O_2$ and DNFB sensitization induced Ni- and DNFB-specific immune responses respectively (section 3.1).

To investigate the reason why such a phenomenon could arise, we assayed the roles played by the T cells and APCs from the different antigen sensitized mice. In short, T cells from LNC of Ni^{low} mice that were either naïve or sensitized with $NiCl_2/H_2O_2$ or DNFB were separated and co-cultured with Ag and irradiated APC isolated from the same mice. This assay was termed "criss-cross restimulation" and the preliminary results in response nickel are summarized in table 4.4.2.2.

	APC		
T CELLS	Naïve	$NiCl_2/H_2O_2$	DNFB
Naive	-	+	-
$NiCl_2/H_2O_2$	+	+	+
DNFB	-	+	+

Table 4.4.2.2. **Summary of preliminary results of "criss-cross" restimulation:** the *in vitro* proliferation to nickel by T cells from $NiCl_2/H_2O_2$-primed mice is a secondary immune response, whereas T cells from DNFB-primed mice show a primary immune response

These results clearly demonstrate that lymphocytes from Ni^{low} mice sensitized to $NiCl_2/H_2O_2$ or DNFB react differently to nickel. In the case of T cells, those isolated from $NiCl_2/H_2O_2$ sensitized mice proliferated to Ni regardless of the APC source. In contrast, T cells from DNFB-sensitized mice only proliferated to Ni when they were co-cultured with APC from sensitized mice. APC from $NiCl_2/H_2O_2$-primed mice could induce all groups of T cells to proliferate to Ni, a characteristic which APC from DNFB-sensitized mice failed to do. Therefore, when Ni^{low} mice are sensitized with $NiCl_2/H_2O_2$, Ni-specific T cells are established, and the proliferation of their T cells to nickel *ex vivo* is a secondary response. In DNFB-sensitized mice, although there is no development of nickel-specific T cells *in vivo*, this sensitization procedure modified both the T cells and APC so that the LNC could be easily primed with Ni *in vitro*. Hence, *in vitro* proliferation measured here actually represents a primary immune response to Ni.

The above results appear to clarify the following points: 1) why LNC from DNFB-sensitized mice proliferate to nickel *in vitro* but fail to produce a DTH response *in vivo*. Quite simply, the proliferation of LNC from DNFB-sensitized mice to Ni was due to an *in vitro* primary response to Ni. 2) why human PBMC isolated from both patch test positive and negative people proliferate *in vitro* to Ni [168]. As in point one, patch test negative PBMC reflect LNC from DNFB-sensitized mice, that is, they respond to nickel *in vitro* because they were somehow modified *in vivo* beforehand.

As discussed before, the induction of a primary immune response requires both signal I (here Ni induced neoantigens) and signal II (costimulatory molecules), but releasing a secondary immune response only needs signal I. Primary immune response are slower than secondary ones; however, in this study the primary responses to Ni by LNC of DNFB- or FITC-sensitized mice *in vitro* were as quick as the secondary responses by $NiCl_2/H_2O_2$ sensitized mice to Ni. The following reasons may explain these findings. First, several independent studies have shown that Ni-specific T cell clones or cell lines can respond to Ni in a processing independent manner [167;170;172]. In fact, some T cell clones were found to proliferate to Ni in the absence of APC leading Nasorri *et al*.[167] to speculate that Ni could be presented by T cells (i.e. T-T presentation), especially when T cells can express MHCII molecules or acquire MHCII molecules from APC under various conditions of activation or inflammation [45;194-197]. Consequently, this processing independent mechanism can shorten the time needed to activate Ni-specific T cells. Second, changes in the local environment induced by

chemical sensitization modify both APC and T cells in the draining lymph nodes, which facilitates the priming of Ni-specific T cells. When an animal is immunized with an antigen, DCs take up the antigen and during migration to local lymph nodes upregulate costimulatory molecules and thus matures. In the lymph nodes mature DC activate Ag-specific T cells which then elicit the activation of bystander T cells by Ag-independent pathways [198;199]. In fact, T cells can also be activated by virus infections [200], cytokines [199;201;202], or previously activated T cells [203], all Ag-independent mechanisms. Therefore, upon chemical immunization, the costimulatory molecules on APC are upregulated and in turn their capacity to activate T cells becomes stronger; simultaneously, T cells, including nickel-specific ones, are modified through Ag-independent pathway(s), which lowers their activation threshold. These cells are then more susceptible to activation *in vitro* even though they cannot be sensitized *de novo*. One could argue that costimulatory molecules are normally upregulated at early time point after sensitization and 10 days after priming, the primary immune response normally could have subsided. To some extent this comment is correct, however, it is also true that, 10 days after priming, the draining lymph nodes of DNFB-, FITC-, and especially $NiCl_2/H_2O_2$-primed mice were still enlarged with significantly higher cell numbers than their naive counterparts (data not shown). Following flow cytometric analysis, the ratio of $MHCII^+$ (i.e. APC): $TCR\beta^+$ (i.e. T cells) in the LNC of DNFB-, FITC- or $NiCl_2/H_2O_2$-primed mice were higher than those found in naive mice (data not shown). This change may sufficient for the *in vitro* priming of nickel-specific T cells but these potential mechanisms need further investigation.

Since nickel-specific T cells can be primed *in vitro* following sensitization with an alternative chemical, one may ask if nickel-specific T cells can also be *de novo* activated *in vivo* by priming the Ni^{low} mice with $NiCl_2$ combining with “non-Ni” antigen such as DNFB or FITC. These investigations are currently being performed. Actually, similar studies have been conducted using guinea pigs breed in a Ni^{low} environment [204]. DTH responses to nickel in these animals could be achieved when nickel was co-administered with the tuberculin purified protein derivative (PPD) after an initial immunization with the mycobacterium. The key to nickel reactivity here was the induced inflammation something that is readily induced following a Ni injection.

4.6 GENETIC AND ENVIRONMENTAL INFLUENCES MAY ACCOUNT FOR THE DIFFERENT SUSCEPTIBILITIES TO NICKEL SENSITIZATION FOUND IN ANIMAL MODELS

The overall threshold for the induction of nickel hypersensitivity in our model is apparently lower than that needed by Hoogstraten et al. [157]. Both groups observed that $Ni^{very\ low}$ mice can be more readily sensitized than Ni^{low} mice. However, while our $Ni^{very\ low}$ mice could be sensitized with Ni^{2+} ions alone, their system required the combination of Ni^{2+} ions and CFA. Moreover, we were also able to readily sensitize our Ni^{low} mice by the co-administration of Ni^{2+} ions and a skin irritant or adjuvant [153]. A possible explanation for this discrepancy may reside in the animal strain, since our Ni^{low} and $Ni^{very\ low}$ mice are C57Bl/6 mice, whereas van hoogstraten et al use BALB/c mice. Another reason could be possible undefined differences in the environmental nickel concentrations in the two animal facilities. If we suppose that the quantity of oral nickel supplied in the food and drinking water or released from the stainless steel items in the animal facility of van Hoogstraten *et al.* [157] exceeded that of ours, this

could account for a generally higher resistance to nickel sensitization of their animals compared with ours.

