Dendritic cells and dietary antigens

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Abbreviations

ANOVA	Analysis of variances
AP	Alkaline phosphatase
AP-1	Activating protein-1
APC	Antigen-presenting cell
APC	Allophycocyanin
APS	Ammoniumpersulfate
ATP	Adenosine triphosphate
BB	Bio-Breeding
BMDC	Bone-marrow-derived dendritic cell
CD	Celiac disease
CP	Cryptopatch
α-CT	α-Chymotrypsin
CTL	Cytotoxic T lymphocytes
CTLA	Cytotoxic-T-lymphocyte-associated antigen
DC	Dendritic cell(s)
DC-SIGN	DC-specific ICAM-3 grabbing nonintegrin
ELISA	Enzyme-linked immunosorbent assay
ERK 1/2	Extracellular signal-regulated kinases
EU	Endotoxin units
Fig.	Figure
FACS	Fluorescence-activated cells sorter
FAE	Follicle-associated epithelium
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Flt3L	Fms-like tyrosine kinase 3 ligand
Fox	Forkhead box
GAD	Glutamic acid decarboxylase
GALT	Gut-associated lymphoid tissue
Gln	Glutamine

GM-CSF	Granulocyte macrophage colony stimulating factor
HC	Hydrolyzed casein
HEPES	N-(2-Hydroxyethyl)piperazine-N`-2-erhane sulfonic acid
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSP	Heat shock protein
HMW	High molecular weight
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase
IEL	Intraepithelial lymphocytes
IENK	Intraepithelial natural killer cells
IFN	Interferon
Ig	Immunoglobulin
IKDC	Interferon-producing killer dendritic cell
IL	Interleukin
IL-1R	Interleukin-1 receptor
ILFs	Isolated lymphoid follicles
JNK	c-Jun NH_2-terminal kinase
KC	Keratinocyte-derived cytokine
LAL	Limulus amebocyte lysate
LAP	Latency-associated peptide
LMW	Low molecular weight
LN	Lymph node
LP	Lamina propria
LPS	Lipopolysaccharide
MALP	Macrophage-activating lipopeptide
MALT	Mucosal-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MIP	Macrophage inflammatory protein
Mkk3	Mitogen-activated protein kinase kinase 3
MLN	Mesenteric lymph node
MAdCAM	Mucosal addressin cell adhesion molecule
MCP	Monocyte chemoattractant protein
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor kappa B
NOD	Non-obese diabetic
NOS	Nitric oxide synthetase
pNA	p-Nitroaniline
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PE	Phycoerythrin
PEP	Prolyl endopeptidase
PmB	Polymyxin B
PP	Peyer's patch
Pro	Proline
PRR	Pattern recognition receptor
RANTES	Regulated on activation, normal T cell expressed and secreted
SDS	Sodium dodecylsulfate
SED	Subepithelial dome
TBS	Tris buffered saline
TBST	TBS/Tween20
TCA	Trichloracetate
T1DM	Type 1 diabetes mellitus
TEMED	N,N,N',N' -Tetramethylethylendiamine
TECK	Thymus-expressed chemokine
tTG	Tissue transglutaminase
TGF	Transforming growth factor

TLR	Toll-like receptor
TMB	3,3’,5,5’-Tetramethylbenzidine
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoeitin
VCAM	Vascular cell adhesion molecule
WF	Wistar-Furth
WG	Wheat gluten

1 INTRODUCTION

1.1 Gut immune system

Striking feature of the mammalian gut immune system is the ability to distinguish between harmful and non-pathogenic flora and to be tolerant to a great variety of food antigens. Mammals and other vertebrates successfully coexist with a diverse and abundant non-pathogenic intestinal microflora: the lower intestine of mammals contains an enormous load of commensal bacteria with a density of 10^{12} organisms per ml of luminal content that are representative of about 1000 species, mostly anaerobes (Mackie, R. et al., 1999; Macpherson, A.J. et al., 2005). The intestine is the biggest lymphoid tissue in the body. There are 10^{12} lymphoid cells per meter of human small intestine, and the number of immunoglobulin (Ig)-secreting cells in murine or human gut exceeds by several fold their amount found in all other lymphoid organs together (Mestecky, J., et. al., 1987; van der Heidjen, P.J., et al., 1987).

Gastrointestinal associated lymphoid tissue referred to as GALT for Gut-Associated Lymphoid Tissue, or MALT for Mucosa-Associated Lymphoid Tissue, can be divided into loosely organized effector sites, i.e. the layer of connective tissue in a mucosa immediately beneath the epithelium called the lamina propria (LP) and intraepithelial lymphocytes (IEL), and more organized structures - mesenteric lymph nodes (MLNs), Peyer's patches (PPs), isolated lymphoid follicles (ILFs) and cryptopatches (CPs) (Newberry, R.D., 2005).

The surface area of intestinal mucosa exceeds in several folds the skin area and is estimated to be 300 m^2 in the human small intestine (Moog, F., 1981). Such a large mucosal surface area in the small intestine is generated by means of evagination into plicae and villi and short tubular invaginations, the crypts. Plicae and villi help to increase gut contact with luminal content, and the crypts provide a protected site for stem cells that are located along the crypt length. The intestinal mucosa is a major immunological surveillance site, and all cell types required for antigen-presenting cell (APC)/T cell interactions are found here. APC are defined as highly specialized cells that can process antigens and display their peptide fragments on the cell surface together with molecules required for T cell activation (Janeway, C.A.Jr., et al., 2001). The immune cells that can express both major histocompatibility complex (MHC) class I and class II

molecules and co-stimulatory cell-surface molecules, capable of activating both $CD8^+$ and $CD4^+$ T cells, such as macrophages, peripheral blood dendritic cells (DCs), splenic DCs, B cells and epidermal Langerhans cells are specified as 'professional APC' (Makala, L.H.C., et al., 2004).

CPs are suggested to be a primary lymphoid organ, participating in extrathymic development of intraepithelial αβ and γδ T lymphocytes. CPs are clusters of approximately 1000 cells located at the base of the intestinal epithelial crypt and randomly distributed throughout the small intestine and colon, where the number of clusters is approximately 1500 and 150, respectively (Newberry, R.D. et al., 2005). Three types of cells are mainly present in CPs: lineage marker negative (lin^-)c-kit^+ cells, $CD11c^+$ DCs and vascular cell adhesion molecule (VCAM)-1^+ stromal cells. There are no mature T and B lymphocytes in CPs or they comprise < 2 %.

PP is the secondary lymphoid organ in the intestine that appears morphologically as a cluster of three or more large lymphoid aggregates with an overlaying follicle-associated epithelium (FAE). In mice PPs are found on the antimesenteric border along the entire length of the small intestine, whereas in humans PPs are mainly located in the ileum (Newberry, R.D. et al., 2005). PPs are composed of B-2 B cell follicles and interving T-cell areas that are separated from the intestinal lumen by a subepithelial dome region and the intestinal epithelium. This lymphoid organ is not served by afferent lymphatics. Specialized layer of epithelial cells, FAE, controls antigen sampling. This FAE is deficient in mucus-secreting goblet cells and anti-bacterial proteins-secreting Paneth cells, has more cuboidal enterocytes, has lower levels of digestive hydrolase enzymes, has decreased brush border enzymes and a very specialized cell type known as microfold (M) cells (Newberry, R.D. et al., 2005). Being typical epithelial cells, M cells orient along the basal lamina, adhere to adjacent enterocytes by apical tight junctions, display polarity with microvilli on their luminal surface, and have basally situated nuclei. M cells can be also seen as discreet clusters in intestinal villi (Newberry, R.D. et al., 2005). Luminal microorganisms and macromolecules are transported across M cells into the underlying lymphoid follicles for initiation of the appropriate immune responses. It is believed that DCs play an important role in the induction of PP immune responses. The

subepithelial dome (SED) region, which is located immediately under the FAE and overlies the lymphoid follicle, contains many DCs with the majority being described as $CD11b^{-}CD8^{-}CD11c^{+}$ (Iwasaki, A, et al., 2000). These DCs capture antigens and migrate directly to the adjacent PP interfollicular T-cell zone, or they may migrate via the draining lymph nodes (LNs) to MLNs, where they can react with naïve T lymphocytes. They then move to the bloodstream via the thoracic duct and subsequently migrate back into the intestinal mucosa. The migration of cells through the draining MLNs and back to the lamina propria of the intestine is dependent on the ligand/receptor pair α4β7/mucosal addressin cell adhesion molecule-1(MAdCAM-1) and CCL25/CCR9 (Berlin, C., et al., 1993; Pabst, O., et al., 2004; Newberry, R.D. et al., 2005).

LNs are highly organized lymphoid organs similar in construction to the spleen. Unlike the spleen they do not contain erythrocytes. The cellular composition comprises T and B lymphocytes and APC, macrophages and DCs, also called veiled cells. Other cell types have restricted access to LNs. B lymphocytes are mainly localized in the periphery of a node, in outer cortex, in a tightly packed nodules called follicles. Some of the B-cell follicles include germinal centers, where B cells undergo intense proliferation after antigenic challenge. The deep cortex, or paracortical area, interfollicular T-cell zone, made up mainly of more diffusely distributed T cells and DCs. The inner part of the lymph node, the medulla, consists of strings of macrophages and antibody-secreting plasma cells known as medullary cord. Via the afferent lymphatics antigens in phagocytic DCs and macrophages enter lymph node (Janeway, C.A., et al., 2001). Via efferent lymphatics in the medulla T cytotoxic and helper cells, and also many APC and some B cells leave the lymph node to perform their functions outside the node after antigen challenge.

MLNs are the largest LNs in the body. They are the first LNs that are developed during embryogenesis (day 10.5-15.5 in mouse).

ILFs are tertiary lymphoid structures with still unclear function. ILFs are 100-200 clusters of lymphocytes predominantly located throughout the length of small intestine. The cells there are mainly B-2 B lymphocytes ($CD23^{+}IgM^{low}\ IgD^{high}\ CD5^{-}CD11b^{-}$, up to 70 %), $CD11c^{+}$ DCs (~ 10 %), $TCR\alpha\beta^{+}\ CD4^{+}$ (~ 10 %) and $TCR\alpha\beta^{+}\ CD8^{+}$ (~ 3 %) T

lymphocytes and c-kit$^+$IL-7R$^+$ cells (~ 15 %) (Newberry, R.D. et al., 2005). ILF formation is suggested to be driven by innate immune response to products present in the intestinal flora. ILFs act as inductive sites for mucosal immune response, i.e. for the generation of IgA responses directed against luminal antigens.

1.2 Dendritic cells

DCs are the most potent APC that have a unique plasticity in the immune system. Being true professional APC, DCs are capable of activating not only memory, but also naïve T cells – a property that is not shared by all APC.

DCs were first identified by Langerhans in 1868 in the epidermis, and were termed Langerhans cells (Langerhans, P., 1868). In 1973 Steinman and Cohn found cells with distinct morphological features in mouse peripheral lymphoid organs (spleen, lymph node and Peyer's patch) and proposed the term 'dendritic cells' (Steinman, R.M., and Z.A. Cohn, 1973). The early 1990s, when it became possible to generate a large number DCs *in vitro* from hematopoietic progenitors, opened a new area in DC research.

In situ, as in the skin and lymphoid organs, immature and mature DC have a stellate shape. After isolation DC display many thin long dendrites (>10 μm) of spiny or sheet-like form. When observed *in vivo*, DCs extend large, delicate processes or veils in many directions from the cell body (Banchereau, J., et al., 1998; Bell, D., et al., 1999). There are three stages of DC development: precursor DC, immature DC and mature DC. DC precursors originate from CD34$^+$ bone marrow progenitors and circulate in the blood as MHC class II$^+$CD11c$^+$ or MHC class II$^+$CD11c$^-$ monocytes. These precursors migrate into tissues to become resident immature antigen-capturing DC. Immature DCs are found within almost all tissues (with possible exception of brain and testis), they are ready to capture antigens, but not yet properly equipped with accessory molecules for potent T cell stimulation. After immature DCs encounter a powerful immunological stimulus, they undergo maturation during migration to secondary lymphoid organs in search for antigen-specific T cells. Migration is mediated by the alteration in the expression pattern of chemokine receptors on DC. DC maturation results in several phenotypical changes that increase the ability to process antigen and activate naïve CD4$^+$ and CD8$^+$ T cells, i.e.

enhanced production of MHC-peptide complexes, increased expression of T cell binding and co-stimulatory molecules, such as CD40, CD80 and CD86, and *de novo* production of growth factors such as interleukin (IL)-2, thiols, chemokines and cytokines (Steinman, R.M., et al., 2003). DCs provide the naïve T cells with two signals required for their activation: the first signal is the antigen-specific signal received as a result of binding of the T cell receptor to peptide presented by MHC molecule; the second signal is provided by costimulatory molecules, such as B7-1 (CD80) and B7-2 (CD86) on DC that trigger CD28 expressed on naïve T cells. Importantly, DCs within lymphoid tissues are able to form MHC-peptide complexes in the steady state without the administration of maturation stimuli. After naïve T cells meet such a DC, they repeatedly divide, but are then deleted, and the animal becomes tolerant to the antigen. In contrast, if the antigen is coadministered with maturation stimuli, immunity develops (Steinman, R.M., et al., 2003).

Immature DCs are characterized by the high level of intracellular MHC class II-rich compartments (MIICs), endocytosis and FcR , low expression of CD 40, 54, 58, 80, 83, 86, p55, IL-12, and high CCR1, CCR5, CCR6 and low CCR7 expression. In contrast, mature DCs have high level of surface MHC class II molecule, low endocytosis and FcR, high expression of CD 40, 54, 58, 80, 83, 86, p55, IL-12, and low CCR1, CCR5, CCR6 and high CCR7 levels (Banchereau, J., et al., 1998; Bell, D., et al., 1999). Immature DCs can internalize a broad spectrum of antigens by different mechanisms. Thus, by phagocytosis they can take up particles and microbes; macropinocytosis enables DC to take up a very large volume of fluid (half the cell's volume per hour); absorptive endocytosis is mediated by C-type lectin receptors like the macrophage mannose receptor and DEC-205, as well as Fc receptors for immunoglobulins Fcγ or Fcε. Macropinocytosis and the use of receptors allow DC effectively present antigens in picomolar and nanomolar concentrations, whereas for other APC micromolar level is usually required (Banchereau, J., et al., 1998). Immature DCs phagocytose apoptotic cells using special receptors $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins, CD36, and the rise of intracellular Ca^{2+} is also required for the process (Rubartelli, A., et al., 1997; Albert, M.L., S.F.A. Pearce et al., 1998; Albert, M.L., B. Sauter et al., 1998). DCs detect the presence of microbes by use of pattern recognition receptors (PRRs) that recognize conserved pathogen-associated

molecular patterns (PAMPs) of microbes. Among PRR expressed by DC are C-type lectins, mannose receptors and toll-like receptors (TLRs). So far, 13 mammalian TLRs have been reported, 10 TLRs (TLR1-TLR10) have been identified in humans, and TLR1-TLR9 + TLR11-TLR13 in mice (Alexopoulou, L., and D. Kontoyiannis, 2005). Different DC subsets express different sets of TLRs (Iwasaki, A., and R. Medzhitov, 2004). Despite the important role of TLRs and PRRs at mucosal sites, their expression on mucosal DC has been poorly investigated.

The presence of abundant intracellular MIICs, a late endosomal structures in immature DCs, allows them quickly produce a large quantities of MHC class II-peptide complexes upon antigen uptake. MIICs contain peptide exchange factors HLA-DM (human) or H-2M (murine) that enhance the peptide binding to MHC class II molecules. During maturation of DC MHC class II-peptide complexes migrate to the surface for antigen presentation to $CD4^+$ T cells, where they remain stable for days (Banchereau, J., et al., 1998). MHC is located on chromosome 6 in humans and on chromosome 17 in the mouse. There are two classes of MHC molecules – MHC class I molecules coded by HLA-A, -B, -C genes in humans and H2-K, -D, -L genes in mice, and MHC class II molecules coded by HLA-DR, -DP, -DQ genes in humans and H-2A and –E genes in mice. MHC class I molecules bind peptides of 8-10 amino acids derived from endogenously synthesized proteins degraded in cytosol by the proteasome. These are either the cells's own proteins or proteins synthesized by invaded pathogens, commonly viruses. This type of antigen presentation is called 'endogenous' pathway. Almost all nucleated cells express MHC class I molecules. Non-nucleated cells, such as red blood cells, usually express no MHC class I molecules (Janeway, C.A., et al., 2001). MHC class II molecules present 'exogenous' antigenic peptides generated in vesicles, which are commonly of 13-17 amino acids, but, in principle, can be much longer (Janeway, C.A., et al., 2001). This is 'exogenous' pathway of antigen presentation. $CD4^+$ and $CD8^+$ T cells differ in their recognition of MHC molecule: CD4 binds to the MHC class II molecule and CD8 to the MHC class I molecules. DCs can process exogenous antigens into the MHC class I pathway to stimulate cytotoxic T lymphocyte (CTL) immunity – the phenomena known as "cross-priming" or "cross-presentation". Among antigens to be cross-presented are soluble proteins, immune complexes, intracellular bacteria, parasites

and, the main group, cellular antigens; the latter may derive from virus-infected cells, tumor cells, transplants and various normal tissue cells (Heath, W.R., et al., 2004). The whole protein is the predominant form of cross-presented protein *in vivo* (Rock, K.L., et al., 2005). Phagocytosis, which internalizes the particles of more than 1 μm in size, is the main mechanism of antigen uptake by cross-presentation, although small particles and soluble antigens in fluid entering by macropinocytosis are also cross-presented but not so efficiently (Rock, K.L. et al., 2005). DC is able to capture antigen material for cross-presentation from dead or dying cells by uptake of apoptotic cells, and also from live cells through intimate cell contact, a process referred to as nibbling, which is mediated via a class A scavenger receptor (Albert, M.L., S.F.A. Pearce et al., 1998; Larsson, M., et al., 2001; Harshyne, L.A., et al., 2001; Harshyne, L.A., et al., 2003; Ramirez, M.C., et al., 2002). The importance of two other mechanisms of antigen uptake by DC for cross-priming was not yet supported *in vivo*: the internalization of heat shock proteins (HSP) as a source of antigen or as a chaperone to antigens and the capture of exosomes, released by many cell types and loaded with proteins (Heath, W.R., et al., 2004). Macrophages, endothelial cells and B cells have also been reported to cross-present, although the role of B cells in this process *in vivo* was suggested to be minimal (Heath, W.R., et al., 2004; Rock, K.L., et al., 2005). Cross-presentation may be a major mechanism of the immune surveillance of tissues, i.e. generation of CTL response to abnormal cells. This process seems to be very important in tumor immunity, self tolerance, viral immunity and DNA vaccination.