4.7 THE UNDERLYING MECHANISMS TO HOW THE ORAL ADMINISTRATION OF NICKEL RENDERS ANIMALS UNRESPONSIVENESS TO NICKEL

From the above discussion in sections 4.4 and 4.5, we can conclude that $NiCl_2$ alone can provide a minimal level of costimulation that is sufficient to prime nickel-specific T cells in $Ni^{very\ low}$ mice. Furthermore, when combined with adjuvant, it can also prime Ni^{low} mice. Therefore, why does the oral administration of nickel render animal's unresponsiveness to nickel? This somewhat contradictory ability of nickel will be addressed in the following sections but in short, a low dose oral uptake of nickel by Ni^{low} animals or humans induces incomplete tolerance whereas a high dose of nickel in Ni^{high} animals induces complete tolerance.

4.7.1 A low oral dose of nickel in adolescents or conventionally reared Ni^{low} animals induces incomplete tolerance to nickel

Epidemiological studies have shown that adolescents who have worn orthodontic braces are less likely to suffer from nickel allergy [173;174], even though the amount of nickel released from the braces is negligible when compared to the total daily uptake in food (20 ng/kg/d vs 2-4 µg/kg/d) [205-207]. In accordance with these findings I find that Ni^{low} mice develop Treg whereas $Ni^{very\ low}$ mice do not (section 3.3.2), although only source of oral uptake of nickel by Ni^{low} mice that extra to $Ni^{very\ low}$ mice comes from the metal lids and drinking water nozzles. Both studies indicate that the protective effects arise from "negligible" amounts of released nickel and the following explanations may account for this phenomenon [208].

First, in contrast to slow-released nickel from dental braces, the rapid ingestion of nickel in food does not allow for the constant exposure of nickel at the mucosal surfaces. Secondly, the anatomic site of nickel uptake might contribute to the greater tolerogenicity of nickel released from the dental braces. For example, food-bound nickel does not remain for extended periods in the oral cavity; it is absorbed via the intestinal mucosa. Nickel which is continuously released from dental braces is absorbed via the oral mucosa in the cheek pouches. In case of Ni^{low} mice, their continuous gnawing at the metal cage parts also exposes their oral mucosa in the cheek pouches to nickel. In hamsters, the cheek pouch is classified as an immune-privileged anatomic site [209], that means that antigen administrated at this location fails to sensitize, but induces a state of tolerance. Conceivably the same applies to humans and mouse cheek pouches. If we consider the oral mucosa in this respect, we can explain why a small amount of nickel entering the body via the oral mucosa can be more tolerogenic that a larger amount passing through the intestine.

4.7.2 Oral uptake high dose of nickel by Ni^{high} animals induce complete tolerance to nickel

Mice reared in the Ni^{low} environment are partially tolerant to nickel because they can still be sensitized with an injection of $NiCl_2/H_2O_2$. However, when Ni^{low} mice are given a high concentration of nickel in their drinking water (10 mM $NiCl_2$ for 4 weeks), Ni^{high}, they enter a state of complete tolerance to nickel(section 3.3.1).

As mentioned in the last section (4.7.1), the exposure of nickel at the oral mucosa is a main contributor in the induction of unresponsiveness to nickel in Ni^{low} mice. However, the intestinal mucosa may also play a role in the induction of complete tolerance to nickel since van Hoogstraten *et al*. could elicit Ni^{high} mice from $Ni^{very\ low}$ mice by intragastric feeding with Ni in drinking water [157]. A steady state of orally administered antigen induces tolerance [140]. In contrast to the immunity raised by the intradermal injections, the oral uptake of nickel favors the development of tolerance. Alongside the reasons summarized in section 1.7.4, we can also conclude that: 1) $NiCl_2$ alone only provides a certain level of costimulation; 2) there is no trauma associated with the oral uptake of nickel and therefore the costimulation induced by the immunization procedure will not occur; 3) Constant nickel exposure at the oral mucosa facilitates the induction of complete tolerance. As mentioned earlier the exposure of nickel at the oral mucosa in Ni^{low} mice leads to the generation of Treg. In this and some other studies [156], Ni^{high} mice were developed by giving nickel salts in the drinking water. These Ni^{high} mice were found to have a much higher concentration of nickel in their body fluids than Ni^{low} mice [177]. From this observation it is conceivable, that the nickel concentration in the saliva, which is in continuous contact with the oral mucosa, is also much higher in Ni^{high} mice. Apparently, this continuous exposure of nickel at the oral mucosa and the intestinal mucosa in Ni^{high} mice leads to the higher frequency and enhanced suppressive capacity of Treg.

4.7.3 Evidence to justify why an increased oral uptake of nickel produces enhance Treg activity

The findings in this study demonstrate that different oral doses of nickel generate populations of Treg with varying suppressive activities. This actually confirms the concept that there exists several levels of peripheral tolerance [211]. *In vitro*, anergic T cells can be induced through T-T presentation, and different anergic states can be achieved by giving different antigen doses in the culture. For instance, a low antigen dose induces hyporesponsiveness, an optimal dose allows the exertion of immunoregulatory effects and a supraoptimal dose antigen can elicit a persistent suppressive state [126]. *In vivo,* the level of peripheral tolerance depends not only on the antigen dose but in the animal model too. Even within a distinct model, the dose of antigen can produce different effects such. For example, Friedman *et al* showed that a high concentration of oral protein antigens induces anergy and increased IL-4 secretion, whereas a low dose generated suppressive T cells that secreted TGF-ß and IL-4 [130]. Another study concluded that tolerant T cells are susceptible to further tolerogenic signals and reach different levels of tolerance depending on the antigen dose [126;212]. When considering these other *in vivo* models, it is reasonable to assume that nickel-specific Treg, with different suppressive abilities, can be induced when animals are orally exposed to different levels of nickel.

4.8 NI-SPECIFIC TREG CELLS

In section 4.4, the possible Treg activity in Ni^{high} and Ni^{low} mice was discussed, the following section will provide a more detailed discussion on these nickel-specific Treg.