DCs are heterogeneous, and at least six different subsets of DC were identified so far (Vremec, D., et al., 2000; Liu, Y.J., 2001; Henri, S., et al., 2001; Shortman, K., et al., 2002; O'Keeffe, M., et al., 2002; Colonna, M., et al., 2004). The first broad subdivision of DCs is into plasmacytoid and conventional subsets made in human and later in the mouse (Grouard, G., et al., 1997; Asselin-Paturel, C., et al., 2001; Nakano, H., et al., 2001; O'Keeffe, M., et al., 2002). Plasmacytoid DCs (pDC) in contrast to conventional DCs express CD45RA and a low level of CD11c (O'Keeffe, M., et al., 2002). The key characteristic of pDC seems to be the production of interferon (IFN)αβ (Cella, M., et al., 1999; Liu, Y.J., 2005). The conventional $CD11c^+$ DCs are further subdivided into the group of blood-derived DCs and the group of tissue-derived DCs (Shortmann, K., et al.,

2002; Heath, W.R., et al., 2004). Because there is no unified classification for DC subsets, we use here the classification described by Heath, W.R. et al. (Heath, W.R., et al., 2004). This is important in order not to confuse, because another subdivision into "lymphoid" ($CD8\alpha^+$) and "myeloid" ($CD11b^+$) DCs also exist (Grabbe, S., et al., 2000). Following this classification, blood-derived conventional DCs are separated into three subsets based on the expression of surface molecules CD4 and CD8α: $CD4^+$ DCs ($CD11b^+$), $CD8^+$ DCs ($CD11b^-$) and $CD4^-CD8^-$ DCs ($CD11b^+$) (Vremec, D., et al., 2000; Heath, W.R., et al., 2004). pDCs can also express CD8α, especially upon activation, but they also have CD45RA on the surface (Henri, S., et al., 2001). The other two tissue-derived subsets, the Langerhans cells and the dermal DCs are characterized by the expression of CD205 (Henri, S., et al., 2001; Heath, W.R., et al., 2004). Blood-derived $CD8^+$ DCs also express CD205, but they are $CD11b^-$, in contrary to tissue-derived $CD8^+$ $CD11b^+$ DCs (Heath, W.R., et al., 2004). Langerhans cells have a high intracellular expression of langerin – the feature that is not seen in dermal DC, although $CD8^+$ DCs also express langerin, but at a moderate level (Valladeau, J., et al., Heath, W.R., et al., 2004). *Shortman K. et al., 2002,* suggested that all DC subsets could derive from common myeloid and lymphoid precursors through a Fms-like tyrosine kinase 3-positive ($Flt3^+$) precursor and described one $CD11c^{int}CD43^+CD45RA^{lo}MHCII^-CD4^-CD8\alpha^-$ isolated from the spleen that could give rise to all classical DC subsets except pDC within three cell divisions (Colonna, M., et al., 2006).

Thus, to date six major DC subsets are identified. The complete understanding of the function of each subset is lacking, although accumulated information allows determining some functions for individual DC populations. pDCs produce large amounts of IFNαβ after stimulation with virions or TLR ligands, such as CpG. This DC subset has been suggested to control viral replication or activation of other DC (Heath, W.R., et al., 2004). Langerhans cells reside in the skin epithelia, where they detect invading pathogens. Under specific conditions they can probably also prime immune response in draining lymph nodes, where they can migrate upon pathogen encounter. Another possibility is that Langerhans cells simply transport antigen, whereas other DC types can capture antigen from migrating Langerhans cells for presentation to T cells. Dermal DCs

of the skin or the interstitial DCs of other tissues do not contain Birbecks granules in contrast to Langerhans cells and they express lower levels of CD205. Dermal DCs can efficiently cross-present soluble antigen and induce $CD4^+$ T-cell immunity to protein antigen (Heath, W.R., et al., 2004). For the three blood-derived DC populations, the functions of $CD4^+$ DCs and $CD4^-CD8^-$ DCs are poorly understood. $CD8^+$ DCs is $CD205^+$ splenic subset that predominates in T-cell areas of lymphoid organs, particularly for the spleen. $CD8^+$ DCs produce IL-12, the cytokine, which is important for CD8 T-cell proliferation. $CD8^+$ DCs preferentially internalize apoptotic cells (Iyoda, T., et al., 2002). $CD8^+$ DCs population is implicated as a primary subset responsible for cross-presentation of cellular antigens to CD8 T cells, although dermal/interstitial DCs may be also involved (Heath, W.R., et al., 2004). Moreover, $CD8^+$ DCs can induce self-tolerance via cross-presentation of tissue-derived self-antigens, the process known as cross-tolerance (Belz, G.T. et al., 2002; Heath, W.R., et al., 2004). It is likely that $CD8^+$ DCs capture antigen from tissue-derived $CD11b^+CD8^-$ DCs, which migrate with cellular antigen to the draining LN, although the possibilities that $CD8^+$ DCs obtain antigen by indirect method from the tissues, e.g. exosomes or HSPs, or that $CD11b^+CD8^-$ DCs can convert to $CD11b^+CD8^+$ DCs, or, finally, that surface antigen molecule can be transferred between the DC subsets, are also suggested (Moron, G., et al., 2002; Morelli, A.E., et al., 2003; Heath, W.R., et al., 2004). Despite the current paradigm that $CD8\alpha^+$ DCs are responsible for cross-priming and cross-tolerance to a wide variety of antigens, recent study suggested that $CD8^-$ $CD11b^+$ DCs, but not $CD8^+$ DCs are responsible for the presentation of intestinal antigens to $CD8^+$ T cells in MLNs, while $CD8^+$ DCs, but not $CD8^-CD11b^+$ DCs exclusively cross-present intravenous soluble antigen in the spleen (Chung, Y., et al., 2005).

DCs play an important role in mucosal immune responses (Kelsall, B.L. and F. Leon, 2005). Following oral antigen exposure diverse $CD4^+$ T cell responses are induced depending on the context of the encountered antigen: Th1 (IFNγ), Th2 (IL-4, IL-13), Th3 (transforming growth factor beta, TGFβ), Tr1 (IL-10) and Treg ($CD25^+Foxp3^+$). All these types of responses can be generated in PP, and it was suggested that specialized DC subsets are responsible for the generation of distinct Th-type responses (Sato, A., and

Iwasaki, A., 2005). In the absence of pathogenic stimuli orally given protein antigens normally induce suppressive immunity. In the murine PPs five DC populations were identified. Three of them express high level of CD11c: $CD11c^{hi}$ $CD8\alpha^{+}CD4^{-}CD11b^{-}$, $CD11c^{hi}$ $CD8\alpha^{-}CD4^{-}CD11b^{+}$, or $CD11c^{hi}$ $CD8\alpha^{-}CD4^{-}CD11b^{-}$ (Iwasaki, A., and B.L. Kelsall, 2001). PP $CD11b^{+}$ DCs produce low level of IL-12p70 and high level of IL-10 and prime naïve T cell to IL-10-producing (Th2) type. By contrast, both $CD8\alpha^{+}$ and $CD8\alpha^{-}CD4^{-}CD11b^{-}$ PP DCs secrete IL-12p70 upon bacterial stimulation, little to no IL-10 and induce differentiation of T cells to interferon (IFN)γ-producing (Th1) type (Iwasaki, A., and B.L. Kelsall, 2001). $CD11c^{hi}$ $CD11b^{+}$ DCs were localized to SED, whereas $CD8\alpha^{+}$ DCs were found exclusively in the T-cell zone IFR, and $CD8\alpha^{-}CD4^{-}CD11b^{-}$ DCs were reported to constitute almost one third of the entire PP DC population (Iwasaki, A., and B.L. Kelsall, 2001). $CD8\alpha^{-}CD4^{-}CD11b^{-}$ DCs were present in both SED and IFR and were scattered throughout the B-cell follicle, except the germinal center; a unique population of these cells was found within the FAE (Iwasaki, A., and B.L. Kelsall, 2000, 2001). DCs from the latter population were immature with bodies located above the basement membrane and dendrites extended to the luminal surface or some of DCs were found in association with M cells within the M cell pocket (Iwasaki, A., and B.L. Kelsall, 2001). It is, therefore, very likely that these DCs capture luminal antigens either directly or early after transport by M cells. pDCs have been identified in IFR of PPs (Asselin-Paturel, C., et al., 2003). They were $B220^{+}Ly6C^{+}$, but also $CD8^{+}$, produced IFNα, but were particularly capable to induce IL-10-producing T cells in mixed lymphocyte assays (Bilsborough, J., et al., 2003). In support of these data another group found pDC in the IFR and SED of PPs, but they were $CD8\alpha^{+}$ and $CD8\alpha^{-}$ $CD11c^{int}B220^{+}Ly6C^{+}$ (Kelsall, B.L. and F. Leon, 2005). Similarly to the studies of mice, DCs have been identified in the IFR, SED and follicle SED of human and macaques PPs, DCs in the IFR and some in the SED expressed DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) (Jameson, B., et al., 2002).

PP DCs express MAdCAM-1, which preferentially binds α4β7, the integrin molecule associated with mucosal homing, thus facilitating the interaction with mucosal T and B cells (Szabo, M.C., et al., 1997; Kelsall, B.L. and F. Leon, 2005). Moreover, PP and

MLN, but not splenic or LN DCs induce the mucosal homing receptor α4β7 as well as the chemokine receptor CCR9 on T cells *in vitro* (Mora, J.R., et al., 2003).

MLN in the mouse contains the same five populations of DCs present in PPs, with an increased amount of $CD8\alpha^{-}CD4^{-}CD11b^{-}$ (Iwasaki, A., and B.L. Kelsall, 2001). $CD8\alpha^{+}$, $CD8\alpha^{-}CD4^{-}CD11b^{-}$ DCs and pDCs have been found in T-cell zones, whereas $CD11b^{+}$ DCs were located primarily outside the T-cell zones (Iwasaki, A., and B.L. Kelsall, 2000; Asselin-Paturel, C., et al., 2003; Kelsall, B.L. and F. Leon, 2005). Similarly to PP DCs, murine MLN DC produce IL-10 and possibly TGFβ and stimulate IL-4, IL-10 and TGFβ production from CD4+ T cells (Akbari, O., et al., 2001; Alpan, O., et al., 2001). MLN DCs are also able to induce the α4β7 integrin on T cells (Stagg, A.G., et al., 2002).

DCs are a major APC population in the intestinal LP of the mouse, rat and human (Kelsall, B.L. and F. Leon, 2005). They are primarily located just below the basement membrane, but in mouse have been shown to extend their dendrites into the intestinal lumen to sample the bacteria (Rescigno, M., et al., 2001; Rescigno, M., G. Rotta et al., 2001; Niess, J.H., et al., 2005; Colonna, M., et al., 2006). DCs were identified within the rat LP epithelium supporting the existence of direct contact of DC with luminal antigens (Maric, I., et al., 1996). Some contradictory data about the phenotype of LP DCs exist. Thus, Mowat A.M. identified the same three $CD11c^{hi}$ DC populations in the LP, which had been previously found in the PP, and also pDCs (Mowat, A.M., 2003). The majority of LP DCs were $CD11b^{+}CD8\alpha^{-}$ with small numbers of $CD11b^{-}CD8\alpha^{+}$ and $CD11b^{-}CD8\alpha^{-}$ DC as well as distinct population of $CD11c^{int}$ class II MHC^{lo} $B220^{+}$ DCs (Chirdo, F.G., et al., 2005). The study of other group showed a predominance of $CD11b^{-}CD8^{-}$ DC, especially in the terminal ileum (Becker, C., et al., 2003). The Kelsall's group identified predominantly $CD11b^{+}$, fewer $CD11b^{-}CD8^{-}CD4^{-}$ and no $CD8^{+}$ DCs in the small intestine and colonic LP of mice (Kelsall, B.L. and F. Leon, 2005). This group could not confirm the presence of intraepithelial DCs in the steady state in the small intestine and colon. Freshly isolated LP DCs in the study from Mowat's group were endocytotic, expressed generally low levels of CD40, CD80 and CD86 and significant levels of mRNA for IL-10 and type I IFN, but not IL-12 (Chirdo, F.G., et al., 2005). In humans and rhesus macaques, DC-SIGN-expressing cells with some CCR5 and CD4-

coexpressing cells are distributed diffusely throughout the rectal LP, although another group suggests the presence of DCs in the LP of small bowel in poorly defined lymphoid aggregates (Jameson, B., et al., 2002; Moghaddami, M., 1998). The population of DC-SIGN$^+$ DCs, which may produce IL-12 and IL-18 during intestinal inflammation from Crohn's disease, was present scattered throughout the colon mucosa (te Velde, A.A., et al., 2003).

1.3 Oral tolerance

Oral antigen administration leads to three major consequences: a local secretory IgA antibody response in the mucosa, the systemic immune response with generation of serum antibodies and cell-mediated immunity, and state of systemic and/or local immunological tolerance. The first two responses comprise the protective immune reaction to invasive pathogens, whereas dietary proteins and commensal bacteria predominantly induce immunological tolerance, or 'oral tolerance' (Faria, A.M.C., and H.L. Weiner, 2005). Oral tolerance is a form of peripheral tolerance, when the cellular and/or humoral immune responses to an antigen are suppressed by prior administration of the antigen by the oral route. There are two main effector mechanisms of oral tolerance: the induction of clonal anergy and deletion or the induction of regulatory T cells. Oral administration of antigen in high doses favors the development of anergy-driven tolerance, whereas low doses of antigen favor the generation of regulatory cell-driven tolerance (Mowat, A.M., et al., 1882; Friedman, A., and H.L. Weiner, 1994). Low doses of orally administered antigens after been presented by APC in the gut stimulate the generation of antigen-specific regulatory T cells that secrete TGFβ, IL-10 and IL-4 and migrate to lymphoid organs, where they suppress the immune response by inhibiting the generation of effector cells, and to target organs, where they suppress disease by releasing antigen-non-specific suppressive cytokines (Faria, A.M.C., and H.L. Weiner, 2005). Several regulatory cells have been described: Th3, Tr1, $CD4^+CD25^+$, $CD4^+CD45Rb^{low}$ and $CD4^+$ latency-associated peptide $(LAP)^+$ T cells. Th3 cells secrete high amounts of TGFβ, Tr1 secrete IL-10 that increases TGF-β production by Th3 cells. TGFβ acts as a growth factor for the generation of Th3 cells and is a critical factor for the

expression of forkhead box P3 (FoxP3) and the development of regulatory function in $CD4^+CD25^+$ T cells (Faria, A.M.C., and H.L. Weiner, 2005). $CD4^+CD25^+$ Tregs are 'innate' thymic Tregs, they have suppressive properties and seem to mediate their regulatory functions by cell contact rather than cytokine release. Activated murine $CD4^+CD25^+$ Tregs have been shown to express surface LAP and TGFβ, and cell-contact-mediated immunosuppression was mediated by membrane-bound TGFβ (Nakamura, K., et al., 2001). LAP expression was also detected on $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in normal mice (Oida, T., et al., 2003). TGFβ-secreting Th3 cells and IL-10-secreting Tr1 cells have been referred as 'adaptive' regulatory T cells. In the model of colitis regulatory T cells sharing properties of the two classes have been described, they expressed $CD45RB^{low}$ and CD25, the high affinity receptor for IL-2, and mediated suppression by production of either TGFβ or IL-10 (Faria, A.M.C., and H.L. Weiner, 2005). A combination of IL-10 and TGFβ induces conventional $CD4^+$ T cells to acquire regulatory functions (Chen, Z.M., et al., 2003). $CD4^+$ T cells have been shown to be important for oral tolerance: oral tolerance can be transferred by $CD4^+$ T cells, depletion of $CD4^+$ T cells at the time of feeding antigen prevents the induction of tolerance to OVA (Faria, A.M.C., and H.L. Weiner, 2005). In induction of oral tolerance $CD8^+$ suppressor T cells can be also involved. Populations of TGF-β-secreting and IL-4 or IL-10-secreting $CD8^+$ T cells have been described (Miller, A., et al., 1992; Ke, Y., and Kapp, J.A., 1996; von Herrath, M.G. et al., 1996). However, some studies demonstrated that oral tolerance may not require $CD8^+$ T cell *in vivo* (Faria, A.M.C., and H.L. Weiner, 2005).

Antigen feeding in high doses results in anergy/deletion of specific T cells in the gut and in systemic antigen presentation after antigen passes through the gut. T cells and epithelial cells dying by apoptosis in the gut are engulfed by macrophages and DC (Fadok, V.A., et al., 1998; Huang, F.P., et al., 2000). It was shown that phagocytosis of apoptotic cells results in the induction of TGFβ production and in the downregulation of pro-inflammatory cytokines in macrophages (Freire-de-Lima, C.G. et al., 2000). In addition, apoptotic T cell can also release TGFβ both in latent and bio-active form (Chen, W., et al., 2001). It was proposed that both mechanisms of oral tolerance induction can overlap at mucosal sites, as clonal deletion of T cells leads to TGFβ secretion that, in

turn, results in the induction of regulatory T cells; anergic cells can produce cytokines, i.e. IL-4 and IL-10, and anergic $CD4^+$ T cells acted as suppressor cells *in vivo* and *in vitro*; from the other site, regulatory T cells were shown to be anergic (Faria, A.M.C., and H.L. Weiner, 2005; Schwartz, R.H., 2003). Thus, after induction of oral tolerance with high doses of antigen antigen-specific $CD4^+CD25^+$ Tregs were induced in Peyer's patches, and clones, derived from these cells produced TGFβ and were anergic (Tsuji, N.M., et al., 2003; Nagatani, K., et al., 2004; Tsuji, N.M., and B. Novak, 2004;).

Despite the appearance of much information in the field of oral tolerance, the mechanisms of its establishment and maintenance are unclear. Intestinal DCs are considered as the key players in this process (Mowat, A.M., et al., 2004). The plasticity of DCs and their ability to collect information and to produce different types of response are now well-known. Activated DC express a high level of co-stimulatory molecules (CD80, CD86) that favors the CD28-mediated recognition by T cells and their productive priming, whereas cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4)-mediated recognition of low levels of CD80/CD86 on resting DC induces tolerance. The presence of 'danger signals', such as pathogen-associated molecule and pro-inflammatory cytokines, in microenvironment determines activation status or lineage of APC (Medzhitov, R., and C. Janeway, 2000; Matzinger, P., 2002). DCs are responsible for the induction of different types of T cell responses, including regulatory, and orally immunized T cells, in turn, may 'educate' DC to give a particular set of signals to a naïve T cells thus transferring tolerance from orally induced Tregs to naïve $CD4^+$ T cells (Alpan, O., et al., 2004). DC from PP and MLN control the localization of activated T cell into the small intestine by inducing the expression of gut-homing receptors α4β7 integrin and CCR9, the receptor for the gut-associated chemokine CCL25 (thymus-expressed chemokine, TECK) (Stagg, A.J., et al., 2002; Mora, J.R., et al., 2003). The expansion of DC numbers in mice by using the cytokine flt3 ligand (flt3L) significantly enhanced their susceptibility to the induction of oral tolerance (Viney, I.L., et al., 1998).

It is unclear, which DC subset is responsible for taking up antigen and for induction of oral tolerance and where it is located. DC populations in PP would be main candidates. But the presence of PP or M cells is not absolutely required for the oral tolerance induction (Enders, G., et al., 1986; Alpan, O., et al., 2001; Spahn, T.W., et al., 2001;

Kunkel, D., et al., 2003; Mowat, A.M., 2003). Mowat's group suggests that DCs in intestinal LP are central for the induction of oral tolerance to dietary proteins (Mowat, A.M., et al., 2004; Chirdo, F.G., et al., 2005). They proposed that LP DCs take up antigen and migrate to the draining MLN before interacting with naïve $CD4^+$ T cells, which is in a good correlation with the presence of antigen-loaded DCs in the draining MLN of antigen-fed rats and mice and absolute requirement of MLN for the induction of oral tolerance (Spahn, T.W. et al., 2001; Spahn, T.W. et al., 2002; Chirdo, F.G., et al., 2005). In support of this hypothesis is an excellent study demonstrated that LP CX_3CR1-positive DCs extend their dendrites through the intestinal epithelium, sample bacteria from the intestinal lumen and transport them to MLN (Niess, J.H., et al., 2005). It was previously shown that intestinal DCs acquire orally delivered Ags and migrate through the mesenteric lymph to draining MLNs (Liu, L.M., and G.G. MacPherson, 1993). MLN DC induce $CD4^+$ T cells to secrete IL-10 and TGFβ after oral administration of protein antigens (Alpan, O., et al., 2001; Akbari, O., et al., 2001). Sato and Iwasaki speculate that this regulatory DC subset in MLN may migrate from PP, LP, isolated lymphoid follicles or that they represent MLN-indigenous DC (Sato, A., and A. Iwasaki, 2005). DC recovered from the MLNs of orally tolerized mice are able to induce *in vitro* differentiation of T cells into cells producing IL-4, IL-10 and TGF-β, a cytokine profile reminiscent of Th2/Th3/Tr1 cells (Alpan, O. et al., 2001; Akbari, O., et al., 2001).

To date, a particular DC subset responsible for tolerogenic response in the intestine was not identified, whereas three DC populations attract an attention (reviewed in Dubois, B., et al., 2005). First two are IL-10-producing DCs: myeloid subset from PPs and $CD11c^{low}CD45RB^+B220^-$ with immature-like phenotype, which have the ability to induce T_{regs} *in vitro* or/and *in vivo* (Iwasaki, A., and B.L. Kelsall, 2001; Wakkach, A., et al., 2003). The third subset is a population of intestinal pDC that in the mouse are defined as $CD11c^{low}B220^+Ly6C^+$ cells and are the main producers of type I IFN in response to virus (Asselin-Paturel, C., et al., 2001). They induce differentiation of IL-10-producing T_{regs} in human and mouse (Gilliet, M., and Y.J. Liu, 2002; Martin, P., et al., 2002; Martin, P., et al., 2002; Bilsborough, J., et al., 2003). MLN pDC support differentiation of Tr1-like cells from naïve $CD4^+$ T cells much more effectively than pDC from spleen (Bilsborough, J., et al., 2003). Interestingly, pDC express a high amount of indoleamine

2,3-dioxygenase (IDO), a tryptophan-catabolizing enzyme that have been suggested to contribute to several forms of tolerance (Fallarino, F., C. Vacca et al., 2002; Grohmann, U., et al., 2003). IDO activity leads to deprivation of tryptophan resulting in suppression of T cell proliferation and generation of kynurenines that can induce apoptosis (Fallarino, F., et al., 2002). Cytokines IL-10 and TGFβ favor stable IDO expression in DC (Munn, D.H., et al., 2002).

1.4 Food antigens

1.4.1 Wheat gluten

Broken immune tolerance to food antigens causes allergic or chronic inflammatory diseases. Intolerance to wheat gluten, a family of storage proteins from endosperm cells, and to related proteins from rye and barley, leads to the development of celiac disease (CD) (Schuppan, D., 2000; Sollid, L.M., 2002; Green, P.H., and B. Jabri, 2006). CD is an enteropathy, where the inflammation of small intestine is followed by villous atrophy, crypt hyperplasia and global malabsorbtion. The crucial role of wheat gluten in CD development was discovered over 50 years ago (Dicke, W.K., et al., 1953). The isolation of wheat gluten was first described more than two centuries ago by Beccari (Beccari, 1745). Wheat gluten, a water-insoluble fraction of wheat flour, is a complex protein mixture that consists of many distinct gliadin monomers and disulfide-linked glutenin polymers. Gliadin, the alcohol-soluble protein fraction of gluten, can be classified according to their electrophoretic mobility at acidic pH to α-, β-, γ- and ω-gliadins and, more recently, according to their N-terminal amino acid sequence to α-, γ- and ω-types (Jones, R.W., et al., 1959; Shewry, P.R., et al., 1983; Wieser, H., 1996; Seilmeier, W. et al., 2001; Wieser, H., 2007). To α- type of gliadin belong approximately 60 % of all gliadin proteins, to γ- type – 30 % and to ω-types – 10 % (Wieser, H. et al., 1994). Glutenins are insoluble in neutral aqueous or saline solution and ethanol. Large glutenin polymers have the final M_r that may exceed several millions, and they are built of the high molecular weight (HMW) and low molecular weight (LMW) glutenin subunits (Wrigley, C.W., 1996). Gliadin, as well as barley- and rye-derived hordeins and secalins, is rich in proline (20 %) and glutamine (30-40 %) residues (Wieser, H., et al., 1983).

Because of this feature the alcohol-soluble storage proteins of the Triticeae (wheat, barley and rye) were called ‘prolamins’. According to a new classification, they consist of three groups: sulfur-rich (S-rich), sulfur-poor (S-poor) and HMW prolamins (Shewry, P.R., and N.G. Halford, 2002).

The incidence of CD is between 0.5 and 1 % in the general population, and about 10 % in families where one member is affected (Koning, F., et al., 2005). CD is a disease with complex genetic. To date, HLA-DQ on chromosome 6p21.3 is the only identified locus for predispoding disease development, and there is a strong association of CD with HLA-DQA1*05-DQB1*02 (DQ2) and DQA1*03-DQB1*0302 (DQ8) (Sollid, L.M., and E. Thorsby, 1993). Approximately 90% of the patients share the *cis*- or *trans*- encoded DQ2.5 variant, and the majority of the remaining patients are HLA-DQ8 (Sollid, L.M., 2002). However, HLA-DQ is a necessary, but not sufficient factor, as it contributes for about 40 % of the risk, and therefore other, non-HLA genes, were suggested for being strong determinants of CD susceptibility (Bevan, S., et al., 1999). Gluten-derived peptides bound to HLA-DQ2/8 molecules initiate $CD4^+$ T cell response that is considered as a main mechanism of the disease development. Indeed, $CD4^+$ T cell specific for gliadin have been isolated from the small intestine of the patients with CD, but not of the controls. The fragments of gluten that can induce $CD4^+$ T cell response were defined as “immunogenic” in contrast to “toxic” fragments, which are able to induce mucosal damage when added in culture to the duodenal mucosal biopsy or when administered *in vivo* on proximal and distal intestine (Lundin, K.E.A. et al., 1993; van de Wal, Y., 1996; van de Wal, 1999; Quarsten, H., et al., 1999; Anderson, R.P. et al., 2000; Arentz-Hansen, H., S.N. McAdam et al., 2000; Arentz-Hansen, H., et al., 2002, Shan, L., et al., 2002; Vader, W. et al., 2002; Molberg, Ø., 2003; Gianfrani, C. et al., 2003; Ciccocioppo, R., et al., 2005; Shan, L., et al., 2005; Howdle, P.D., et al., 1981; Wieser, H., et al., 1982; de Ritis, G., et al., 1988; Ellis, H.J. et al., 2001). The peptides defined as a toxic are not necessarily immunogenic and vice versa, but some peptides have both capacities (Martucci, S., et al., 2003; Fraser, J.S., et al., 2003).

Some of the immunogenic peptides are immunodominant, they are the most effective in the induction of antibody and T cell response and specifically stimulate T cells from

intestinal biopsies of nearly all patients in contrast to less immunogenic epitopes that are stimulatory only for T cells from some patients (Ciccocioppo, R., et al., 2005). Some of gluten peptides that are inactive or simply immunogenic in native form, have strongly increased T cell reactivity after their treatment with tissue transglutaminase (tTG or TG2; E.C.2.3.2.13) (Molberg, Ø., et al., 1998; van de Wal, Y., et al., 1998). Indeed, HLA-DQ2 and HLA-DQ8 molecules require negative charge in the peptides for their proper binding (Johansen, B.H., et al., 1996; van de Wal, Y., et al., 1996; Kwok, W.W., et al., 1996; Godkin, A., et al., 1997). HLA-DQ2 prefers negative charges in bound peptides at either p4, p6 or p7 position in the peptide, and Lys-β71of DQ2 plays an important role in it, HLA-DQ8 prefers negative charges at position p1 or p9 (Johansen, B.H., et al., 1996; van de Wal, Y., et al., 1996; Kim, C.Y., 2004; Qiao, S.-W., et al., 2005; Koning, F. et al., 2005). Gliadin-derived peptides are almost devoid of amino acids with negative charge, they are rich in uncharged glutamine and proline residues and display only a low affinity for DQ molecules (Johansen, B.H., H.A. Gjertsen et al., 1996). Glutamine in the context of proline at certain positions is a good substrate for TG2 that converts it into the negatively charged glutamic acid by deamidation (Molberg, Ø., et al., 1998; van de Wal, Y., et al., 1998). The deamidation of gliadin peptides specifically increases their recognition by circulating antibodies, but is not absolutely required for T cell recognition and reactivity *in vitro* (Aleanzi, M., et al., 2001; Vader, W., et al., 2002; Senger, S., et al., 2005). TG2 was identified as endomysial autoantigen in CD (Dietrich, W., et al., 1997). The highly specific IgA and IgG autoantibodies against TG2 and gliadin are sensitive markers of CD (Reeves, G.E.M. et al., 2000; Schilling, J., et al., 2005). However, anti-tTG response may be gluten-independent, as it was shown in NOD mice on a gluten-free diet (Sblattero, D., et al., 2005).

The presence of multiple proline and glutamine residues in gluten epitopes makes them exceptionally resistant to enzymatic digestion by gastric, pancreatic and intestinal proteases and peptidases (Hausch, F., et al., 2002). The current CD treatment is life-long gluten-free diet. But recently found possibility to effectively degrade gliadin peptides by bacterial prolyl endopeptidase (PEP) alone or together with intestinal brush border enzymes *in vitro* or *in vivo* suggests an oral peptidase therapy for CD (Hausch, F., et al.,

2002; Shan, L., et al., 2002; Piper, J.L., et al., 2004). In addition, PEP, in combination with gastric and pancreatic enzymes as well as brush-border membrane enzymes, could detoxify food-grade gluten as it was accessed on patient-derived intestinal T cells (Marti, T., et al., 2005). Among other therapeutic options for CD are intranasal administration of α-gliadin, inhibition of intestinal TG2, blocking the binding of gluten peptides to the HLA-DQ2 or HLA-DQ8 molecules, cytokine therapy and inhibition of selective adhesion molecules (Maurano, F., et al., 2001; Sollid, L.M., and C. Khosla, 2005).

1.4.2 Hsp 60.

HSPs are the highly conserved proteins that are present in all prokaryotes and eukaryotes (Linquist, S., and E.A. Craig, 1988; Tsan, M.-F., and B. Gao, 2004). Because of their helper functions in the folding, unfolding, refolding of misfolded or translocations of proteins, in the assembly or disassembly of protein complexes they have been termed 'molecular chaperones'. HSPs are involved in MHC class I and II antigen presentation inside the cell (Li, S., et al., 2002). In addition, the exogenous antigenic peptides chaperoned by HSPs can be presented on MHC class I of APC to cytotoxic T cell after their internalization via endocytic receptor of APCs, a process, called cross-presentation (Li, S., et al., 2002). HSPs are expressed both constitutively (cognate proteins) and in inducible forms, in response to different kinds of stress: heat shock, ultraviolet radiation, heavy metals, free radicals, ischemia, hypoxia, infections, malignancies, growth factors, cell differentiation etc. The constitutive expression is the most prominent in mammalian tissues. Being mainly regarded as intracellular molecules, HSPs can be also found extracellularly upon necrotic cells death and in response to stressful conditions (Tsan, M.-F., and B. Gao, 2004). HSPs are subdivided into main groups according to their molecular mass: small HSPs, HSP40, 60, 70, 90 and 110. Their commonly used nomenclature is adopted after the Cold Spring Harbor meeting of 1996 (Hightower, L.E., and L.M. Hendershot, 1997).

The members of the HSP60 family are among the most conserved protein families in evolution (Brocchieri, L., and S. Karlin, 2000). Prokaryotic (microbial) Hsp60 and eucariotic (human) Hsp60 have local sequence homology over 70% (Karlin, S., and L.

Brocchieri, 2000). The HSP60 (chaperonin) family in mammalian species consists of mitochondrial Hsp60 (mt-Hsp60) and cytosolic Hsp60 (T-complex polypeptide-1) (Linquist, S., and E.A. Craig, 1988; Tsan, M.-F., and B. Gao, 2004). The monomer of mt-Hsp60 assembles into heptamers and tetradecamers, the latter requires the presence of mt-Hsp10 and ATP (Levy-Rimler, G., et al., 2001). The cytosolic Hsp60 forms heterooligomeric ring structures and helps to fold cytoskeletal proteins actin and tubulin (Llorca, O., et al., 2000). In normal heart and muscle cells 25 to 30% of total Hsp60 is cytosolic (Gupta, S., and A.A. Knowlton, 2005). The HSP60 proteins play a significant role in apoptosis, which can be both pro- and anti-apoptotic depending on the stimulus and cell type (Gupta, S., and A.A. Knowlton, 2005). In mitochondria of T cells Hsp60 together with its co-chaperone Hsp10 forms a complex with apoptotic protease pro-caspase-3 and controls its activation (Samali, A., et al., 1999; Xanthoudakis, S., et al., 1999). Cytosolic Hsp60 co-localizes with pro-apoptotic protein Bax, sequestering Bax in cytosol, preventing its translocation to mitochondria and therefore apoptosis (Kirchhoff, S.R., et al., 2002; Gupta, S., and A.A. Knowlton, 2005). Upon stress, cytosolic hsp60 redistributes to the plasma membrane, freeing Bax to move to the mitochondria that leads to the release of cytochrome c, the formation of apoptosome and subsequent activation of caspase cascade (Gupta, S., and A.A. Knowlton, 2002; Gupta, S., and A.A. Knowlton, 2005). Under severe conditions cytosolic Hsp60 can be also imported into mitochondria by cytoplasmic Hsp70 (Itoh, H., et al., 2002).

The presence of Hsp60 on plasma membrane may be considered as a danger signal for immune system that marks stressed, activated or damaged cells, targeting them for clearance by cytotoxic cells or macrophages as part of the tissue repair process (Belles, C., et al., 1999; Gupta, S., and A.A. Knowlton, 2002). Another possibility is that Hsp60 might stabilize membrane structure thus protecting it (Török Z., et al., 1997). Surface-expressed Hsp60 was identified on macrophages, dendritic cells, endothelial cells, γδ T cells, B cells, oligodendrocytes, B-lymphoma (Daudi) cells (Wand-Württenberger, A., et al., 1991; Stanislawska, J., et al., 2004; Xu, Q., and G. Wick, 1993; Belles, C., et al., 1999; Fisch, P., et al., 1990; Freedman, M.S., et al., 1992; Jarjour, W., et al., 1990; Pfister, G., et al., 2005). In the mucosa of patient with inflammatory bowel disease B7-positive monocytes strongly express human Hsp60 (Peetermans, W.E., et al., 1995). In

murine pancreatic β cells Hsp60 is localized to the mitochondria (80-85%) and to the extramitochondrial sites, including secretory granules, to the central core of mature insulin secretory granules, in particular, in synaptic-like microvesicles and microtubules, while during insulitis the antigen rapidly accumulated in cytoplasm and on the surface membrane, and this correlated with the induction of Hsp60 autoantibodies (Brudzynski, K., et al., 1992; Brudzynski, K., 1993; Brudzynski, K., et al., 1995; Soltys, B.J., and R.S. Gupta, 1996).