4.8.1 Evaluation of the effectiveness of the Treg cells of Ni^{high} and Ni^{low} mice

Two general conclusions can be drawn from the results presented in sections 3.3.1 to 3.3.4:

First, the higher the oral uptake of nickel, the stronger the suppressive effectiveness of Treg cells. This conclusion is based upon the following four observations:

(i) the splenic T cells of Ni^{high} mice were able to prevent the nickel-specific immune responses of both Ni^{low} and $Ni^{very\ low}$ mice, irrespective of the recipients' mode of immunization;

(ii) in contrast, the splenic T cells from Ni^{low} mice only suppressed the responsiveness of $Ni^{very\ low}$ recipients and, moreover, were only able to do so when the recipients were subsequently sensitized with $NiCl_2$ alone, but not with $NiCl_2/H_2O_2$;

(iii) T cells from $Ni^{very\ low}$ mice are unable to prevent the sensitization to nickel; and

(iv) the number of donor T cells that sufficed to preclude nickel hypersensitivity in the $Ni^{very\ low}$ recipients was much lower when the cells were obtained from Ni^{high} donors rather than Ni^{low} donors.

(v) Confirming the observations made *in vivo*, T cells from Ni^{high} mice and Ni^{low} mice were able to suppress *in vitro*, nickel-specific restimulation of responder T cells from $Ni^{very\ low}$ mice that had been primed with $NiCl_2$ alone. However, the suppressive effects of the two sources of Treg differed when they were asked to suppress the nickel-specific restimulation of T cells from $Ni^{very\ low}$ mice that had been immunized with $NiCl_2/H_2O_2$.

Second, the nickel-specific effector T cells of $Ni^{very\ low}$ mice sensitized with $NiCl_2/H_2O_2$ are more resistant to the suppression exerted by Treg cells than those of the same type of mouse sensitized with $NiCl_2$ alone. This conclusion in turn is based on the following three findings:

(i) Ni^{low} T cells were able to suppress the elicitation of nickel hypersensitivity in the $Ni^{very\ low}$ mice previously sensitized with $NiCl_2$ alone, but not in those previously sensitized with $NiCl_2/H_2O_2$;

(ii) in order to prevent the primary response to nickel of $Ni^{very\ low}$ mice, the number of Ni^{high} Treg cells required was higher when the priming was performed with $NiCl_2/H_2O_2$ than with $NiCl_2$ alone;

(iii) 10^7 nylon wool enriched-splenic T cells of Ni^{high} donors were able to completely suppress the secondary response to nickel of $Ni^{very\ low}$ mice, when the $Ni^{very\ low}$ recipients were immunized with $NiCl_2$ alone, but not when they were immunized with $NiCl_2/H_2O_2$.

4.8.2 Classification of nickel-specific Treg

A transfer of $CD4^+$ Treg from either Ni^{high} or Ni^{low} donors was sufficient to prevent hypersensitivity induced in $Ni^{very\ low}$ mice after immunization $NiCl_2$ alone. In contrast, both $CD4^+$ and $CD8^+$ Ni^{high} Treg were required to prevent the hypersensitivity induced after immunization with $NiCl_2/H_2O_2$ which confirmed our previous results obtained after the adoptive transfer of Ni^{high} Treg into Ni^{low} recipients (Figure 2 of ref. [177]). In other words, the tolerant state provided by $CD4^+$ Treg can be broken by vigorous immunization, whereas that provided by both $CD4^+$ and $CD8^+$ Treg cannot. As we shall discuss below, this conclusion can be adopted to other situations of 'incomplete' tolerance.

Murine and human $CD4^+$ Treg express a variety of additional surface markers, such as $CD5^{high}$, $CD45RB/RC^{low}$, and/ or $CD25^+$, which indicate that the cells have been primed [213]. Primed T cells have a reduced requirement for accessory cell costimulation, and in generally TCR signaling allows their differentiation to effector function [214]. The same principle applies for the activation of Treg. In the case of $CD4^+CD25^+$ Treg the specific antigen (signal I) is needed for their activation but in distinction to naïve Th cells, their requirement for costimulation (signal II) is lower or unnecessary [56;215-218]. Furthermore, even the strength of signal I (antigen dose) required for elicitation of $CD4^+CD25^+$ Treg is lower than that required for Th stimulation [56]. Hence, we may deduce that $CD4^+CD25^+$ Treg can be more easily activated than naive $CD4^+CD25^-$ Th cells. Activated $CD4^+CD25^+$ Treg may exert suppression by enhancing the activation threshold of Th cells [210]. Interestingly however, their suppressive activity can be overruled by a more vigorous signal I or signal II [54;219;220]. These concepts may explain why Ni^{low} mice can be immunized with $NiCl_2/ H_2O_2$ but not $NiCl_2$ alone. In the latter case, where a strong signal II is absent, the signal I provided by $NiCl_2$ would rapidly and successfully trigger the suppressor-effector function of $CD4^+$ Treg which would in turn enhance the activation threshold of nickel-specific effector T cells. When $NiCl_2/H_2O_2$ is injected a higher expression of CD80/CD86 on the APC is achieved [189], which overcomes the high threshold necessary for nickel-specific effector T cell activation and therefore leads to the successful sensitization of Ni^{low} mice. In other words, a vigorous signal II, in conjunction with signal I, is able to break the incomplete tolerance state of Ni^{low} mice.

The Treg found in Ni^{high} mice comprise of $CD8^+$ T cells [157;177] which require "help" of $CD4^+$ T cells in order to exert their suppressor activity (Figure 6 of ref. [177]). Synergism between suppressor-inducer $CD4^+$ T cells and suppressor-effector $CD8^+$ Treg cells was described repeatedly, however, the mechanism of their interaction remained obscure [221]. Recently, in the TNP tolerance model, Ferguson *et al.* [222] was able to induce tolerance in recipients, only after an adoptive transfer of $CD8^+$ Treg and TNP-specific $CD4^+$ T cells from the same donor. In our experiments, we have conclusively identified nickel-specific $CD8^+$ Treg in Ni^{high} mice (Figure 3.3.4.1 and Figure 6 of ref. [177]), indicating that in Ni^{high} mice, a sufficient number of nickel-induced neoantigens is presented to

$CD8^+$ T cells [223]. In order to be cross-presented by MHC-I molecules, extracellular antigen has to be present at a high concentration [35;224;225] suggesting that cross-presentation can occur more readily in Ni^{high} mice than in Ni^{low} mice.