Bacterial Hsp60 is highly immunogenic, can induce antibody production and T-cell activation (Zügel, U., and S.H.E. Kaufmann, 1999). The priming of the immune system to Hsp60 is a common phenomenon, occurring early in life, because the level of antibodies against Hsp60 in infants was significantly increased after their vaccination with a trivalent vaccine against tetanus, diphtheria and pertussis (Del Giudice, G., et al., 1993). Hsp60 has a high degree of sequence homology among various pathogenic and nonpathogenic bacteria (Shinnick, T.M., 1991). Hsp60, together with Hsp70 and Hsp90, represents a major antigen in a wide spectrum of infections, i.e. in infectious diseases caused by bacteria, protozoa and fungi, as well as in various experimental infection models (Zügel, U., and S.H.E. Kaufmann, 1999). Due to cross-reactivity the antibodies and T cells against bacterial Hsp60 are capable to recognize also mammalian Hsp60 (Kissling, R., et al., 1991). Homology between self and foreign Hsp60 molecules can lead to the activation of potentially self-reactive T cells in the periphery during infections and, consequently, to the development of autoimmune disease (Zügel, U., and S.H.E. Kaufmann, 1999). A minor population of T cells, γδ T cells, is considered to contribute to the first line of the defense against pathogens. Activated Hsp60-reactive γδ T cells are protective against bacterial infection and parasites (Zügel, U., and S.H.E. Kaufmann, 1999). The isolation of mycobacterial Hsp60-reactive γδ T cells from the thymus of newborn mice and the capacity of these cells to respond to a mammalian peptide with a partial homology to mycobacterial Hsp60 peptide allowed to propose the involvement of these cross-reactive γδ T cells in both protection against mycobacterial infection and autoimmunity (Born, W., et al., 1990; O'Brien, R.L., et al., 1992). Hsp60 has been implicated in the pathogenesis of a number of autoimmune diseases, such as type 1

diabetes, Crohn's disease, juvenile chronic arthritis, atherosclerosis, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, Kawasaki disease, psoriasis, chronic gastritis, Behcet's disease, Hashimoto's thyroiditis (Elias, D., et al., 1991; Szewezuk, M.R., and W.T. Depew, 1992; Res, P.C., et al., 1988; Xu, Q., 2002; Zügel, U., and S.H.E. Kaufmann, 1999). It was shown in transfer experiments on germ-free mice that mycobacterial Hsp60-specific CD8 T cells caused autoimmune inflammatory disease in small intestine indicating the cross-recognition of host-derived Hsp60 (Steinhoff, U., et al., 1999). Moreover, transfer of a non-cross-reactive, bacterial hsp60-specific T cell clone did not induce pathology (Steinhoff, U., et al., 1999). The pathology was characterized by massive infiltrates of Hsp60-specific CD8 T lymphocytes, firstly, in the LP in the vicinity of self-Hsp60-expressing APC, and later, during disease progression, in the epithelial layers of the villi. Interestingly, infiltrating Hsp60-specific CD8 T cells partly replaced γδ T cells, which are involved in the maintenance of the intestinal epithelium (Steinhoff, U., et al., 1999).

However, healthy individuals have also antibodies as well as T cells cross-reactive for Hsp epitopes shared between pathogen and mammalian Hsp (Munk, M.E., et al., 1989; Lamb, J.R., et al., 1989). It is very likely therefore that in healthy individuals permanent encounter of cross-reactive Hsp epitopes derived from food and commensal organisms represents an additional mechanism of tolerizing of self-Hsp-specific T cells that escaped thymic selection (Mowat, A.M., 1987).

HSPs have also a direct effect on the innate immune system. The study of this issue allows better understanding of the regulatory role of HSPs in various inflammatory diseases. Bacterial Hsp60 was shown to activate innate immunity by eliciting pro-inflammatory response in human monocytes and murine macrophages and by increasing the adhesiveness to monocytes and granulocytes of endothelial cells (Friedland, J.S., et al., 1993; Peetermans, W.E., J.I. Raats et al., 1995; Wallin, R.P.A., et al., 2002). Mammalian Hsp60 activated macrophages, dendritic cells, B cells, vascular endothelium and smooth muscle cells (Skeen, M.J., et al., 1996; Kol, A., et al., 1999; Chen, W. et al., 1999; Vabulas, R.M., et al., 2001; Moré, S.H. et al., 2001; Wallin, R.P.A., et al., 2002). Thus, human Hsp60 induced rapid release of tumor necrosis factor (TNF)α, nitrite production and gene expression of the Th1-promoting cytokines IL-12 and IL-15 in

murine macrophages (Chen, W., et al., 1999). The human Hsp60-induced TNFα and NO signaled through TLR4, however in the binding of human Hsp60 to the cell surface of murine macrophages another stereospecific receptor is engaged that is distinct from the receptors for Hsp70, Hsp90 or gp96 (Ohashi, K., et al., 2000; Habich, C., et al., 2002). The single C-terminal epitope, aa481-500, was responsible for the binding of human Hsp60 to J774A.1 macrophages, and three epitopes, aa241-260, aa391-410 and aa461-480 of human Hsp60 were necessary for the binding to bone-marrow macrophages from C57BL/6J mice (Habich, C., et al., 2004; Habich, C., et al., 2006). According to another study, bacterial Hsp60-induced IL-6 production by murine macrophages was not mediated by TLR2, TLR4 or myeloid differentiation factor 88 (MyD88) (Gobert, A.P., et al., 2004). CD14 was shown to be an essential receptor for the activation of human PBMC and monocyte-derived macrophages by recombinant human (rh) Hsp60 (Kol, A., et al., 2000). Human monocyte-derived immature DC internalized hHsp60 through receptor-mediated endocytosis that did not involve CD14 or TLR4 (Lipsker, D., et al., 2002). However, another group showed the necessity of both TLR2 and, to a lesser extent, TLR4 for TNFα production from BMDC of C3H/HeJ mice in response to bacterial Hsp60 (Vabulas, R.M., et al., 2001). It was recently found that human Hsp60 is also able to activate naïve mouse B cells stimulating their proliferation, secretion of IL-10 and IL-6 and up-regulation of the expression of MHC class II and accessory molecules CD69, CD40 and CD86 (Cohen-Sfady, M., et al., 2005).

1.5 Diabetes, food antigens and gut immune system.

1.5.1 Type 1 diabetes mellitus (T1DM)

T1DM is an immune-mediated disease characterized by a specific loss of most insulin-producing β cells in the pancreatic islets of Langerhans. During the development of islet inflammation (insulitis) macrophages, DCs, CD8 and CD4 T cells and B lymphocytes infiltrate pancreatic islets with a predominance of CD8 T cells. Cells in inflamed islets show hyperexpression of MHC class I molecules (Atkinson, M.A., and G.S. Eisenbarth, 2001; Yoon, J.-W., and H.-S. Jun, 2001). In spite of insulin therapy and tight control of blood glucose there is still a significant risk of neuropathy, retinopathy, nephropathy and

hypoglycemia. In Europa and North America more than 2 million individuals have T1DM. With an annual increase by 3-4 % the T1DM incidence is expected to be about 40 % higher in 2010 than in 1997 (Atkinson, M.A., and G.S. Eisenbarth, 2001). T1DM is a multifactorial disorder. It develops in genetically predisposed individuals and depends on environmental factors, such as viral infection, e.g. coxsackievirus and cytomegalovirus, and/or diet. The genes within the MHC HLA class II region on chromosome 6p21, termed *iddm1*, account for about 45 % of genetic susceptibility for the disease. The risk of disease incidence is associated with HLA-DR3 and HLA-DR4 haplotypes, in particular with DQα- and DQβ alleles, while the HLA-DR2 haplotype is protective. DQ2(DQA1*05-DQB1*02)/DQ8(DQA1*03-DQB1*0302) heterozygous individuals are of the highest risk for T1DM (Undlien D.E., et al., 1997). In addition, about 20 non-HLA loci contribute to disease susceptibility (Atkinson, M.A., and G.S. Eisenbarth, 2001).

Diabetes-prone BioBreeding (BB) rats and non-obese diabetic (NOD) mice are the two most commonly used rodent models of spontaneous diabetes that is similar to human T1DM (Nakhooda, A.F. et al., 1977; Makino, S., et al., 1980; Mordes, J.P., et al., 2001; Mordes, J.P. et al., 2004; Rees, D.A., and J.C. Alcolado, 2005). Among β-autoantigens identified in humans, NOD mice and BB rats are insulin, glutamic acid decarboxylase (GAD), islet cell autoantigens, the insulin receptor, Hsp 60/65, the glucose transporter, IA-2, 37/40 kD tryptic fragments of a 64-kD antigen (different from GAD), a 52-kD protein, a 69-kD protein carboxypeptidase H and a 38-kD autoantigen, from which the first two, insulin and GAD, are considered to be the most important (Bach, J.F., 1995; Yoon, J.-W., and H.-S. Jun, 2001).

1.5.2 BB rats

In 1974 outbred Wistar rats from a commercial breeding company, the Bio Breeding Laboratories in Ottawa, Canada, developed spontaneous hyperglycemia and ketoacidosis thus giving rise to BBdp rats (Nakhooda, A.F., et al., 1977). Hyperglycemia in these animals is of autoimmune origin (Mordes, J.P., et al., 1996; Mordes, J.P., et al., 2001). BBdp rats of both sexes develop insulitis between 30 and 90 days of age rapidly followed

by β-cell destruction and overt diabetes (Guberski, D.L., 1994). Insulitis in BBdp rats is morphologically similar to that observed in human T1DM with a predominance of Th1-type lymphocytes (Kolb, H., et al., 1996; Zipris, D., et al., 1996). All spontaneously diabetic BB rats are lymphopenic, they have severe reduction of $CD4^+$ T cell numbers and a nearly complete absence of CD8 T $cells^+$ (Mordes, J.P., et al., 2004). BB rats are also deficient in $ART2^+$ regulatory T cells, and transfusion with $CD4^+$ $ART2^+$ T cells can prevent diabetes in BB rats (Bortel, R., et al., 2001; Mordes, J.P., et al., 2004). Coisogenic diabetes-resistant BBc rats were developed from normoglycemic BBdp rats. The immune system of BBc rats is normal, they do not become diabetic spontaneously, but can develop insulitis and hyperglycemia after *in vivo* treatment with activators of the immune system or after depletion of $ART2^+$ regulatory T cells (Mordes, J.P., et al., 1996; Mordes, J.P., et al., 2001).

1.5.3 Association between T1DM and celiac disease (CD)

T1DM is often associated with other immune-mediated diseases, such as thyroid disease or CD (Bigazzi, P.E., 1990; Cronin, C.C., and F. Schanahan, 1997; Atkinson, M.A., and G.S. Eisenbarth, 2001; Narendran, P., et al., 2005). There is a strong association between T1DM and CD (Walker-Smith, J.A., et al., 1969; Savilahti, E., et al., 1986; Cronin, C.C., and F. Schanahan, 1997). Both have an increased frequency of HLA-DQ2 and DQ8 locuses of the MHC, celiac disease-specific autoantibodies are found in type 1 diabetic patients and vice versa (Cronin, C.C., and F. Schanahan, 1997; Bonifacio, E., et al., 1998). But on the contrary to CD that is usually detected after onset of T1DM in 5-10% of patients, T1DM development is a very rare case after the diagnosis of CD, even if islet autoantibodies are present (Cronin, C.C., and F. Schanahan, 1997; Bonifacio, E., et al., 1998). The reported prevalence of CD in type 1 diabetic patients varies between studies and countries (Saukkonen, T., et al., 1996; Rensch, M.J., et al., 1996; Carlsson, A.K., et al., 1999; Lampasona, V., et al., 1999; Hansen, D., et al., 2001; Aktay, A.N., et al., 2001; Jaeger, C., et al., 2001; Barera, G., et al., 2002; Spiekerkoetter, U., et al., 2002; Gillett, P.M., et al., 2001). Probably, it reflects the differences in technical approach (the choice of autoantigens and autoantibodies), genetic/environmental background and the age of

patients. Thus, IgG tTG C autoantibodies alone or together with IgA tTG C were found in 41% of new-onset type 1 diabetic patients (n=287) (Lampasona, V., et al., 1999). According to another studies 16.8% of recent-onset type 1 diabetes patients (n=197) had one or more CD-associated antibodies (anti-gliadin IgG and IgA, anti-tTG IgA), 6.3% or 7.7% of children with T1DM were tTGA-positive (Jaeger, C., et al., 2001; Spiekerkoetter, U., et al., 2002; Gillett, P.M., et al., 2001). The prevalence of tTG antibodies is associated in particular with DQ2 homozygosity: one third of DQ2 homozygous type 1 diabetic patients expressed IgA tTG autoantibodies (22/68) vs. 11.6% in non-selected population of diabetic patients (98/847) and less than 2% in those lacking DQ2 or DQ8 (Bao, F., et al., 1999). The first-degree relatives, mostly siblings and parents, of type 1 diabetic patients, do not have an increased level of CD-associated antibodies, whereas in offsprings of type 1 diabetic patients the risk to develop CD is high (Jaeger, C., et al., 2001; Saukkonen, T., et al., 2001; Hummel, M., et al., 2000).

1.5.4 Diet is a risk factor for T1DM

In BB rats and NOD mice the incidence of spontaneous diabetes is broadly modulated by diet (Scott, F.W., 1996; Elliott, R.B., and J.M. Martin, 1984; Elliott, R.B. et al., 1988; Coleman, D.L. et al., 1990). Thus, food, containing hydrolyzed casein as the only source of protein, decreases the development of diabetes in BB rats or NOD mice (Scott, F.W. et al., 1985; Elliott, R.B. et al., 1988; Coleman, D.L. et al., 1990). In contrary, wheat gluten in the diet promotes diabetes incidence in NOD mice and BB rats (Scott, F.W. et al., 1988; Hoorfar, J. et al., 1993; Funda, D.P. et al, 1999; Schmid, S. et al., 2004). Gut immune system is responsible for the first immune contact with a food. It was shown that type 1 diabetic patients and diabetes-prone animals may have abnormalities in the intestine, such as increased permeability and immune activation (Carratu, R., et al., 1999; Savilahti, E., et al., 1999; Meddings, J.B., 1999; Westerholm-Ormio, M., et al., 2003; Flohé, S.B., et al., 2003; Hardin, J.A., et al., 2002; Malaisse, W.J., et al., 2004). Mucosal vascular addressin MAdCAM-1 that normally homes lymphocytes to Peyer's patches in the gut through interaction with their adhesion molecule $\alpha 4\beta 7$ integrin, is also expressed in the islets of diabetic patients and NOD mice, and treatment with monoclonal

antibodies against MAdCAM-1 and β7 integrin protects against diabetes and insulitis in NOD mice (Streeter, P.R., et al., 1988, Vaarala, O., 1999; Yang, X.-D., et al., 1994). T lymphocytes, bearing α4β7 integrin, infiltrate islets of NOD mice (Hänninen, A., et al., 1996; Yang, X.-D. et al., 1994). It was suggested that gut-derived β autoreactive T cells infiltrate the islets (Vaarala, O., 1999; Jaakkola, I., et al., 2003). Indeed, it was shown that lymphocytes from MLN of 3 weeks-old NOD mice transferred diabetes (Hänninen, A., et al., 1998; Vaarala, O., 1999; Jaakkola, I., et al., 2003).

Mucosal autoantigen administration was suggested as a possible therapy for autoimmune diseases, including T1DM (Weiner, H.L., et al., 1994; Vaarala, O., 1999). A single intranasal administration of GAD65 peptides induced Th2 response and prevented diabetes in NOD mice (Tian, J., 1996). Oral administration of insulin together with bacterial adjuvant suppressed the development of diabetes in NOD mice due to the deviation of cytokine balance in the islets of Langerhans towards Th2 type (Hartmann, B., et al., 1997). Oral dosing of bacterial immunostimulants LPS alone or Escherichia coli extract OM-89, containing mainly acidic glycoproteins including HSP65, also lead to a Th2 shift of pancreatic mRNA expression in BB rats and gut cytokine gene expression in NOD mice (Bellmann, K., et al., 1997). It was suggested that extract OM-89 has suppressive effect in autoimmunity due to the presence of bacterial heat shock proteins, namely, that induction of peripheral tolerance occurs at the level of regulatory T cells with specificity to HSPs (Bloemendal, A., et al., 1997; Wendling, U., and J.C. Farine, 1998). Although oral tolerization with autoantigens often gives a successful result, it has to be approached with many precautions, because of possible adverse effect (Bellmann, K., et al., 1998; Hänninen, A., 2000; Weiner, H.L., 2004).

Gluten-free diet is the only effective therapy for CD to date, but it was also effective in preventing diabetes or reducing its incidence in NOD mice and BB rats (Elliot, R.B., and J.M. Martin, 1984; Hoorfar, J., et al., 1993; Funda, D.P., et al., 1999). Delayed diabetes onset and reduced diabetes incidence were also seen in NOD mice after exposure to wheat and barley protein-free diet (Schmid, S., et al., 2004). In preclinical T1DM in humans, gluten-free diet did not change disease-associated autoantibody titers, but improved insulin secretion and reduced IgG gliadin antibodies (Hummel, M., et al., 2002; Pastore, M.-R., et al., 2003). In one recent study in diabetic children with CD gluten-free

diet improved growth, in contrast to several previous observations in children and adults with both CD and T1DM showing no improvement in growth or metabolic control of diabetes (Saadah, O.I., et al., 2004; Savilahti, E., et al., 1986; Acerini, C.L., et al., 1998; Westman, E., et al., 1999; Kaukinen, K., et al., 1999; Amin, R., et al., 2002).

1.6 Aims of the current study

This study explores the hypothesis that the gut immune system and APCs are important in promoting the development of autoimmunity.

The experimental design:

1. To investigate the interaction of bone marrow-derived DC (BMDC) with wheat gluten and rhHsp60 in vitro.

Working hypothesis is that direct contact of DCs with dietary wheat gluten or rhHsp60 leads to DC stimulation and induction of Th1-scewed immune response. The study of DC maturation markers, DC cytokine and chemokine production, selected candidate receptors and signaling pathways involved in these interactions was performed.