Interestingly, the suppressive activity of $CD8^+$ Treg from Ni^{high} mice was only seen when: 1) the Ni^{low} and $Ni^{very\ low}$ recipients received a co-transfer of $CD4^+$ T cells from Ni^{high} donors and 2) the recipients were sensitized with $NiCl_2/H_2O_2$, but not $NiCl_2$ alone. These observations indicate that although $CD8^+$ Treg require the presence of $CD4^+$ T cells they do not need to be activated by $NiCl_2/H_2O_2$. In further experiments I showed that the suppression of *in vivo* secondary immune responses could be elicited by the activation of CD8 Treg by $NiCl_2$ alone (Figure 3.3.2.2). In these experiments, donor T cells from Ni^{high} mice were transferred into recipients which had been immunized with $NiCl_2/H_2O_2$ 10 days before (bar 5, Figure 3.3.2.2). The donor cells then protected the recipients from DTH responses elicited one day after transfer with $NiCl_2$ alone, confirming that Treg cells were activated by the re-challenge and not the priming. In addition, only the co-transfer of Ni^{high} donor $CD8^+$ and $CD4^+$ T cells can prevent the primary immune responses in $Ni^{very\ low}$ recipients that are immunized with $NiCl_2/H_2O_2$ (Figure 3.3.4.1).

The transfer $CD4^+$ Treg from Ni^{high} or Ni^{low} donors can suppress DTH responses in $Ni^{very\ low}$ mice sensitized with $NiCl_2$ alone (Figures 3.3.4.2A and B, respectively). However, the suppressive capacities of $CD4^+$ T cells from Ni^{high} and Ni^{low} mice are different: in contrast to a transfer Ni^{low} T cells (10^6), only 10^3 splenic T cells from Ni^{high} mice are necessary to suppress DTH responses (Figures 3.3.3C and 3.3.3A respectively). Indicating that the suppressive capacity of $CD4^+$ T cells from Ni^{high} mice are 1000 times higher than that of Ni^{low} mice. So far, it is undetermined whether the different suppressive capacities of these $CD4^+$ T cell populations are due to differences in numbers or the quality of Treg.

4.8.3 Comparisons of Ni-specific Treg cells in mice and in human

The characterization of nickel-specific Treg in humans has been well studied. *In vitro*, nickel-specific $CD4^+$ T cells from skin lesions of nickel allergic patients or PBMC from nickel-allergic and non-allergic individuals, were all able to suppress secondary immune response to nickel [178]. These $CD4^+$ Treg (Tr1 cells) produced high amounts of IL-10, variable amounts of TGF-β, no IL2 and low to undetectable levels of INF-γ. When co-cultured with nickel and DC, these Tr1 cells blocked the maturation and function of DC in a cell-cell contact-independent, but IL-10-dependent fashion. These DC then had an impaired capacity to activate specific Tc1 and Th1 effector cells for nickel or other antigens [178]. Furthermore, in contrast to $CD4^+CD25^+$ T cells obtained from non-allergic individuals, those obtained from allergic individuals readily proliferated in response to this agent and failed to suppress nickel-specific effector T cells and naive $CD45RA^+$ T cells to nickel [179]. Interestingly, whereas the $CD4^+CD25^+$ Treg from non-allergic individuals can suppress nickel allergy in a cell-cell contact-dependent manner [179], nickel-specific Tr1 cells regulate the allergic contact dermatitis (ACD) by releasing cytokines [178]. As reviewed in section 1.7.2, $CD4^+CD25^+$ Treg can infectiously spread tolerance to naïve $CD4^+$ T cells and turn these naïve T cells into Tr1 cells [90;91]. Recently,

$\alpha_4\beta_7$ intergrin-expressing $CD4^+CD25^+$ Treg proved to be able to induce IL-10 secreting Tr1 cells [76;226]. Therefore, in humans, the nickel-specific effector Treg are $CD4^+$ Treg (i.e. Tr1 and $CD4^+CD25^+$ Treg).

As discussed before, whereas $CD4^+$ T cells mice act as effector Treg in Ni^{low}, $CD4^+$ and/or $CD8^+$ Treg can be effector Treg in Ni^{high} mice. We assume that the difference is not due to a species difference between man and mouse, but rather nickel exposure. The concentration of nickel provided to Ni^{high} mice in the drinking water is about 10^6 times higher than that for humans, whereas the nickel uptake between humans and Ni^{low} mice are comparable [227]. If indeed the involvement of anergic $CD8^+$ and/or $CD4^+$ Treg in Ni^{high} mice were due to their higher oral uptake of nickel, $CD4^+CD25^+$ Treg and/or Tr1 cells would be the predominant suppressor-effector cells in Ni^{low} mice, as it was shown for humans by Cavani *et al.*

4.9 THE ROLE OF TOLEROGENIC APC IN NI^{HIGH} MICE

In Ni^{high} mice, Treg and tolerogenic APCs are generated. In comparison to Ni^{low} mice, splenic APCs from Ni^{high} mice exhibited an increase in DEC-205 expression on DC, an increase in CD38 expression on B cells, and a striking decrease in the expression of CD40 on DC and B cells (ref. [189], Figure 1). This tolerogenic phenotype of DC and B cells in the spleens of Ni^{high} mice conforms not only to the remarkable efficiency of their APCs to adoptively transfer the tolerance (Figure 3.4.2), but also with their reduced allostimulatory capacity (Figure 3.4.1). DEC-205 is mainly expressed by $CD8\alpha^+$ DCs in spleen [228] and this population of DC are associated with tolerance induction. In the absence of maturation stimuli *in vivo*, DC were shown to present Ag in a tolerogenic fashion to $CD4^+$ and $CD8^+$ T cells by targeting the DEC-205 receptor. Thereafter, the T cells underwent an abortive activation, and the few cells that survived appeared to be anergic [229-231]. Another marker, CD40, whose expression was reduced on APC in Ni^{high} mice, is constitutively expressed on B cells, macrophages, and DC. When it interacts with CD40 ligand (CD40L) on $CD4^+$ T cells, the APCs start to up-regulate the costimulatory activity and cytokine production required for priming and expansion of Ag-specific $CD4^+$ and $CD8^+$ T cells [232]. The CD40-CD40L interaction required for T cell priming has been noted for its importance in mouse models of contact hypersensitivity [233]. Generally, when the CD40-CD40L interaction is interrupted, the administration of Ag induces tolerance [234]. The spontaneous down-regulation of CD40, which we noted on the APC of Ni^{high} mice, is comparable with that described for other models of T cell unresponsiveness in mice and humans [235;236]. For instance, in an *in vitro* system with human PBMC [236], $CD8^+CD28^-$ Treg were found to inhibit CD40 upregulation on the APC which included B cells. $NiCl_2$, which fails to upregulate the costimulatory molecules CD80/CD86 in Ni^{low} mice [189] (Figure 7 of ref. [189]), apparently promotes the induction of tolerogenic, nickel-presenting APC. Consequently, the reduced costimulation of these APC after activation could restrict T cell priming towards nickel. When applied to the situation that develops upon oral administration of nickel it is conceivable that nickel-specific Treg are induced through nickel exposure without costimulation. Activation of these Treg by injection of $NiCl_2/H_2O_2$ might then prompt them to inhibit the upregulation of costimulatory signals [66;236;237] or induce co-inhibitory signals [238] on APC. Consistent with this, it has been noted recently that the injection of $NiCl_2/H_2O_2$

into orally tolerized mice fails to upregulate CD80/CD86 expression on the APC in the draining lymph nodes (M. Fang, unpublished result). The induction of oral tolerance towards nickel also results in a notable upregulation of CD38 expression on splenic B cells. CD38 is widely primarily expressed on B cells and T cells [239] and its ligation induces Ca^{2+} influxes and has been shown to enhance cell proliferation [239;240]. A $CD38^{+}$ T cell population with suppressive properties has been reported [241], but in our model an alteration in CD38 expression by T cells could not be detected. While the functional significance of the enhanced CD38 expression by B cells of Ni^{high} mice is unknown, this observation in consistent with the other findings reported here. All point to an active contribution by B cells to initiate and transfer tolerance towards nickel.

APC from Ni^{high} mice also proved capable of transferring nickel tolerance, and this was not simply a passive transfer of nickel but required intact cells that actively contributed to the induction of tolerance in the recipients (Figure 3 of ref. [189]). Nickel ions are haptens which differ qualitatively and quantitatively from conventional Ag in the following way; 1) they distribute ubiquitously within the body [177], 2) they do not need to be processed, 3) they do not enter into peptide competition for MHC binding sites because most nickel ion-induced neoantigens result from exogenous attachment of the metal ions to MHC molecules and those self-peptides are presented anyway [153;233]. Theoretically, each nickel ion could form a neoantigen so virtually all APC from Ni^{high} mice could carry those neoantigens. Therefore, both the number of tolerogenic APC and the density of nickel ion-induced neoantigens carried by them are probably much higher in tolerant hosts. The unusually low number of both bulk T cells and APC that are capable of transferring specific tolerance accentuates their enormous infectivity capacity and, hence, the dominance of T cell tolerance towards nickel. We are aware of only one other investigation, in which a comparatively small number of tolerogenic APC sufficed to induce tolerance. In that experiment, an injection of only 20 peritoneal exudate cells, which were treated *in vitro* with Ag and TGF-β, induced tolerance in the recipient mice [242]. Interestingly, these cells also showed a reduced CD40 expression [209].

Amongst the donor APC fraction, it was the B cells were primarily carried the tolerogenicity [189]. Previous studies have demonstrated that B cells from tolerant donor mice can induce Treg upon adoptive transfer [116;243]. For instance, in the ACAID (anterior chamber-associated immune deviation) model B cells were found to become tolerogenic through contact with tolerogenic macrophages. Even though those B cells were unable to directly suppress the development of DTH, they were capable of inducing specific Treg cells [116]. For this to occur, B cells were required to present the Ag, which they had acquired from the tolerogenic macrophages, in the context of Qa-1 [116]. Qa-1 is a MHC class Ib molecule known to guide the suppressive activity of $CD8^{+}$ T cells in a variety of different experimental models [244]. Also in the ACAID model, the eye-derived tolerogenic macrophages and splenic B cells in the marginal zone needed to express CD1 [115]; only then were CD1d-reactive NKT cells in the spleen sufficiently activated to produce IL-10 which, in turn, promoted the differentiation of specific Treg [245], [209]. It remains to be seen whether or not splenic B cells in orally-induced nickel tolerance are also rendered tolerogenic through contact with tolerogenic macrophages and/or DC which then subsequently induce Qa-1 restricted $CD8^{+}$ T cells. The similarity between the ACAID model and oral tolerance to nickel is further supported by the fact that in both models, mice which lack NKT cells, fail to become tolerant [209;246].

4.10 INFECTIOUS TOLERANCE IS A POWERFUL AMPLIFICATION MECHANISM

Here, the term infectious tolerance describes the ability of Ag-specific donor T cells to adoptively transfer tolerance to Ag-specific T cells [247]. In our nickel model, infectious tolerance was, indeed, found to account for the spread of tolerance from Ni^{high} donors to Ni^{low} recipients. The infectious tolerance pathway was found to follow a spread of tolerance from Ni^{high} $Ly5.1^+$ donor Treg to Ni^{low} $Ly5.2^+$ host APC, and, vice versa from tolerogenic $Ly5.1^+$ donor APC to $Ly5.2^+$ T cells of the Ni^{low} host (Figure 3.4.4). For the successful spread of tolerance Ni^{low} hosts had to be immunized with $NiCl_2/H_2O_2$ before transferring their cells to the second set of Ni^{low} recipients [189]. As far as Treg are concerned, *in vivo* experiments have shown these cells require activation before they can act as Treg effector cells [222;244]. In fact, prior immunization of animals is an essential element in all assay systems to detect functional Treg *in vivo* [116;243]. The requirement for immunization in order to spread the tolerance from Treg to APC *in vivo* has not been reported before. As far as the potentially tolerogenic Ni^{high} donor APC are concerned, there is only one other model that shows a requirement for recipient immunization in order for the tolerance to spread from APC to T cells [248;249]. However, in that model a Th2 response was suppressed and not a Th1 response, as in the present investigation. How in our model very small numbers of dormant, potentially tolerogenic APC (from the Ni^{high} $Ly5.1^+$ donors) succeeded to tolerize the naive T cells of the Ni^{low} recipients, if the recipients were deliberately immunized, needs to be unraveled. The phenomenon, however, is of general interest because it can explain how an otherwise immunizing maneuver may be converted into its contrary, tolerization. These observations might have implications, for instance, in attempts aiming at immunotherapy of tumors.