2. To study the effect of wheat gluten diet on the immunity of small intestine of BBdp rats.

Working hypothesis is that wheat gluten diet causes early pro-inflammatory changes in the gut of BBdp rats, which lead to gut inflammation, thus predisposing to β-cell damage in the pancreas and development of T1DM later in life. For testing this hypothesis diabetes prone BBdp and control BBc and WF rats were fed diabetogenic wheat gluten or NTP-2000 diets or diabetes-retardant hydrolyzed casein (HC) diet from weaning, and the level of Th1 cytokine IFNγ, Th2 cytokine IL-10 and chemokines monocyte chemoattractant protein (MCP)-1/CCL2 or macrophage inflammatory protein (MIP-2)/CXCL2 was analyzed in the upper small intestine (duodenum) of BBdp rat at 10, 23, 30, 45, 70, 95, 120 days of age.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

2.1.1.1 Chemicals

Albumin (bovine serum, fraction V)	Sigma, Taufkirchen, Germany
Ammoniumpersulfate (APS)	Sigma, Taufkirchen, Germany
Bromphenol blue sodium salt	Sigma, Taufkirchen, Germany
α-Chymotrypsin	Sigma, Taufkirchen, Germany
Glucose	Merck, Darmstadt, Germany
Glycerol	ICN Biochemicals, Cleveland, USA
Glycine	Merck, Darmstadt
HEPES (N-(2-Hydroxyethyl)piperazine-N'-2-erhane sulfonic acid)	Serva, Heidelberg, Germany
β-Mercaptoethanol (β-ME)	Sigma, Taufkirchen, Germany
Methanol p.A.	AppliChem, Darmstadt, Germany
n-Octyl-β-D-glycopyranoside	Calbiochem, Merck Biosciences GmbH, Schwalbach/Ts., Germany
Paraformaldehyde	Fluka, Taufkirchen, Germany
Phenol Red	Sigma, Taufkirchen, Germany
Sodium chloride (NaCl)	Fluka, Taufkirchen, Germany
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4xH_2O$)	Merck, Darmstadt, Germany
Sodium dodecylsulfate (SDS)	Merck, Darmstadt, Germany
Sodium hydrogencarbonate ($NaHCO_3$)	Merck, Darmstadt, Germany
Sodium hydrogen phosphate dihydrate ($NaHPO_4x2H_2O$)	Merck, Darmstadt, Germany
Sodium orthovanadate (Na_3VO_4)	Sigma, Steinheim, Germany
5-Sulfosalicylic acid dihydrate	Roth, Karlsruhe, Germany
Tris(hydroxymethyl)-aminomethan ($H_2NC(CH_2OH)_3$)	Merck, Darmstadt, Germany
Trichloracetate (TCA)	Merck, Darmstadt, Germany
Tween 20 (Polyoxyethylensorbitanmonolaurat)	Merck, Darmstadt, Germany

2.1.1.2 Inhibitors, agonists and antagonists

Protease-inhibitor cocktail Complete (Roche, Mannheim, Germany)

Pefabloc SC Plus (Roche, Mannheim, Germany)

4-(2-Aminoethyl)-benzenesulfonyl-flouride, hydrochloride (AEBSF) with PSC-protector

Sodium orthovanadate solution (Na_3VO_4)

Stock solution of 0.5 M Na_3VO_4 was prepared in H_2O, pH was adjusted to 9-10 with conc. HCl. Thereafter the solution was heated at +95°C till the full appearance of yellow colour and pH was again adjusted to 9-10 after cooling. The exact concentration of the solution was determined photometrically using the coefficient of molar extinction ε 2925 l/mol x cm at λ = 265 nm. The aliquots were stored at -20°C.

2.1.2 Materials for SDS-PAGE and Western blot

2.1.2.1 Antibodies

Anti-phospho-SAPK/JNK	Cell Signaling Technology Inc., Beverly, MA, USA; distributor New England Biolabs, Frankfurt am Main, Germany
The antibodies were diluted in TBST/BSA 1:1000.	
Anti-phospho-p44/42 MAPK	Cell Signaling Technology Inc., Beverly, MA, USA
The antibodies were diluted in TBST/skim milk 1:1000.	
Anti-phospho-p38 MAPK	Cell Signaling Technology Inc., Beverly, MA, USA
The antibodies were diluted in TBST/BSA 1:1000.	
Anti-phospho-NFκB p65	Cell Signaling Technology Inc., Beverly, MA, USA
The antibodies were diluted in TBST/BSA 1:1000.	
Goat anti-rabbit IgG-horseradish peroxidase(HRP)	Dianova, Hamburg, Germany
These antibodies were used as second one for a-phospho-SAPK/JNK, a-phospho-p38, a-phospho-p65 NFκB and were diluted in TBST/skim milk 1:5000.	

Goat anti-mouse IgG-HRP	Dianova, Hamburg, Germany

These antibodies were used as second one for a-phospho-p42/44 and were diluted in TBST/skim milk 1:1000.

2.1.2.2 Markers

Precision Plus Protein Standards, unstained	Bio-Rad Laboratories GmbH, Munich, Germany
Precision Strep Tactin-HRP Conjugate	Bio-Rad Laboratories GmbH, Munich, Germany

This solution is used for the identification of Precision Plus Protein Standards and is diluted 1:5000 in TBST/skim milk .

2.1.2.3 Others

Nitrocellulose membrane, Hybond-ECL	Amersham Biosciences UK Limited, Amersham place, Little Chalfon, UK
POD (BM Chemiluminiscence Blotting Substrate)	Roche, Mannheim, Germany
Ponceau S solution for electrophoresis	Serva, Heidelberg, Germany
ProSieve Gel Solution	Cambrex Bio Science Rockland Inc., USA
Skim milk powder	Fluka, Taufkirchen, Germany
SuperSignal West Pico Chemiluminescent Substrate	Pierce, Bonn, Germany

2.1.3 Antibodies for Fluorescence Activated Cell Sorting (FACS) analysis

All antibodies were purchased from BD Biosciences (Heidelberg, Germany).

2.1.3.1 Primary antibodies

Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse I-A/I-E

(isotype control FITC-conjugated rat IgG_{2a}, κ)

FITC-conjugated mouse anti-mouse $I\text{-}A^k$

(isotype control FITC-conjugated mouse IgG_{2a}, κ)

R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD86 (B7-2)

(isotype control PE-conjugated rat IgG_{2a}, κ)

R-PE-conjugated hamster anti-mouse CD54

(isotype control PE-conjugated hamster IgG_1, κ)

R-PE-conjugated rat anti-mouse CD14

(isotype control PE-conjugated rat IgG_1, κ)

Biotinylated rat anti-mouse CD40

(isotype control biotinylated rat IgG_{2a}, κ)

Biotinylated hamster anti-mouse CD11c (Integrin α_χ chain)

(isotype control biotinylated hamster IgG, group 1, λ)

Allophycocyanin (APC)-conjugated rat anti-mouse CD11b

(isotype control APC rat IgG_{2b}, κ)

APC-conjugated rat anti-mouse CD45R/B220

(isotype control APC rat IgG_{2a}, κ)

PE-conjugated rat anti-mouse Ly-6G (Gr-1)

(isotype control PE-conjugated rat IgG_{2b}, κ)

2.1.3.2 Secondary antibody

Streptavidin-conjugated Cy-Chrome

2.1.3.3 Blocking antibody

Purified anti-mouse CD16/CD32 (FcγIII/II Receptor) (Fc Block)

2.1.4 Materials for Enzyme-Linked Immunosorbent Assay (ELISA)

2.1.4.1 ELISA Sets

Rat IFNγ OptEIA Set	BD Biosciences, Heidelberg, Germany

Capture antibody: anti-rat IFN-γ monoclonal antibody
Detection antibody: biotinylated anti-rat IFN-γ monoclonal antibody
Enzyme reagent: avidin-horseradish peroxidase conjugate
Standard: baculovirus-expressed recombinant rat IFN-γ

Rat IL-10 OptEIA Set	BD Biosciences, Heidelberg, Germany

Capture antibody: anti-rat IL-10 monoclonal antibody
Detection antibody: biotinylated anti-rat IL-10 monoclonal antibody
Enzyme reagent: avidin-horseradish peroxidase conjugate
Standard: baculovirus-expressed recombinant rat IL-10

Rat MCP-1 OptEIA Set — BD Biosciences, Heidelberg, Germany

Capture antibody: anti-rat MCP-1 monoclonal antibody
Detection antibody: biotinylated anti-rat MCP-1 monoclonal antibody
Enzyme reagent: avidin-horseradish peroxidase conjugate
Standard: baculovirus-expressed recombinant rat MCP-1

Mouse TNFα OptEIA Set — BD Biosciences, Heidelberg, Germany

Capture antibody: anti-mouse TNFα monoclonal antibody
Detection antibody: biotinylated anti-mouse TNFα monoclonal antibody
Enzyme reagent: avidin-horseradish peroxidase conjugate
Standard: baculovirus-expressed recombinant mouse TNFα

Mouse IL-10 OptEIA Set — BD Biosciences, Heidelberg, Germany

Capture antibody: anti-mouse IL-10 monoclonal antibody
Detection antibody: biotinylated anti-mouse IL-10 monoclonal antibody
Enzyme reagent: avidin-horseradish peroxidase conjugate
Standard: baculovirus-expressed recombinant mouse IL-10

Mouse MCP-1 OptEIA Set — BD Biosciences, Heidelberg, Germany

Capture antibody: anti-mouse MCP-1 monoclonal antibody
Detection antibody: anti-mouse MCP-1 monoclonal antibody conjugated to horseradish peroxidase
Standard: baculovirus-expressed recombinant mouse MCP-1

Mouse IL-1β DuoSet — R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany

Capture antibody: rat anti-mouse IL-1β
Detection antibody: biotinylated goat anti-mouse IL-1β
Enzyme reagent: streptavidin-horseradish peroxidase conjugate
Standard: recombinant mouse IL-1β

Mouse IL-12 p40 DuoSet — R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany

Capture antibody: rat anti-mouse IL-12p40
Detection antibody: biotinylated goat anti-mouse IL-12p40
Enzyme reagent: streptavidin-horseradish peroxidase conjugate
Standard: recombinant mouse IL-12p40

Mouse IL-12 p70 Quantikine Kit — R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany

Capture antibody: anti-mouse IL-12 p70 monoclonal antibody
Detection antibody: anti-mouse IL-12 p70 polyclonal antibody, conjugated to horseradish preoxidase
Standard: recombinant mouse IL-12 p70

Mouse MIP-2 DuoSet — R&D Systems GmbH, Wiesbaden-, Nordenstadt, Germany

Capture antibody: rat anti-mouse MIP-2
Detection antibody: biotinylated goat anti-mouse MIP-2
Enzyme reagent: streptavidin-horseradish peroxidase conjugate
Standard: recombinant mouse MIP-2

Mouse MIP-1α DuoSet — R&D Systems GmbH, Wiesbaden-, Nordenstadt, Germany

Capture antibody: goat anti-mouse MIP-1α
Detection antibody: biotinylated goat anti-mouse MIP-1α
Enzyme reagent: streptavidin-horseradish peroxidase conjugate
Standard: recombinant mouse MIP-1α

Mouse KC DuoSet — R&D Systems GmbH, Wiesbaden-, Nordenstadt, Germany

Capture antibody: rat anti-mouse KC
Detection antibody: biotinylated goat anti-mouse KC
Enzyme reagent: streptavidin-horseradish peroxidase conjugate
Standard: recombinant mouse KC

Mouse RANTES DuoSet — R&D Systems GmbH, Wiesbaden-, Nordenstadt, Germany

Capture antibody: rat anti-mouse RANTES
Detection antibody: biotinylated goat anti-mouse RANTES
Enzyme reagent: streptavidin-horseradish peroxidase conjugate
Standard: recombinant mouse RANTES

2.1.4.2 Others

96-well Falcon microplate — BD Labware, NY, USA

96-well Nunc-immunoplate — Nunc, Wiesbaden, Germany

2.1.5 Materials for Limulus Amebocyte Lysate (LAL) Assay

LAL Kit	BioWhittaker Molecular Applications, Walkersville, USA

2.1.6 Materials for protein determination

Bio-Rad protein assay kit	Bio-Rad Laboratories GmbH, Munich, Germany

2.1.7 Buffers, solutions and medium

2.1.7.1 Media and serum for cell culture

Phosphate-buffered saline (PBS) Dulbecco's w/o Ca^{2+} and Ma^{2+}, w/o sodium bicarbonate	Invitrogen, Karlsruhe, Germany
Fetal calf serum (FCS)	PAA Laboratories, Pasching, Austria

2.1.7.1.1 Media for dendritic cell culture

Gentamicin	Sigma, Taufkirchen, Germany
L-glutamine	Biochrom AG, Berlin, Germany
Penicillin	Sigma, Taufkirchen, Germany
Recombinant murine Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)	R&D Systems GmbH, Wiesbaden Germany
RPMI 1640 medium, low endotoxin	Biochrom AG, Berlin, Germany

2.1.7.1.2 Media for macrophage culture

Ampicillin	Serva, Heidelberg, Germany
Accutase	PAA Laboratories, Pasching, Austria
L-glutamine	PAA Laboratories, Pasching, Austria
Non essential amino acids (MEM)	PAA Laboratories, Pasching, Austria

Penicillin G K-salt	Serva, Heidelberg, Germany
Recombinant murine interferon gamma (rmIFN-γ)	R&D Systems GmbH, Wiesbaden, Germany
RPMI 1640 medium	PAA Laboratories, Pasching, Austria
Streptomycin	Serva, Heidelberg, Germany

Solution A:

NaCl	0.9 %
HEPES	10 mM
Phenol Red	1:1000 v/v
adjust pH to 7.5	
KCl	10 mM
Glucose	0.1 %

2.1.7.1.3 Investigated stimuli for dendritic cell culture and macrophage culture

Lipopolysaccharide (LPS) from *E. coli* O26:B6	Sigma, Taufkirchen, Germany
Macrophage-activating lipopeptide-2 (MALP-2) MALP-2 was diluted in 25 mM n-octyl-β-D-glycopyranoside, Calbiochem, in PBS	Provided by Dr. P. Muhlradt from German Research Center for Bio-Biotechnology, Immunobiology Research Group, Braunschweig, Germany
Polymyxin B PmB was incubated with the stimuli 1 h at 4°C before the addition to the cells	Sigma, Taufkirchen, Germany
Protein G Agarose	Sigma, Taufkirchen, Germany
Recombinant murine interleukin 1β (rmIL-1β)	R&D Systems GmbH, Wiesbaden Germany
Recombinant human Hsp60 (rhHsp60)	Peptor, Rehovot, Israel
Wheat gluten	ICN Biochemicals, Cleveland, OH, USA

Synthetic gliadin peptides:

H2N-LGQQQPFPPQQPY-COOH (GP_{31-43})	Eurogentec, Seraing, Belgium
H2N-FQQPQQQYPSSQ-COOH ($GDA7W_{242-253}$)	Eurogentec, Seraing, Belgium
H2N-QLQPFPQPQLPYPQPQS-COOH (GP_{57-73})	Eurogentec, Seraing, Belgium
H2N-LQLQPFPQPQLPYPQPQLPYPQPQLPY-PQPQPF-COOH (GP_{56-88}) The peptides were dissolved in sterile water aqua ad injectabilia DeltaSelect to the concentration 1 mg/ml before use	Eurogentec, Seraing, Belgium

Antibodies:

Rat anti-mouse IL-1 receptor type I (CD121a)	BD Biosciences, Heidelberg, Germany
Isotype control rat IgG_1, κ	BD Biosciences, Heidelberg, Germany

Enzymes:

Proline specific endopeptidase (PEP) from *Flavobacterium meningosepticum*	United State Biological, Swampscott, MA, USA
90 g of PEP (53 U/mg) were dissolved in 1 ml of 0.05 M phosphate buffer, pH 7.0, and sterilized by filtering through 0.2 μm Supor Acrodisk 32 syringe filter (x2). Then to 0.3 ml of this solution 2.274 ml of 0.1 M phosphate buffer, pH 7.0, were added to reach PEP activity 556 mU/ml	
Proteinase K-Agarose from *Tritirachium album*	Sigma, Taufkirchen, Germany
Proteinase K- or protein G-agarose were incubated overnight in 10 % ethanol in PBS at +4°C, then washed 3 times with RPMI w/o FCS and antibiotics, centrifuged 30 ces at 500 g, RPMI was added to the pellet. The stimuli were incubated 3.5 h at 37°C in thermoshaker with proteinase K at final concentration 30 U/mg of the stimuli, centrifuged 1min at 8 000 g, filtered through 0.2 μm Supor Acrodisk 32 syringe filter and added to the cells.	

2.1.7.2 Buffer for cell lysate preparation

Lysis buffer:

HEPES	25 mM
NaCl	150 mM
Glycerol	10 %
Triton X-100	1 %
$Na_2H_2P_2O_7$	10 mM
β-Glycerophosphate	20 mM
Na_3VO_4	2 mM
NaF	10 mM

The buffer sterile filtered, aliquoted and stored at –20ºC. Prior to use aliquots of protease-inhibitors cocktail Complete (1 tablet is dissolved in 2 ml dist. H_2O), 40 mM Pefabloc SC Plus and Na_3VO_4 were taken from –20ºC and added 1:50, 1:100 and 1:500, respectively, to buffer, aliquot of PSC-Protector were taken from +4ºC and added 1:200 to buffer.

2.1.7.3 Solutions, buffers and gels for SDS-gel electrophoresis and electrotransfer

Buffer for electrophoresis
(running buffer, cathode-buffer, 10-times concentrated):

Tris/HCl, pH 8.3	250 mM
Glycin	1.92 M
adjust pH to 8.3 with 5N HCl	
SDS	1 % (w/v)

Sample buffer by Laemmli:

Tris/HCl, pH 6.8	50 mM
SDS	2 % (w/v)
Glycerol	10 %
β-Mercaptoethanol	100 mM
Bromphenol Blue	0.1 %

Laemmli buffer is stored at -20°C.

Buffer for resolving gel :

Tris/HCl, pH 8.8	1 M

Buffer for stacking gel :

Tris/HCl, pH 6.8	0.5 M

SDS-PAGE-gel mixtures:

Following gel mixtures are valid for 1 mini gel (size 10 cm x 7.5 cm)

Resolving gel 10 %:

Tris/HCl 1 M, pH 8.8	0.938 ml
Pro Sieve	0.75 ml
APS 10 %	37.5 µl
TEMED	2 µl
Milli Q water	2.025 ml

Stacking gel 5 %:

Tris/HCl 0.5 M, pH 6.8	1.875 ml
Pro Sieve	0.25 ml
APS 10 %	15 µl
TEMED	2.5 µl
Milli Q water	1.875 ml

2.1.7.4 Solutions for immunological determination of proteins by Western blot

Blotting buffer (transfer buffer):

Tris/HCl	25 mM
Glycine	192 mM
Methanol	20 % (v/v)
Milli Q water	till 1 L

Store at +4°C.

Ponceau S stock solution:

Ponceau S	2 % (w/v)
TCA	30 % (w/v)
Sulfosalicylic acid	30 % (v/v)

Store at +4°C. Stock solution is diluted 1:10 in H_2O prior to use.

Tris-Buffered Saline (TBS):

Tris/HCl, pH 7.6	20 mM
NaCl	150 mM
Milli Q water	till 1 L

TBS/Tween 20 (TBST)

Tween 20	0.1 % (v/v) in TBS

Blocking solution

Skim milk powder	5 % (w/v) in TBST

Solution for the dilution of antibodies

Albumin (bovine serum, fraction V)	5 % (w/v) in TBST
Skim milk powder	5 % (w/v) in TBST

2.1.7.5 Solutions for FACS analysis

CellWash (BD, Erembodegem, Belgium)

Paraformaldehyde (Fluka, Taufkirchen, Germany)

For the cell fixation 1 % pharmaldehyde is used. The solution is kept at +4°C and prepared from the stock solution, 20 % paraformaldehyde in PBS (stored at -20 °C).

2.1.7.6 Buffers and solutions for ELISA

PBS Dulbecco's w/o Ca^{2+} and Ma^{2+} Biochrom AG, Berlin, Germany

10 x PBS

9.55 g of PBS Dulbecco are dissolved in 100 ml milli Q H_2O. The buffer is stored at room temperature.

Coating buffer for rat IFNγ and rat MCP-1 OptEIA Sets (BD Biosciences):

0.1 M carbonate, pH 9.5:

$NaHCO_3$	0.84 g
Na_2CO_3	0.356 g
milli Q H_2O	to 100 ml
adjust pH to 9.5	

The buffer is freshly prepared or used within 7 days of preparation after storage at 2-8°C.

Coating buffer for other OptEIA Sets (BD Biosciences) and for DuoSet (R&D Systems) for mouse IL-12p40:

0.2 M sodium phosphate, pH 6.5:

Na_2HPO_4 x 2 H_2O	1.48 g
NaH_2PO_4 x H_2O	1.85 g
milli Q H_2O	to 100 ml
adjust pH to 6.5	

The buffer is freshly prepared or used within 7 days of preparation after storage at 2-8°C.

Coating buffer for other DuoSets (R&D Systems) is PBS

Assay diluent:

1 x PBS with 10 % FCS (PAA Laboratories, Pasching, Austria, aliquots are stored at -20°C).

The solution is freshly prepared or used within 3 days of preparation after storage at 2-8°C.

Wash buffer:

1 x PBS with 0.05 % Tween-20 (Merck, Darmstadt, Germany)

The solution is freshly prepared or used within 3 days of preparation after storage at 2-8°C.

Block buffer for all OptEIA Sets (BD Biosciences) and for DuoSet for mouse IL-12p40 (R&D Systems):

Assay diluent is used as block buffer.

Block buffer for all DuoSets (R&D Systems):

BSA	1 %
Sucrose	5 %
NaN_3	0.05 %
in PBS	

Substrate solution:

Tetramethylbenzidin (TMB) Substrate Reagent Set	BD Biosciences, Heidelberg, Germany

The solution is stored at 2-8°.

Prior to use pre-warmed to room temperature Substrate Reagent A (H_2O_2 in buffered solvent) and B (3,3´,5,5´-tetramethylbenzidine in organic solvent) are mixed in equal volumes.

Stop solution:

H_2SO_4 x H_2O	2 N

2.1.8 Labware

Petri dishes 100 mm x 15 mm	Falcon BD Labware, Franklin Lakes, NJ, USA
Petri dishes 60 mm x 10 mm	Falcon BD Labware, Franklin Lakes, NJ, USA
Plates 24-well	Falcon BD Labware, Franklin Lakes, NJ, USA
Pipette 2 ml, 5 ml,10 ml, 25 ml	Falcon BD Labware, Franklin Lakes, NJ, USA
Syringe filter 0.2 µm Supor Acrodisk 32	Pall Gelman Sciences, USA
Syringe filter 1.2 µm Supor Acrodisk 32	Pall Gelman Sciences, USA
Tissue culture bottle 75 cm^2, 150 cm^2	Falcon BD Labware, Franklin Lakes, NJ, USA
Tube 15 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Tube 50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Tube polystyrene round-bottom 5 ml	Falcon BD Labware, Franklin Lakes, NJ, USA

2.1.9 Equipment

Analytical balances “Sartorius analytic A200S”	Sartorius GmbH, Göttingen, Germany
Autoclave “Tuttnauer Systec 2540 EL”	Peter Oehmen GmbH, Essen, Germany
Balances “Sartorius universal U3600”	Sartorius GmbH, Göttingen, Germany
Dryer “Hearaeus Instruments B15”	Heraeus Instruments, Hanau, Germany
Eppendorf centrifuge “Centrifuge 5810R”	Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany
Eppendorf centrifuge “Centrifuge 5415C”	Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany
FACSCalibur Flow Cytometry System	BD Biosciences, Heidelberg, Germany
Fume cupboard hood “LaminAir HB 2472”	Holten LaminAir A/S, Alleroed, Denmark
Homogeniser „Ultra-Turrax T25“	Janke & Kunkel GmbH & Co. KG, IKA-Labortechnik, Staufen, Germany
Incubator “Heraeus 6000”	Heraeus Instruments, Hanau, Germany
Lumi-Imager	Roche Applied Science, Boehrninger Mannheim, Mannheim, Germany
Magnetic stirrer „Ikamag RCT“	Janke & Kunkel GmbH & Co. KG, IKA-Labortechnik, Staufen, Germany
Microplate reader “Titertek Multiscan MCC”	Joint Ventire Company of Labsystems and Flow Laboratories, Helsinki, Finland
Microscope “Axiovert 25”	Carl Zeiss AG, Oberkochen, Germany
Microscope “Axiolab”	Carl Zeiss AG, Oberkochen, Germany
Mini-PROTEAN Electrophoresis Cell	Bio-Rad Laboratories GmbH, Munich, Germany
Mini Trans-Blot Cell	Bio-Rad Laboratories GmbH, Munich, Germany
Overhead stirrer “Heidolf”	Progen Scientific Ltd., Mexborough, UK
Power supply “Model 1000/500”	Bio-Rad Laboratories GmbH, Munich, Germany
Precision microplate reader “Emax”	Molecular Devices, Sunnyvale, CA, USA
Spectrophotometer “DU 650”	Beckman Instruments GbmH, Munich, Germany
Thermoshaker “Schutron”	Schutron Labortechnik, Quedlinburg, Germany

2.1.10 Animals

Female BALB/c mice	M&B A/S, Ry, Denmark
Female C3H/HeN mice	Harlan Winkelmann GmbH, Borchen, Germany
Female C3H/HeJ mice	Harlan Winkelmann GmbH, Borchen, Germany
Female TLR2$^{-/-}$ 129/Sv x C57BL/6J mice	Tularik Inc., South San Francisco, CA, USA; were bred in Technical University of Munich, Munich, Germany
Female C57BL/6J mice	Jackson Laboratories, Bar Harbor, Maine, USA
Male and female BioBreeding diabetes-prone (BBdp) rats	Animal Resources Division of Health Canada, Ottawa, Canada
Male and female BioBreeding control (BBc) rats	Animal Resources Division of Health Canada, Ottawa, Canada
Male and female Wistar-Furth (WF) rats	Charles River, St. Constant, Quebec, Canada

All animals were raised under specific pathogen-free conditions and were weaned at 23 day of age. They were given free access to food and water.

2.1.11 Diet

From the 23rd day after birth BBdp, BBc and WF rats were fed one of three diets:

NTP-2000 diet (Zeigler Bros., Gardners, PA, USA) (Rao, G.N., 1996)

37.3 % ground wheat, 22.2 % ground corn, 5 % soybean meal, 8.5 % oat hulls, 4 % of fish meal, 7.5 % of alfalfa meal, 5.5 % purified cellulose, 3 % corn oil and 3 % soy oil. This nonpurified diet was irradiated before use and contained low levels of chemical and microbial contaminants.

The other two diets were isocaloric and isonitrogenous semi-purified ones:

Wheat gluten (WG) diet

22.5 % wheat gluten (ICN Biochemicals, Cleveland, OH, USA), 50.2 % corn starch, 12 % sucrose, 5 % corn oil, 5 % fiber (Solka-Floc), 3.5 % AIN-76 (or AIN-93G) mineral

mix (ICN), 1 % AIN-76A (or AIN-93G) vitamin mix (ICN), supplemented with 0.2% choline bitartrate, 0.02% DL-methionine, 0.5% L-lysine, and 0.08% L-threonine to compensate for low sulphur amine acids in wheat proteins.

Hydrolised casein (HC) diet

20 % casein hydrolysate (pancreas S enzymatic hydrolysate; RedStar Bioproducts, Mississauga, Ontario, Canada), other components: 50.2 % corn starch, 12 % sucrose, 7 % soybean oil, 5 % fiber, 3.5 % AIN-76 (or AIN-93G) mineral mix, 1 % AIN-76A (or AIN-93G) vitamin mix, 0.2% choline bitartrate, and 0.3% L-cystine.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Isolation and culture of dendritic cells (DCs)

DCs were generated according to the method described previously (Lutz M.B. et al., 1999). Briefly, bone marrow cells (2 x 10^6) from tibiae and femurs of 7-12 weeks-old female BALB/c mice, C3H/HeN and C3H/HeJ were seeded in Petri dishes (100 x 15 mm) in 10 ml of culture medium containing 20 ng/ml of GM-CSF. As culture medium, very low endotoxin-RPMI 1640 medium, supplemented with 10 mM HEPES, 2 mM L-glutamine, 20 µg/ml gentamicin, 60 µg/ml penicillin, 50 µM 2-ME and 10 % heat-inactivated FCS was used. On the 3rd day 10 ml of culture medium containing 20 ng/ml of GM-CSF were added to the dishes. On the 7th day 10 ml of the medium were replaced by fresh culture medium supplemented with 20 ng/ml of GM-CSF. On day 9 non-adherent cells contained 90-94 % of DC or their progenitors ($CD11c^+$), < 0.5 % of macrophages ($CD14^+CD11c^-$), < 5 % of granulocytes ($Ly6G^+CD11c^-$) and < 1% of B cells ($B220^+CD11c^-$). 1 x 10^6/ml of these cells was seeded in 1 ml of culture medium supplemented with 10 ng/ml of GM-CSF in 24-well plates. After 20 h of stimulation with 1, 10 or 100 µg/ml of α-chymotrypsinized wheat gluten, or 0.3, 3 or 30 µg/ml of rhHsp60, or 10 ng/ml of LPS from *Escherichia coli* O26:B6, or 1, 10 or 100 ng/ml of recombinant murine interleukin 1β (rmIL-1β). BMDC were harvested and used for FACS analysis. Cell culture supernatants were collected and stored at -20°C before the determination of cytokines and chemokines.

2.2.1.2 Culture of macrophages

The mouse macrophage cell line J774 A.1 was purchased from the German Collection of Microorganism and Cell Culture (Braunschweig, Germany). J774 A.1 cells were cultured in RPMI 1640 medium supplemented with 10 % v/v FCS, 25 mg/L ampicillin, 120 mg/L peniciliin, 270 mg/L streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine and 10 ml/L non-essential amino acids (100x), 24 mM $NaHCO_3$ and 10 mM HEPES. Macrophages were grown in tissue culture bottle at 37ºC, 5 % CO_2. After reaching confluency, macrophages were washed with Solution A (see "Materials") and detached from the bottom by using accutase. After centrifugation of the cell suspension 5 min at 250 g the macrophages were resuspended in RPMI. The cell number was counted and adjusted to 1 x 10^6 cells/ml. The macrophages were seeded in a volume 200 µl per well in 96-well plate and incubated or not 23 h with 25 U/ml of rmIFN-γ. After that the cells were stimulated for 6 h with 1 or 10 ng/ml of LPS, 1, 10, 100 µg/ml of wheat gluten and different gliadin-peptides at concentration 100 µM. The cell culture supernatant was collected and stored at -20°C before the determination of TNF-α.

2.2.2 Biochemical methods

2.2.2.1 Solubilization of wheat gluten

Wheat gluten was digested with α-chymotrypsin (α-CT) according to the method described previously (Arentz-Hansen, E.H., et al., 2000). Briefly, wheat gluten powder and α-CT were mixed 100:1 (w/w) in 174 mM Tris-base (pH 7.8) and incubated overnight at 37°C. After centrifugation at 1 500 x g for 15 min at a room temperature, the supernatant was sterilized by filtering through 0.2 µm Acrodisc Syringe Filter. Protein concentration was determined by Bio-Rad protein assay, and aliquots of digested wheat gluten were stored at -20°C.

2.2.2.2 Isolation of gut tissue samples and preparation of tissue homogenates

The gut samples were isolated from rats in Dr. Fraser Scott´s lab (Ottawa Health Research Institute, Ottawa, Canada) according to the procedure described previously (Courtouis, P., et al., 2004) and sended on dry ice. They were stored at –80°C prior to

homogenisation. After thawing of 1cm of G2 gut sample (duodenum) was homogenized in 2 ml of PBS Dulbecco´s w/o calcium and magnesium + protease inhibitor cocktail Complete (Roche, Mannheim, Germany, 1 tablet is dissolved in 25 ml of PBS) on ice using Ultra-Turrax T25 homogenizer. After centrifugation at 1000 g for 10 min the supernatant was collected and filtered through 1.2 μm syringe filter Supor Acrodisk 32 (Pall Gelman Sciences, USA). The supernatant was stored at -80 ºC.

2.2.2.3 Preparation of cell lysates

7.0 x 10^6 DCs were seeded in 2 ml RPMI in Petri dishes 60 mm x 10 mm and incubated 15 or 45 min with the stimuli at 37ºC, 5 % CO_2. Thereafter the cells were harvested, transferred into 15-ml tube on ice with 8 ml of PBS Dulbecco´s w/o calcium and magnesium and w/o sodium bicarbonate. After shaking the tube was centrifuged 5 min at 500 g at 4ºC (Eppendorf centrifuge 5810 R, Germany), the supernatant was discarded and the cells were washed with ice-cold PBS as described before two times. After the last washing the pellet was vigorously resuspended with a pipette in 100 μl of ice-cold lysis buffer (see "Materials"). After that the lysate was transferred into 1.5-ml Eppendorf tube, rotated in overhead rotor "Heidolf" (Progen Scientific Ltd., UK)) 30 min at 4ºC and centrifuged 15 min at 10 000 g at 4ºC. The supernatant was transferred into ice-cold 1.5–ml Eppendorf tube and the aliquot was taken for protein determination by Bio-Rad assay. The rest of supernatant was quickly frozen on dry ice and then stored at –80ºC until use. The protein concentration was in the range 3-4 mg/ml.

2.2.2.4 Protein determination

Protein concentration in the lysates was determined by Bio-Rad protein assay and in the gut tissue samples by measuring OD at 280 nm on Beckmann spectrophotometer (Beckmann Instruments, Fullerton, CA, USA) using BSA calibration curve (0-5 mg/ml).

2.2.2.5 SDS-Polyacrylamide Gel Electophoresis (SDS-PAGE) of proteins

SDS-PAGE is used to separate polypeptides according to their molecular weight. Before loading to the gel, proteins are dissociated, most commonly, by means of strongly anionic detergent SDS (Laemmli, U.K., 1970) in combination with a reducing agent and heat.

The amount of bound SDS is proportional to the molecular weight of polypeptide. After binding to SDS denaturated poplypeptides become negatively charged, and SDS-polypeptide complexes are moved in the gel in discontinuous buffer system (Ornstein, L., 1964; Davis, B.J., 1964) after the application of the gel to an electric current. After migrating through a stacking gel of high porosity, the complexes are deposited in a very thin zone (or stack) on the surface of the resolving gel. The sample and the stacking gel contains Tris·Cl (pH 6.8), running buffer contains Tris-glycine (pH 8.3) and 1 % SDS (w/v), and the resolving gel contains Tris·Cl (pH 8.8). The leading edge of the moving boundary is formed by the chloride ions in the sample and stacking gel, while the trailing edge is composed of glycine molecules. After ionization of glycine resulting glycine ions migrate through the stacked polypeptide and travel through the resolving gel immediately behind the chloride ions. The SDS-polypeptide complexes, which are now free from the moving boundary, move through the resolving gel and are separated according to size by sieving. ProSieve gel used in our study is a unique modified acrylamide formulation which can be used as a gradient polyacrylamide gel. 10 % ProSieve gel gives optimal separation for polypeptides of 15-100 kDa. TEMED catalyzes the formation of free radicals from APS thus accelerating the polymerization of acrylamide.

For our mini format vertical gel electrophoresis two gels can be prepared at a time. For preparation of each gel two mini-glass plates (short plate 10.1 x 7.3 cm and spacer plate 10.1 x 8.2 cm) with spacers are placed into a casting frame in a way that their bottom surface is smooth. This position is fixed with screws, and the glass plates are firmly placed in a casting stand. The space between the glass plates is quickly filled with 10 % resolving gel solution (see “Materials”) right after addition of APS and TEMED. On the top of the gel ca. 800 µl of water was added. When the resolving gel is ready (in ca. 30 min.), the water is discarded, the 15-well comb is placed between the glass plates and the solution for 5 % stacking gel (see “Materials”) is added on the top of the resolving gel. After the stacking gel is ready (in ca. 30 min.) the comb is taken out, and the wells are washed with water or buffer. Thereafter the gel is transferred to electrophoresis tank, and SDS-running buffer (see “Materials”) is poured inside till the glass plates are covered (ca. 800 ml of the buffer). As it was mentioned before, proteins prior they are loaded to the gel, should dissociate into their polypeptide subunits by means of detergent SDS,

reducing agent and heat. Each sample should have the same concentration of protein in lysis buffer (2 mg/ml in our experiments). Diluted in Laemmli buffer (which contains SDS and reducing agent β-mercaptoethanol, see "Materials") samples are denaturated during 5-min. incubation at 95°C. Marker (see "Materials") is diluted 1:50 in Laemmli buffer without heating. Cold samples containing 14 μg of protein and marker are injected into the wells in the stacking gel. Thereafter the tank is connected to the power supply in a way that the positive pole (red plus) is at the far end and the negative pole (black minus) is at the closer end. As all the proteins have strong negative charge because of SDS, they will all move towards bottom of the gel. Electrophoresis starts with voltage 50 V for 20-30 min. When the samples reach the resolving gel, the voltage is changed to120 V for ca. 100 min. The electrophoresis is finished when the moving boundary is close to the bottom of a glass plate.

2.2.2.6 Electrophoretic transfer

To immobilize and further characterize the polypeptides which are separated by SDS-PAGE, they are transferred from polyacrylamide gel to nitrocellulose membrane by wet electrophoretic transfer (Towbin H. et al., 1979). Negatively charged proteins from gel drive over to the positively charged nitrocellulose membrane. We performed electrotransfer using Mini Trans-Blot Cell (Bio-Rad Laboratories Inc.). After the end of electrophoresis the stacking gel is cut off and the residual resolving gel is taken from the glass plate on a dry Whatman filter of gel size. In the cassette for electrotransfer the gel is placed in a following order: briefly wetted with blotting buffer (see "Materials") Whatman filter, dry Whatman filter, gel, Hybond-ECL nitrocellulose membrane (Amersham Biosciences) and two briefly wetted with blotting buffer Whatman filters. The air bubbles are carefully removed. The gel holder cassette is placed first into electrophoresis blotting module and then in the buffer tank in a way that nitrocellulose membrane faces the cathode. The Bio-Ice cooling unit in the buffer tank absorbs the heat generated during rapid transfers. After addition of blocking buffer the buffer tank is covered with lid and connected to the power supply: 100 V for 1 h. After the end of electrotransfer the nitrocellulose membrane is stained with Ponceau S (Serva) to visualize the polypeptide bands. The membrane can be stored at -20°C or further processed.