Our concept that nickel-specific Treg need APC as intermediate cells, in order to confer suppressive activity on new cohorts of T cells in the recipients, was further substantiated by the following observation. While one-time immunization of recipient mice sufficed to spread the tolerance from donor T cells to host APC and, vice versa, the tolerance spread from donor T cells to host T cells or from donor APC to host APC, actually required two immunizations of the recipients (Figure 9 of [189]). This observation suggests that the tolerance spread from T cells to T cells or from APC to APC, did not simply occur via direct cell-cell contact, but first required ‘infection’ of the respective opposite cell type, i.e., APC in the former case and T cells in the latter. Recently, the existence of an inhibitory feedback loop between tolerogenic APC and regulatory T cells *in vivo* has been suggested by Wei-Ping *et al.* [250]. However, although these authors induced transplantation tolerance *in vivo*, the capacity of the regulatory T cells and tolerogenic APC to infectiously spread the tolerance to the opposite cell type was restricted to *in vitro* experiments. Moreover, since T cell responsiveness to MHC alloantigens were studied in their system, it was not possible to experimentally dissect signal I from signal II and, hence, to demonstrate a requirement for costimulation for the spread of tolerance. In the *in vitro* models of infectious tolerance studied by Dieckmann *et al.* [91] and Jonuleit *et al.* [90], human $CD4^+CD25^+$ Treg required pre-activation (provided in an Ag-nonspecific manner) to enable them to spread the tolerance through cell-cell contact. However, in contrast to those *in vitro* studies, for the different Ag-reactive T cells to meet *in vivo* and specifically suppress or be suppressed, APC are apparently needed to act as bridges and, as discussed before, also act as mediators of suppression.

Collectively, our results demonstrate that infectious tolerance *in vivo* involves a reciprocal interplay of specific Treg and tolerogenic APC that are driven by immunization. With regard to the consequences of immunization, however, we noted a striking difference between the induction phase of T cell suppression (which can be defined as the time period of oral nickel treatment) and the effector phase (defined here as the time period following adoptive cell transfer and the subsequent immunization of the recipients). Prior to, or early on in the induction phase, Ag administration together with enhanced costimulation, as inducible by H_2O_2, would obviate the tolerization. The opposite effect is achieved, when Ag and a source of 'danger' [251], such as H_2O_2, intrude into an immune system that harbors a few anergic Treg cells or tolerogenic DC and B cells: in the effector phase of suppression, immunization with $NiCl_2/H_2O_2$ leads to a dramatic spread of tolerance. Thus, once Treg and tolerogenic APC are induced by oral administration of nickel, the tolerance becomes self-enhancing and self-maintaining when the Treg and tolerogenic APC are exposed to Ag in the presence of danger. Under these conditions tolerogenic APC and Treg effector cells can engage naive T cells and normal APC into the tolerization process so that unresponsiveness prevailed.

4.11 CORRELATIONS BETWEEN MICE AND MEN

In this study, we noted an inverse correlation between the amount of nickel taken up orally and the susceptibility to be sensitized to nickel. The underlying mechanism here can be seen as a direct correlation between the oral nickel load and the suppressive strength or number of nickel-specific Treg.

By analogy to the observations made in mice, oral exposure of humans to different concentrations of nickel might also lead to different levels of tolerance, ranging from complete, partial and none. The existence of nickel-specific Treg in non-allergic humans [179] indicates that their immune system is not just indifferent or naive towards nickel, but, instead, responds to this ubiquitous environmental agent by generating Treg. As a corollary, this implies that the bodily concentration of nickel, which is engulfed and absorbed from food and beverages, is high enough to be perceived by human T cells and to prime them to act as Treg.

With regard to the daily amount of nickel per kg body weight, which is taken up from food and drinking water, there are hardly any differences between human and the Ni^{low} mice [206;207;227]. Therefore, it is conceivable that there is also no difference between non-allergic humans and the Ni^{low} mice with regard to their Treg. Both Ni^{low} mice and non-allergic humans possess certain nickel-specific Treg and, as a consequence, fail to be sensitized by dermal exposure to Ni^{2+} ions *alone*. At least the Ni^{low} mice, however, are still susceptible to sensitization when they encounter nickel in the context of 'danger' [153;177;189;246]. Currently, it appears that the nickel-specific Treg in most non-allergic humans are inferior to those of the Ni^{high} mice as far as number and/or suppressive strength is concerned. This presumably explains the susceptibility of humans to sensitization when the nickel exposure occurs in the context of 'danger', such as ear-piercing or an irritated skin. If the oral nickel

uptake of humans were as low as that of our $Ni^{very\ low}$ mice, they probably would lack nickel-specific Treg and, as a consequence, the incidence of human nickel allergy would be presumably much higher.

We hypothesize that the lower the oral uptake of nickel, the lower the suppressive capacity of Treg and, hence, the easier it is to sensitize to nickel. If this hypothesis can be corroborated by the results of future experiments, it would have direct consequences concerning the way by which environmental nickel concentrations should be regulated by the authorities. Although reducing the nickel concentration in items of frequent *dermal* contact has been shown to lower the incidence of *de novo* sensitization to nickel in humans [252], a reduction of maximal threshold concentrations of nickel in the drinking water and/or food might actually be counter-productive in that it would increase, rather than decrease, the number of humans sensitized to nickel (albeit beneficial for individuals already sensitized and suffering from severe nickel allergy). Supposing that the oral nickel intake of humans were reduced to the level of the $Ni^{very\ low}$ mice, humans presumably would be sensitized even when exposed to Ni^{2+} ions in the absence of 'danger'. In other words, if the oral nickel intake of humans were reduced, we should expect an increased incidence of nickel allergy.

Another interesting finding in this study is nickel-specific T cells which can be primed *in vitro* from "non-Ni" antigen-primed LNC. This may explain why LTT with human PBMC can not be used in clinic diagnosis. Lisby *et al* found that human PBMC from both patch test positive and negative people proliferate *in vitro* to Ni [160;168]. This is not surprising, because humans are not only vaccinated but also possibly infected with different microbes. These vaccinations or infections could modify both T cells and APCs, and facilitate bystander T cells be primed with Ni. During inflammation, this *in vitro* priming is even easier to establish. So, the proliferation detected when non-allergic individuals are cultured *in vitro* with nickel represents secondary immune response. Of course, during inflammation, due to the change of costimulatory molecules and/or other accessory molecules, the requirement of antigen (here, Ni-neoantigen) concentration for priming antigen-specific T cells is reduced [253-255]. Simultaneously, inflammation provides an environment that makes Ni ion release easier from a variety of items [256]. Therefore, the priming of nickel-specific T cells is easier in irradiated skin compared to normal skin. *In vivo* however, exposure to nickel at such a high concentration does not often occur, therefore there are far less patch test positive individuals than *in vitro* LTT positive individuals.