2.2.3 Immunological methods

2.2.2.1 Immunological determination of proteins by Western blot

To identify the protein of interest on the membrane, the specific primary antibodies are used. To avoid their binding to nitrocellulose membrane, it is blocked with 5 % skim milk in TBST (see "Materials") for 45 min. After washing three times for 5 min in TBST the nitrocellulose membrane is incubated with the respectively diluted primary antibodies (see "Materials") 1 h at room temperature or overnight at +4°C. Thereafter the membrane is washed three times for 5 min in TBST, and respectively diluted horseradish peroxidase (HRP)-conjugated secondary antibodies are added (see "Materials") for 1 h at room temperature. The membrane is washed two times for 5 min with TBST and 1 time for 5 min with TBS. To visualize the polypeptide bands, chemiluminescent detection is used. In the presence of hydrogen peroxide enzyme HRP bound to the second antibody catalyzes the oxidation of dyacylhydrazides like luminol producing an activated intermediate reaction product which decays to the ground state by emitting the light. Light emission is greatly enhanced by the presence of radical transmitter between the formed oxygen radical and luminol, 4-iodophenol. The light is detected by X-ray film. In our experiments nitrocellulose membrane was exposed 1, 3 or 10 min to the chemiluminescent reagent POD (Roche, see "Materials") and the light emission was visualized using Lumi-Imager (Roche, Germany). The quantification of the final image was performed by means of the programme "Lumianalyst" (Roche, Germany).

2.2.3.2 FACS analysis

The immune cells display a certain range of surface molecules which can be specifically labelled with fluorochrome-conjugated monoclonal antibodies. This makes possible cell analysis and sorting by flow cytometry. We used FACSCalibur Flow Cytometry System from BD Biosciences (Heidelberg, Germany). BD FACSCalibur is a four-color, dual-laser system that is capable of both cell analysis and sorting. The cells are analyzed while passing one at a time through a focused laser beam. Three of the used fluorochromes, fluorescein (FITC), phycoerythrin (PE) and Cy5-PE are excited by 488-nm light of an Argon ion laser, and allophycocyanin (APC) is excited by 595-nm of red diode laser. The emission spectrum of each fluorochrome is detected by a unique fluorescence channel.

The specificity of detection is controlled by the wavelength selectivity of optical filters. For the simultaneous work of multiple fluorescence channels, the emitted light is sorted by placing the filter at a 45° angle to a light source. In this case light which would have been transmitted by that filter is still transmitted but light that would have been blocked is reflected (at a 90° angle) and goes to another detector. Before starting multicolor FACS, a proper *compensation* should be done (detailed information is on the website *www.drmr.com*). The goal of compensation is to remove the spillover fluorescence of a particular probe from the "wrong" channel. Proper compensation requires that the median fluorescence of the stained population (positive) in every other unspecific for the stain channel is equal to the median fluorescence of unstained population (negative) in those channels. Normally autofluorescence of the positive and negative population should be the same in all channels. To make the correct compensation a population of cells stained with only a single color in a saturating amount is mixed, in roughly equal proportions, with an unstained population (or the population stained with isotype control), and the compensation is set using BD CellQuest software. Only after that multicolor FACS analysis of the cells of interest can be started. We used 2-, 3- and 4-color analysis.

The staining of surface molecules on BMDC with fluorochrome-conjugated monoclonal antibodies is performed on ice. After preincubation for 5 min with FcBlock to avoid non-specific binding, 2-4x10^5 BMDC are stained for 30 min with saturating amounts of the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-mouse I-A^d or anti-mouse I-A^k , R-phycoerythrin (R-PE)-conjugated anti-mouse CD86, anti-mouse CD54 or anti-mouse CD14, biotinylated anti-mouse CD40 or anti-mouse CD11c, allophycocyanin (APC)-conjugated anti-mouse B220 (see "Materials"). For each antibody an isotype control of appropriate subclass (BD Biosciences) was used. After washing twice with Cell Wash, BMDC were incubated for 30 min with streptavidin-conjugated Cy-Chrome for staining of biotinylated primary antibodies. Cells were washed twice with CellWash, fixed with 1 % paraformaldehyde and analyzed by flow cytometry.

2.2.3.3 ELISA

ELISA is a highly sensitive and specific immunoassay that uses enzyme-conjugated antibodies for measuring of antigen. The term "enzyme-linked immunosorbent assay" (ELISA) was first introduced by Engvall and Perlmann in 1971 (Engvall, E., and P. Perlmann, 1971). There are three main types of ELISA: direct, indirect and "sandwich ELISA". The latter is the most commonly used method. It allows measuring the amount of antigen between two layers of antibodies and quantifying of antigen in low concentrations and in the presence of high amounts of contaminated proteins. Cytokine sandwich ELISA can specifically detect and quantitate the concentration of soluble cytokine and chemokine proteins. The basic cytokine sandwich ELISA is a five-step procedure with an obligatory washing steps in between: 1) coating of the microtiter plate wells with highly purified anti-cytokine antibodies (capture antibodies) which are noncovalently absorbed onto plastic microwells, primarily as a result of hydrophobic interactions; 2) addition of block solution to reduce non-specific binding to uncoated region of the plate and non-specific binding sections of the absorbed proteins to prevent false positive results; 3) addition of antigen (soluble cytokine protein present in the sample) to the wells; 4) addition of biotin-conjugated anti-cytokine antibodies (detection antibodies) followed by an enzyme-labelled avidin or streptavidin; 5) addition of substrate that after reaction with the enzyme produces coloured product (in case of chromogenic substrate) which is measured spectrophotometrically by ELISA-plate reader at an appropriate optical density (OD) or light (in case of chemiluminescent substrate) which is detected by luminometer.

The concentration of cytokine of interest in the sample can be interpolated from the standard curve. Standard curve is incorporated into a sandwich ELISA assay by making serial dilutions of a standard cytokine protein solution of known concentration (typically ng or pg of cytokine/ml). The OD of unknown samples should fall within the linear portion of the standard curve. If the concentration of cytokine in the sample is higher, the appropriate dilution of the sample is necessary to do. The data are analyzed using ELISA computer software programme where also different standard curve fit analysis (linear, linear-log, log-log, or four parameter transformations) can be performed (Davies C., 1994). Because of the enzyme-mediated amplification of the detection antibody signal,

the sandwich ELISA can measure physiologically relevant (i.e., > 5-10 pg/ml) concentrations of specific cytokine and chemokine proteins in mixed cytokine milieus. Although many different types of enzymes for ELISA are known to date, HRP and alkaline phosphatase (AP) are the most common. For our experiments we used OptEIA Sets from BD Biosciences, where enzyme reagent was avidin-HRP conjugate, and DuoSets from R&D Systems with streptavidin-HRP conjugate as enzyme reagent. The chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB) yields a blue color when oxidized with H_2O_2 (catalyzed by HRP) with major absorbances at 370 nm and 652 nm (Josephy P. et al, 1982). The colour then changes to yellow with the addition of sulphuric acid with maximum absorbance at 450 nm. The procedure for BD and RD ELISA assays that we used in our study were slightly different.

The protocol for OptEIA ELISA assay (BD Biosciences):

1. Coat microwell with 50 µl/well of capture antibody diluted in coating buffer. Seal plate and incubate overnight at 4°C.
2. Aspirate wells and wash 3 times with > 300 µl/well of wash buffer. After last wash invert plate and blot on absorbent paper to remove any residual buffer.
3. Block plate with with 100 µl/well assay diluent. Incubate at room temperature for 1 h.
4. Aspirate/wash as in step 2.
5. Prepare standard dilutions in assay diluent (for the dilution of the samples we used RPMI in case of cell culture and ice-cold Complete in PBS for gut homogenates). Pipette 50 µl of each standard, sample and control into appropriate wells. Seal plate and incubate for 2 h at room temperature. As a positive control for all cell culture ELISAs pool of DC culture after stimulation with 1 µg/ml of LPS was used, the aliquots were stored at -20°C; for gut homogenate ELISAs pool of different homogenates was used, the aliquots were stored at -80°C.
6. Aspirate/wash as in step 2, but with 5 total washes.
7. Add 50 µl of detection antibody + avidin-HRP reagent to each well. Seal plate and incubate for 1 h at room temperature.
8. Aspirate/wash as in step 2, but with 7 total washes.
9. Add 50 µl of TMB + H_2O_2 to each well. Incubate plate without sealer for 30 min at room temperature in the dark.
10. Add 25 µl of 2 N H_2SO_4 to each well.
11. Read absorbance at 450 nm within 30 minutes of stopping reaction, subtract absorbance at 540 nm (reference filter) from the absorbance at 450 nm.

The protocol for DuoSet ELISA assay (R&D Systems):

1. Coat microwell with 50 µl/well of capture antibody diluted in PBS. Seal plate and incubate overnight at room temperature.
2. Aspirate wells and wash 3 times with > 300 µl/well of wash buffer. After last wash invert plate and blot on absorbent paper to remove any residual buffer.

3. Block plate with with 100 µl/well assay diluent. Incubate at room temperature for 1 h.
4. Aspirate/wash as in step 2.
5. Prepare standard dilutions in assay diluent (for the dilution of the samples we used RPMI in case of cell culture and ice-cold Complete in PBS for gut homogenates). Pipette 50 µl of each standard, sample and control into appropriate wells. Seal plate and incubate for 2 h at room temperature.
6. Aspirate/wash as in step 2.
7. Add 50 µl of detection antibody diluted in reagent diluent to each well. Seal plate and incubate for 2 h at room temperature.
8. Aspirate/wash as in step 2.
9. Add 50 µl of streptavidin-HRP to each plate. Cover the plate and incubate for 20 min at room temperature in the dark.
10. Aspirate/wash as in step 2.
11. Add 50 µl of TMB + H_2O_2 to each well. Incubate plate without sealer for 20 min at room temperature in the dark.
12. Add 25 µl of 2 N H_2SO_4 to each well.
13. Read absorbance at 450 nm within 30 minutes of stopping reaction, subtraction setting wavelength correction at 540 nm.

The absorbance was read at microplate reader "Titertek Multiscan MCC" (Labsystems, Helsiniki, Finland), and the data were calculated and analyzed by using the programme "MicroWin" version 3.0. The four parameter model was chosen for standard curve fit analysis.

2.2.4 LAL assay

This assay allows determining the low amount of endotoxin from gram-negative bacteria in a solution. In 1956 Bang F.B. (Bang, F.B., 1956) observed that a gram-negative infection of *Limulus polyphemus*, the horse-shoe crab, resulted in fatal intra-vascular coagulation. Later Levin J. and Bang F.B. (Levin, J., and F.B. Bang, 1964) demonstrated that this clotting was the result of the enzymatic reaction between endotoxin and a clottable protein in the circulating abemocytes of *Limulus*. They prepared a lysate from washed amebocytes which was an extremely sensitive indicator of the presence of endotoxin. The principle of present test is as follows: endotoxin from gram-negative bacteria catalyzes the activation of proenzyme in LAL, the activated enzyme in turn catalyzes the splitting of p-nitroaniline (pNA) from a colourless synthetic substrate Ac-Ile-Glu-Ala-Arg-pNA. After the reaction is stopped with stop reagent, the pNA release is measured photometrically at 405-410 nm. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1-1.0 EU/ml range. The concentration of endotoxin in a sample is calculated from the endotoxin standard curve.

Test procedure:

1. Into the appropriate well of microplate, pre-equilibrated in the heating block at 37°C, dispense 50 µl of blank (LAL Reagent Water), sample or endotoxin standard (1.0, 0.5, 0.25, 0.1, 0.05, 0.025 or 0.01 EU).
2. At time T = 0 add 50 µl of LAL to the first column of microplate and begin timing. Briefly take the mictoplate from the heating block, mix and return the plate to the heating block, cover the microplate.
3. At T =10 min pipette 100 µl of pre-warmed substrate solution to each well in the same manner as in step 2.
4. At T = 16 min add 50 µl of stop reagent to each well in the same manner as in steps 2 and 3.
5. Read the absorbance of each microplate well at 405-410 nm on microplate reader (the absorbance of milli Q water is zero).

The absorbance was read on a microplate reader "Emax" (Molecular Devices, Sunnyvale, CA, USA) and the data were calculated and analyzed by using the programme "SOFTmax". The four parameter model was chosen for standard curve fit analysis.

2.2.5 Statistics and graph computer programmes

The data were expressed as means ± SEM. Statistical analysis of the data (one-, two-way ANOVA and Student's two-tailed unpaired *t* test) and graph building were performed by using GraphPad Prism 3.0 software. Differences were considered statistically significant with $p < 0.05$.

3 RESULTS

3.1 In vitro study of the effects of food components on innate immunity

3.1.1 Wheat gluten

3.1.1.1 Wheat gluten stimulates maturation of BMDC

Incubation of BMDCs from BALB/c mice with 1, 10 or 100 μg/ml of the gluten preparation resulted in a significant dose-dependent increase of the surface expression of MHC class II molecule I-A^d, CD86, CD40 and CD54 on BMDC.

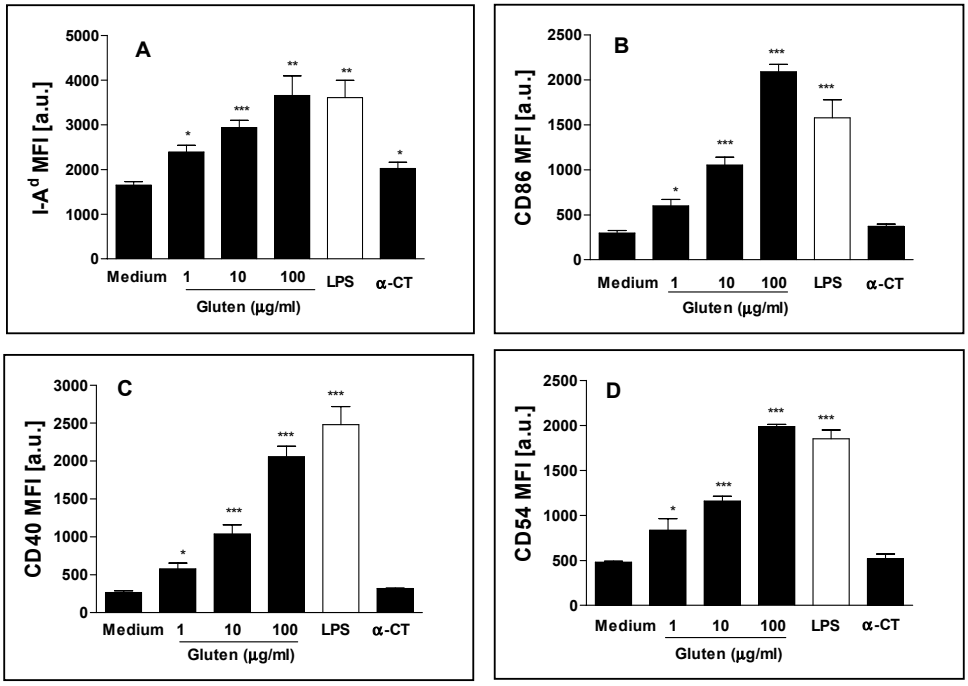

Fig. 3-1 Expression of surface molecules on BMDC after treatment with wheat gluten, LPS or α-CT. BMDC from BALB/c mice were incubated for 20 h with 1, 10 or 100 μg/ml of gluten, 10 ng/ml of LPS or 28 μg/ml of α-CT. Non-adherent cells were collected, stained for I-A^d (*A*), CD86 (*B*), CD40 (*C*) or CD54 (*D*), and analyzed by FACS. The data represent mean fluorescence intensities (MFI) ± SEM of three to six independent experiments. MFI is expressed in arbitrary units, [a.u.]. Significant differences to medium are indicated as $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$, Student's unpaired *t* test. One-way ANOVA for wheat gluten data shows $p < 0.001$ (*A*), $p < 0.0001$ (*B*), $p < 0.0001$ (*C*) and $p < 0.0001$ (*D*).

The mean fluorescence intensities (MFI) of these maturation markers after exposure to 100 μg/ml of gluten were 2.2-, 7.0-, 7.8- and 4.1-fold higher, respectively, as compared to medium control. Up-regulation of surface expression of maturation markers was similar to that seen on DC incubated with 10 ng/ml of LPS: 2.2-, 5.3-, 9.3-, 3.9-fold increase, respectively, compared to LPS. α-CT at the concentration of 28 μg/ml, corresponding to the enzyme content in 100 μg/ml of gluten preparation, did not enhance the expression of the investigated surface molecules on BMDC with the exception of I-A^d (23 % increase) (Fig. 3-1).

Double staining for MHC class II and co-stimulatory molecules, which are regulated partially independently, showed that stimulation of BMDCs with LPS caused parallel increase of I-A^d and co-stimulatory molecules CD86, CD40 or CD54. Incubation of BMDCs with gluten also induced coordinated up-regulation of I-A^d and co-stimulatory molecules, i.e. cells with moderate or high density expression of MHC class II also exhibited moderate or high expression of co-stimulatory molecules, respectively.