4.12 CONCLUSIVE MARK.

In 1991, an effort to reduce the high sensitization rates involved the introduction of legislations in 1991 in Denmark to limit nickel release from items intended for direct and prolonged contact with the human skin, for example, jewelry, piercing materials and watches. The European Union instituted similar law in 2000. These laws have significantly decreased the incidence of newly sensitized nickel allergic individuals [252]. However, there is controversial epidemiological evidence concerning the exposure conditions that favor the induction of nickel-specific Treg and also nickel tolerance. Although the reduction of dermal nickel exposure has decreased the incidence of newly sensitized nickel allergic individuals [252], other epidemiological observations have suggested the opposite,

namely a reduced incidence of nickel hypersensitivity due to an increased environmental exposure to this potential sensitizer [257] .

By analyzing the effects of different nickel-exposed breeding conditions we have been able to investigate in the mouse, the relationship of exposure conditions to nickel-allergy and nickel-induced tolerance as well as the mechanisms. This was achieved by focusing upon the following points: a relationship between oral nickel uptake and the susceptibility to become sensitized to nickel (4.1-4.4); *in vitro* priming of nickel-specific T cells (4.5); the mechanisms of oral administration of nickel to render mice tolerance (4.7); nickel-specific Treg induction (4.8); the tolerogenic APCs in Ni^{high} mice (4.9); and infectious tolerance as an amplification mechanism in nickel tolerance (4.10). This is the first time that $Ni^{very\ low}$ mice have been reported to be primed by Ni ions alone, and also the first direct evidence that Treg exist in Ni^{low} mice.

5 APPENDIX A: EQUIPMENT AND MATERIALS

Micrometer gauge (Oditest D 1000 gauge)	The Dyer company, Lancaster, PA, USA
Neubauser-Kammer heamocytometer	Germany
DAIPLAN, light microscope	Leitz, Germany
MEGAFUGE, centrifuge	Kendro Laboratory Products GmbH, Rodenbach, Germany
Cytoperm incubators	Heraues, Germany
Sterile flow benchs	Gelaire flow laboratories GmbH, Meckenheim, Germany
MACS, magnetic cell sorters and columns	Miltenyi Biotec, Bergisch Gladbach, Germany
AutoMACS, magnetic cell sorter and columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Gammacell 2000 cell irradiator	Molsgaard Medical, Denmark
FACScalibur	Becton Dickson, Germany
96 well plate ELISA reader	Dynex Technologies, Germany
PHD Cell Harverster Model 200A/290	Cambridge Technology, MA, USA
Ready Filters with Xtalscint	Beckman coulter, Fullerton, CA
Liquid Scintillation System, Model: LS 6000 IC	Beckman Instruments, CA, USA
Inotech Sample Harvesting System	Inotech, Dottikon, Switzerland
Printed filtermat A	Wallac Oy, Turku, Finland
Meltilex^{TM} AA, Scintillator Sheets	PerkinElmer life sciences, Wallac Oy, Turku, Finland
96-well Nunc Maxisorb ELISA plates	Nunc GmbH & Co. KG, Wiesbaden, Germany
1450 MicroBeta TriLux Liquid Scintillation and Luminescence Counter	Wallac Oy, Turku, Finland
pH meter	Beckman, CA, USA
Heat block	Germany
-80^{o}C freezers	Peter Oehmen GmbH, Essen, Germany
ONCE syringes	Fabricante: Becton Dickinson C.A., Madrid, Spain
Microlance needles, variety of gauges	Becton Dickinson & Co. Ltd., Co Louth, Ireland

Nylon Wool (Typ 200 L)	Robbins Scientific Corp., Sunnyvale, CA, USA
Pipette	Eppendorf-Netheder-Hinz GmbH, Hamburg, Germany

6 APPENDIX B:

$NiCl_2 \cdot 6H_2O$	Sigma-Aldrich Chemie, Steinheim, Germany
DNFB	Sigma-Aldrich Chemie, Steinheim, Germany
DNBS	ICN Biomedicals Inc., Aurora, OH, USA
FITC	Sigma-Aldrich Chemie, Steinheim, Germany
Acetone	E. Merck, Darmstadt, Germany
Oliver Oil	Sigma diagnostics, St. Louis, MO, USA
Dibutylpthalate	Sigma-Aldrich Chemie, Steinheim, Germany
30% H_2O_2	E. Merck, Darmstadt, Germany
DMSO	Sigma-Aldrich Chemie, Steinheim, Germany
Concavalin A	Sigma-Aldrich Chemie, Steinheim, Germany
H_2SO_4	E. Merck, Darmstadt, Germany
PEG 1500	Boehringer, Freiburg, Germany
Streptavidin-Horseradish Peroxidase (AV-HRP)	Amersham Pharmacia Biotech, Freiburg, Germany
FITC-labed Streptavidin	BD Biosciences, Heidelberg, Germany
TMB plus, ready to use	Kem-EN-Tec A/S, Denmark
Fetal Calf Serum (FCS)	Sigma-Aldrich Chemie, Steinheim, Germany
BSA	SERVA Electrophoresis GmbH, Heidelberg, Germany
HAT	PAA Laboratories GmbH, Linz, Austria
HT	PAA Laboratories GmbH, Linz, Austria
RMPI 1640 medium	PAA Laboratories GmbH, Linz, Austria
DMEM medium	PAA Laboratories GmbH, Linz, Austria
Penicillin/Streptomycin	PAA Laboratories GmbH, Linz, Austria
0.9% Saline	Fresenius Kabi Deutschland GmbH, Homburg, Germany
Commercial PBS	Sigma-Aldrich Chemie, Steinheim, Germany
Glutamine (100x)	PAA Laboratories GmbH, Linz, Austria
Sodium pyruvate (100 mM)	PAN Biotech GmbH, Aidenbach, Germany
Non essential amino-acids MEM (100x)	PAN Biotech GmbH, Aidenbach, Germany
Essential amino-acids (50x)	PAA Laboratories GmbH, Linz, Austria
$NaHCO_3$	E. Merck, Darmstadt, Germany
ß-Mercapto-ethanol	Sigma Chemical Company, St. Louis, MO, USA
Ficoll Plaque	Pharmacia, Freiburg, Germany
Ethanol	J.T.Baker, Germany
NaCl	E. Merck, Darmstadt, Germany

KCl	E. Merck, Darmstadt, Germany
Tryptan blue	Sigma-Aldrich Chemie, Taufkirchen, Germany
EDTA	SERVA Electrophoresis GmbH, Heidelberg, Germany
Tris	SERVA Electrophoresis GmbH, Heidelberg, Germany
NH_4Cl	E. Merck, Darmstadt, Germany
Na_2HPO_4	E. Merck, Darmstadt, Germany
Na $H_2PO_4 \cdot 2H2O$	E. Merck, Darmstadt, Germany
KH_2PO_4	E. Merck, Darmstadt, Germany
Gentamycinsulphate	ICN Biomedicals Inc., Aurora, OH, USA
[^{3}H] thymidine	ICN Biomedicals Inc., Aurora, OH, USA
Tween-20	Merck-Schuchardt, Hohenbrunn, Germany
NaN_3	Sigma-Aldrich Chemie, Steinheim, Germany

7 APPENDIX C

7.1 FETAL CALF SERUM (FCS)

After heated at 56°C for 1 hour, the FCS (Sigma) was aliquotted to 25 ml or 50 ml and stored at -20°C.

7.2 PENICILLIN AND STREPTOMYCIN (PEN/STREP)

After thawing, Pen/Strep (10000U/10mg/ml) were aliquotted into 2.5 ml portion and stored at -20°C until required. One 2.5 ml aliquot was to add to 500 ml of medium to provide 50 IU/ml penicillin and streptomycin.

7.3 PEN/STREP AND ß-MERCAPTO-ETHANOL (PEN/STREP/ß-ME)

After thawing 100 ml Penicillin/Streptomycin (10000U/10mg/ml) and supplemented with 80 µl ß-mercapto-ethanol, the above solution were aliquotted into 2.5 ml portion and stored at -20°C until required. One 2.5 ml aliquot was to add to 500 ml of medium to provide 50 IU/ml penicillin and streptomycin and 50 µM ß-mercapto-ethanol.

7.4 MEDIUMS AND THEIR SUPPLEMENTS

7.4.1 *SC and "SC medium*"

SC was prepared as following

100 ml 0.2M Glutamine (x100)
10 ml Penicillin/Streptomycin (10000U/ml)
100 ml 0.1 M Sodium pyruvate
100 ml non essential amino-acids MEM (x100)
100 ml RMPI 1640
50 µl ß-Mercapto-ethanol

After sterile filtration, the above solution was aliquotted in 21 ml portion and frozen at -20°C until required.

"SC medium" was then prepared by adding one aliquot of SC, one aliquot of Penicillin/Streptomycin and one aliquot of FCS (50 ml for 10% FCS; 25 ml for 5% FCS. In this study, only 10% FCS was used) to 500 ml naked RMPI 1640 medium.

7.4.2 "*DMEM medium*"

"DMEM medium" war prepared by supplementing 500 ml naked DMEM medium with one aliquot of FCS (50 ml) and one aliquot of Pen/Strep/ß-Me.

7.4.3 *TC and "TC medium"*

TC was prepared as following

500 ml RPMI 1640
59.5 ml essential amino acids(x50)
29.8 ml non essential amino-acids MEM (x100)
79.4 ml 0.1 M Sodium pyruvate
6.7g $NaHCO_3$
7.9 ml gentamycinsulphate
7.9 ml H_2O
27 µl ß-Mercapto-ethanol

After sterile filtration, the above solution was aliquotted in 48 ml portion and frozen at -20^{o}C until required.

"TC medium" was then prepared by adding one aliquot of TC, one aliquot of Penicillin/Streptomycin and one aliquot of FCS (50 ml for 10% FCS; 25 ml for 5% FCS. In this study, only 5% FCS was used) to 500 ml naked RMPI 1640 medium.

7.4.4 "*HAT medium"*

500 ml "TC medium" was supplemented with an aliquot of sterile HAT (50x) so that the "HAT medium" contained 100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine.

7.4.5 "*HT medium*"

500 ml "TC medium" was supplemented with an aliquot of sterile HAT (50x) so that the "HT medium" contained 100 µM hypoxanthine and 16 µM thymidine.

8 APPENDIX D

8.1 PBS (10X)

80g	NaCl
2g	KCl
11.5g	$Na_2HPO_4 \cdot 2H_2O$
2g	KH_2PO_4

Dissolved in 1L water and adjust pH to 7.4, then diluted it in 1:10 with distilled water and sterilely filtered through a 0.2µM filter.

8.2 FREEZING BUFFER

10% DMSO in FCS

8.3 FACS BUFFER(10X):

391.5g NaCl
14g KCl
13g KH_2PO_4
178g $Na_2HPO_4 \cdot 2H_2O$

Dissolved in 5 Liter distilled water. Before use, 1:10 diluted with distilled water and added 0.36g EDTA/liter and then filtered it with 0.2 µl filter.

8.4 ACT SOLUTION

2.06g	Tris(Base)
8.55g	NH_4Cl

Dissolved in 600 ml water and adjust pH to 7.2.
Added distilled water to 1 liter, filter sterilize through a 0.2µM filter.

8.5 TRYPTAN BLUE SOLUTION

Stock solutions were diluted with sterile PBS
50 ml: 31.25 ml Tryptan blue
18.75 ml PBS

8.6 BUFFER AND SOLUTIONS FOR ELISA:

8.6.1 *Binding solution*

0.1M Na_2HPO_4, adjust pH to 9.0 with 0.1M NaH_2PO_4.

8.6.2 *PBS/tween*

0.5ml of tween-20 in 1 L PBS

8.6.3 *Blocking Buffer*

10%FCS or 1% BSA in PBS

8.6.4 *Blocking Buffer/tween*

Adding 0.5ml tween-20 to 1 L blocking buffer.

8.7 BUFFER FOR SEPARATING CELLS BY MACS:

8.7.1 *Rinsing Buffer (only be used in autoMACS*)

2 mM EDTA dissolved in PBS, sterilized by passing through a 0.2 µl filter.

8.7.2 *Running Buffer (for the cell sorting by all MACS sorter*)

Adding sterile FCS to Rinsing buffer, made the final concentration of FCS 1%. Otherwise, 2 mM EDTA and 0.5% BSA dissolved in PBS, sterilized by passing through a 0.2 µl filter.

8.7.3 *Cleaning Buffer (only be used in autoMACS*)

70% ethanol.

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Hiermit erkläre ich an Eidesstatt, dass ich die vorliegende Arbeit mit dem Titel “Contact hypersensitivity to nickel: experimental induction of allergy and tolerance, respectively“ selbständig angefertigt und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe.

Ich habe die vorgelegte Dissertation weder in dieser noch in ähnlicher Form bei einer anderen Fakultät eingereicht

Xianzhu Wu

Düsseldorf, im Februar 2005

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