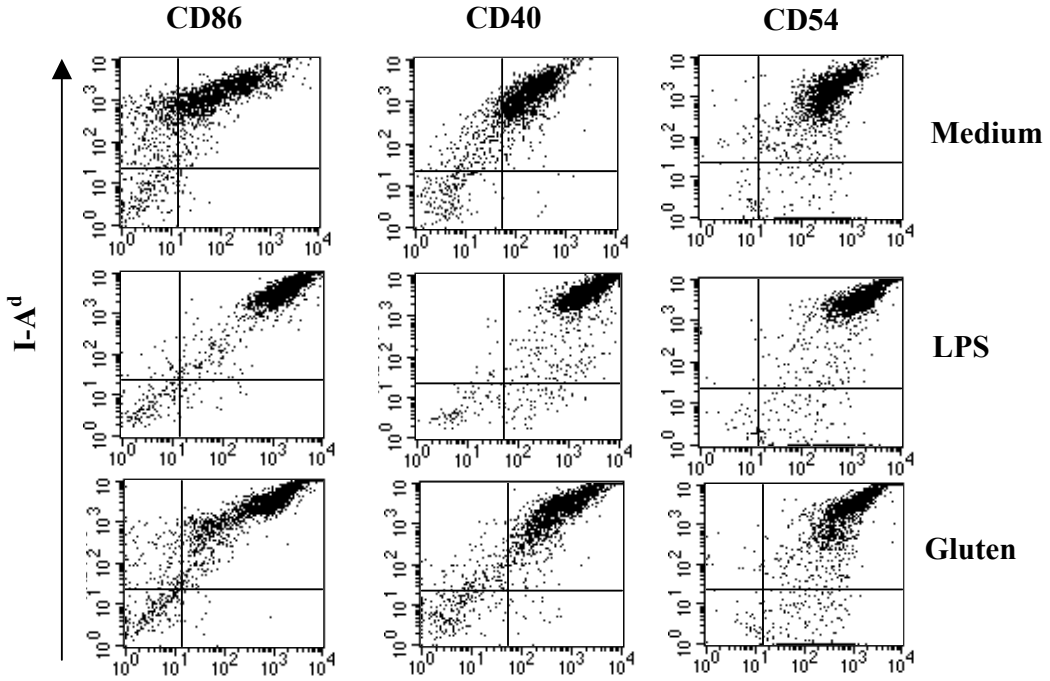

Fig. 3-2 Analysis of BMDC maturation after exposure to wheat gluten or LPS. BMDCs from BALB/c mice were cultured in medium or medium supplemented with 10 µg/ml of wheat gluten or 10 ng/ml of LPS for 20 h. After incubation, non-adherent cells were collected, stained for I-A^d, CD86, CD40 or CD54 and analyzed by FACS. Isotype controls were used to set quadrant lines (solid lines) in such a way, that the number of false positive cells was less than 5 % of all cells tested.

3.1.1.2 Wheat gluten-induced maturation of BMDC is not due to bacterial contamination

Our study shows that the crude preparation of wheat gluten has a stimulatory effect on BMDCs. As it is possible that gluten preparation can be contaminated with bacteria or their products, we have undertaken several approaches to clarify this point. First of all, wheat gluten pool was checked for the presence of LPS by using LAL assay. The average endotoxin content appears to be 0.016 endotoxin units (EU) (equivalent to 1.2 pg of LPS) per microgram of protein: in 1 µg/ml of wheat gluten preparation it was 0.023 EU (equivalent to 1.7 pg of LPS), and in 10

µg/ml – 0.102 EU (equivalent to 7.7 pg of LPS). We then studied whether LPS in such a small concentrations affects BMDC maturation and found out that at 10 pg/ml of LPS from *E.coli* (Sigma) the expression of maturation markers on BMDCs was not different from the control.

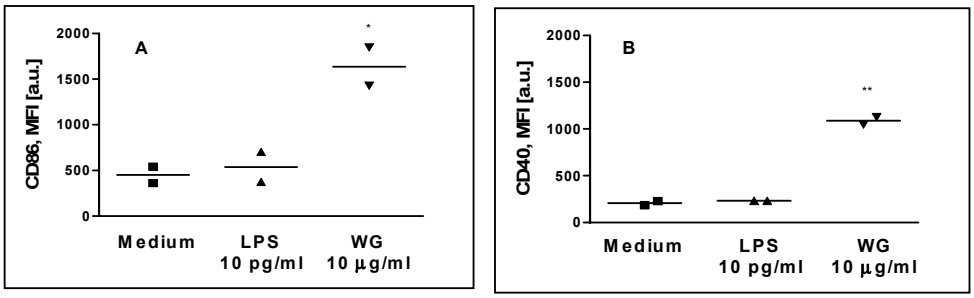

Fig. 3-3 The effect of low concentration of LPS on maturation of BMDCs. Wheat gluten (10 µg/ml) or LPS (10 pg/ml) were incubated with BMDCs from BALB/c mice for 20 h. Thereafter non-adherent cells were harvested, stained for CD86 (*A*) and CD40 (*B*) and analyzed by FACS. The data represent MFI of two independent experiments. Significant effects were indicated as $^{*}p < 0.05$ and $^{**}p < 0.001$, Student's unpaired *t* test.

Because LPS can derive from different bacterial sources it was worth using another approach to check for endotoxin contamination of wheat gluten preparation. Antibiotic peptide PmB is widely used as a blocker of LPS from Gram-negative bacteria, it binds to the lipid A portion of LPS (Storm D.R. et al., 1977; Morrison D.C. et al., 1976). We preincubated wheat gluten with PmB 1 h at 4°C for neutralization of endotoxin, LPS plus PmB served as a control.

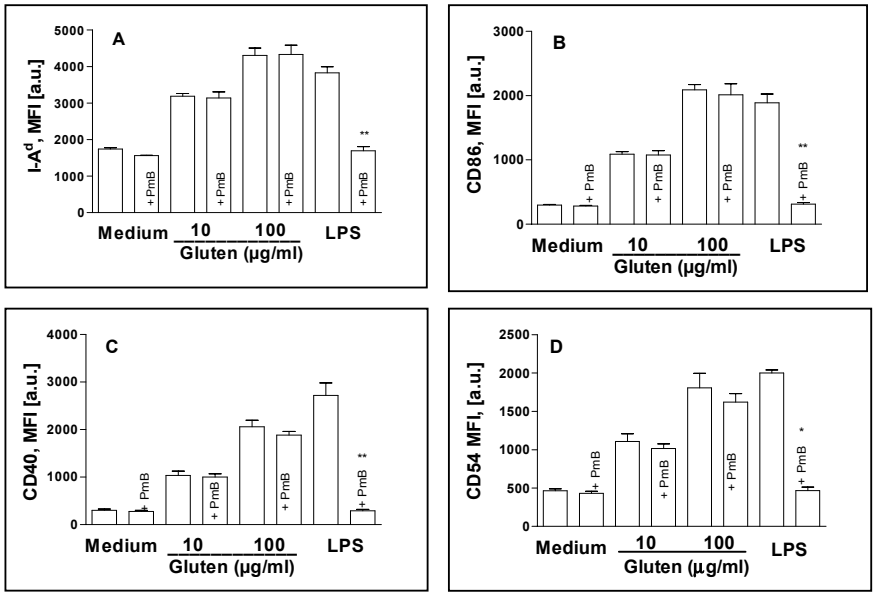

Fig. 3-4 The effect of PmB on wheat gluten- or LPS-induced maturation of BMDCs. Wheat gluten (10 and 100 µg/ml) or LPS (10 ng/ml) were incubated with 10 µg/ml PmB (1 h, 4°C) prior to the addition to BMDCs from BALB/c mice for another 20 h. Thereafter non-adherent cells were harvested, stained for I-A^d (*A*), CD86 (*B*), CD40 (*C*) or CD54 (*D*) and analyzed by FACS. The data represent MFI ± SEM of three independent experiments. Significant effects of PmB treatment are indicated as $^{*}p < 0.05$ and $^{**}p < 0.001$, Student's unpaired *t* test.

As expected, preincubation with PmB completely abrogated LPS-induced expression of I-A^d, CD86, CD40 and CD54 molecules. In contrast, when gluten was preincubated with PmB, no suppression of BMDC maturation was observed. This held true for all four maturation markers analyzed.

Next we investigated the effect of gluten on the maturation of BMDCs from C3H/HeJ mice that express a mutated, largely non-reactive LPS receptor TLR4 (Poltorak, A., et al, 1998). BMDCs from wild type C3H/HeN mice served as control.

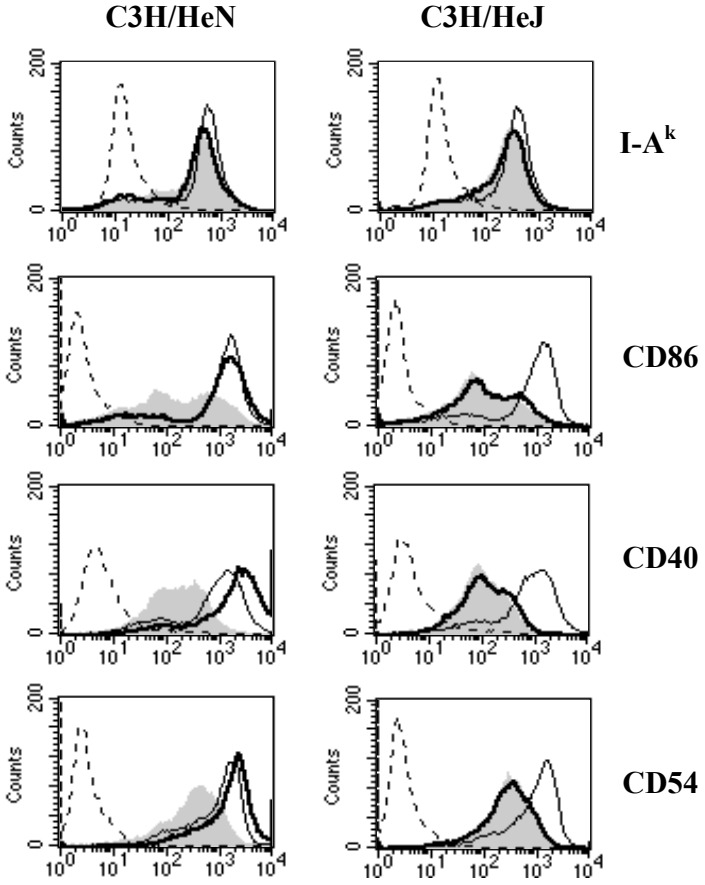

Fig. 3-5 TLR4-independent maturation of BMDC in response to wheat gluten. BMDCs from C3H/HeN and C3H/HeJ mice were incubated with or without 10 µg/ml of wheat gluten (standard solid line) or 1 ng/ml LPS (thick solid line) for 20 h. Thereafter non-adherent cells were harvested, stained for I-A^k, CD86, CD40 or CD54 and analyzed by FACS. The untreated medium control is shown as a shaded grey area and the isotype control as broken line, respectively.

BMDCs isolated from the two strains were incubated with gluten or LPS for 20 h. Our results show that fluorescence intensity curves for gluten versus LPS- stimulated BMDCs largely overlapped in C3H/HeN mice. By contrast, BMDCs from C3H/HeJ were unresponsive to LPS, whereas gluten retained its property to induce cell maturation. In BMDCs from both strains, MHC class II expression on non-stimulated cells was already high and could not be further enhanced by LPS or gluten treatment. As in BALB/c mice, the effect of gluten was dose-dependent in both strains. Both, background and induced expression of maturation markers was

consistently higher in BMDCs from C3H/HeN mice as compared to BMDCs from TLR4-defective mice.

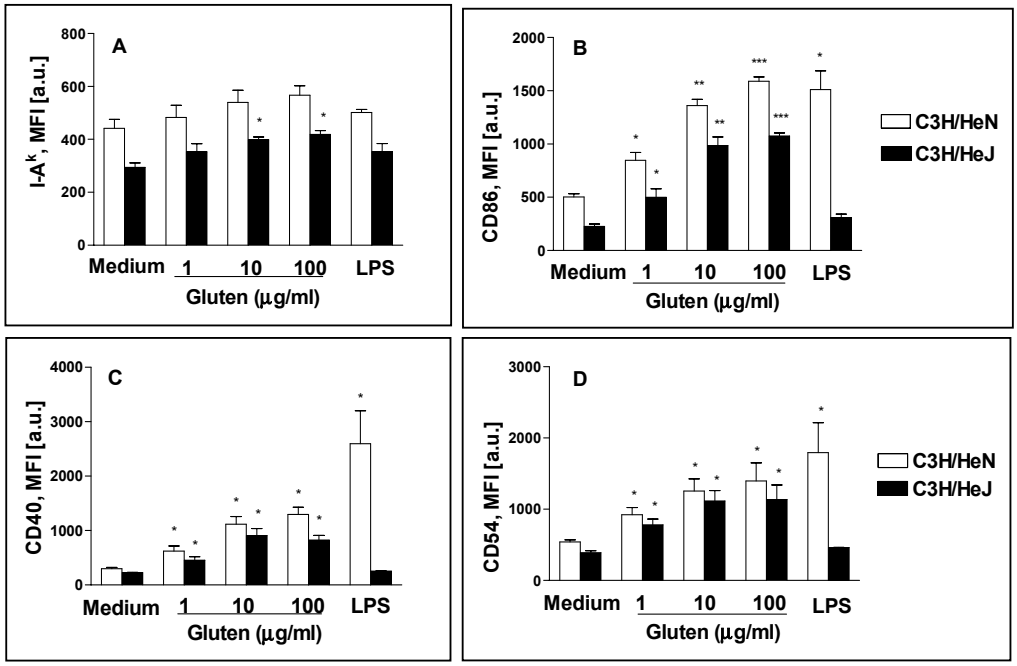

Fig. 3-6 Dose-dependency of wheat gluten-induced BMDC maturation in C3H/HeN versus C3H/HeJ mice. BMDCs were incubated with 1,10 or 100 µg/ml of wheat gluten or 1 ng/ml LPS for 20 h. Thereafter non-adherent cells were harvested, stained for I-A^k (*A*), CD86 (*B*), CD40 (*C*) or CD54 (*D*) and analyzed by FACS. The data represent mean MFI ± SEM of three independent experiments. Significant differences to the medium control of corresponding strains are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

These results indicate that TLR4 is not involved in gluten-induced BMDC maturation.

Finally, our last approach was to study whether TLR2 is involved in stimulation of BMDCs by wheat gluten. TLR2 is the main receptor for the majority of bacterial ligands, namely, bacterial lipoproteins and lipopeptides, LPS from *Leptospira and Prophyromonas,* yeast cell walls (zymosan), whole mycobacteria, mycobacterial lipoarabinomannan (LAM), whole Gram-positive bacteria, peptidoglycan (PGN), *Treponema* glycolipid and *Trypanosoma cruzi* glycophosphatidylinositol anchor; TLR2 together with TLR1 recognizes triacylated (at the NH2-terminal cysteine residue) lipopeptides, and together with TLR6 – diacylated lipopeptides, Gram-positive bacteria, PGN and zymosan (Akira S. et al., 2001; Akira S., 2003).

We isolated BMDC from female TLR2$^{-/-}$ 129/Sv x C57BL/6J mice and female C57BL/6J mice, the latter were used as a control. Then we stimulated BMDCs with different concentrations of wheat gluten preparation or 1 ng/ml of MALP-2 (Mühlradt P.F. et al., 1997)

or 1 ng/ml of LPS. LPS signals mainly through TLR4 and MALP-2 as diacylated at the NH_2-terminal cysteine residue lipopeptide – through TLR2+TLR6 (Takeuchi O. et al., 2001). In our study wheat gluten, irrespectively from the strain used, induced dose-dependent maturation of BMDCs, LPS also caused high expression of the maturation markers on BMDCs from TLR2$^{-/-}$ mice, whereas the stimulatory effect of MALP-2 was completely abrogated in TLR2$^{-/-}$ mice. Therefore, maturation of BMDCs in response to α-chymotryptic wheat gluten digest is also TLR2-independent.

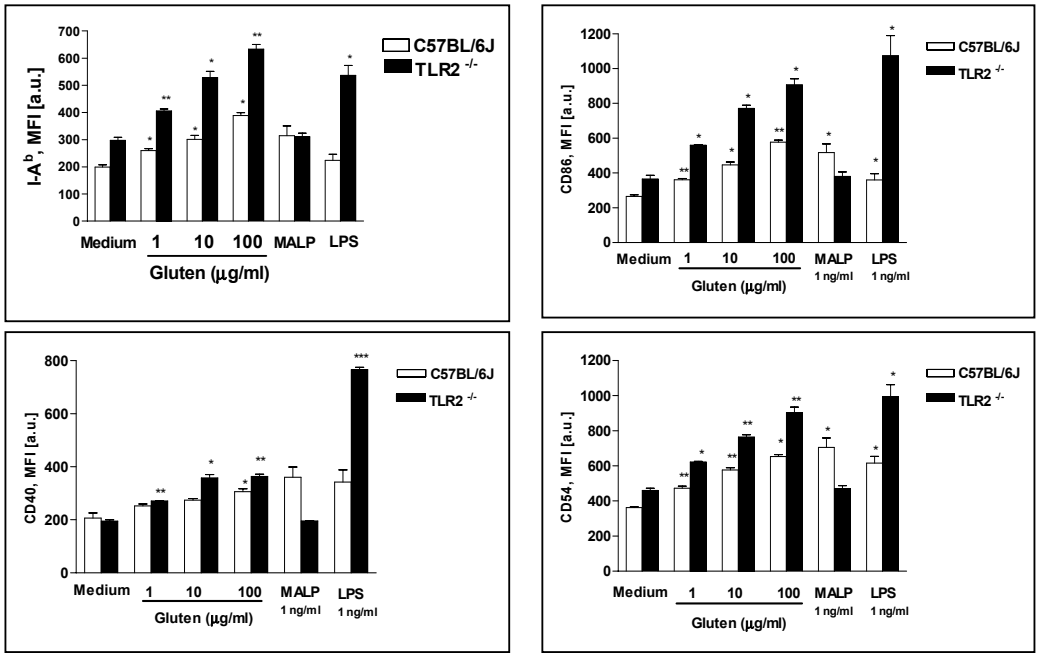

Fig. 3-7 Effect of TLR2 on maturation of mouse BMDCs. BMDCs from C57BL/6J (white bars) and TLR2$^{-/-}$ 129/Sv x C57BL/6J (black bars) mice were incubated with 1,10 or 100 µg/ml of gluten or 1 ng/ml MALP-2 or 1 ng/ml LPS for 20 h. Thereafter they were stained for I-A^b, CD86, CD40 or CD54 and analysed by FACS. The data represent mean values ± SEM of four independent experiments (for CD40 – n = 3-4). Significant differences to respective medium control are indicated as * $p < 0.05$, ** $p < 0.001$, Student's unpaired *t* test.

3.1.1.3 Wheat gluten induces cytokine and chemokine production in BMDC

We investigated whether wheat gluten digest induces the production of cytokines or chemokines in BMDC, in addition to its effect on maturation.

Gluten stimulated the dose-dependent release of IL-1β from BMDCs. Maximal IL-1β release was seen at the gluten dose of 100 µg/ml (337.3 ± 32.1 pg/ml), which was about 40 % of the IL-1β levels seen in response to LPS (Fig. 3-8*A*). TNFα secretion was only minimally induced by the highest concentration of gluten, although it was strongly induced by LPS (Fig. 3-8*B*). An even more striking disparity between LPS and gluten was observed for IL-10 production, which

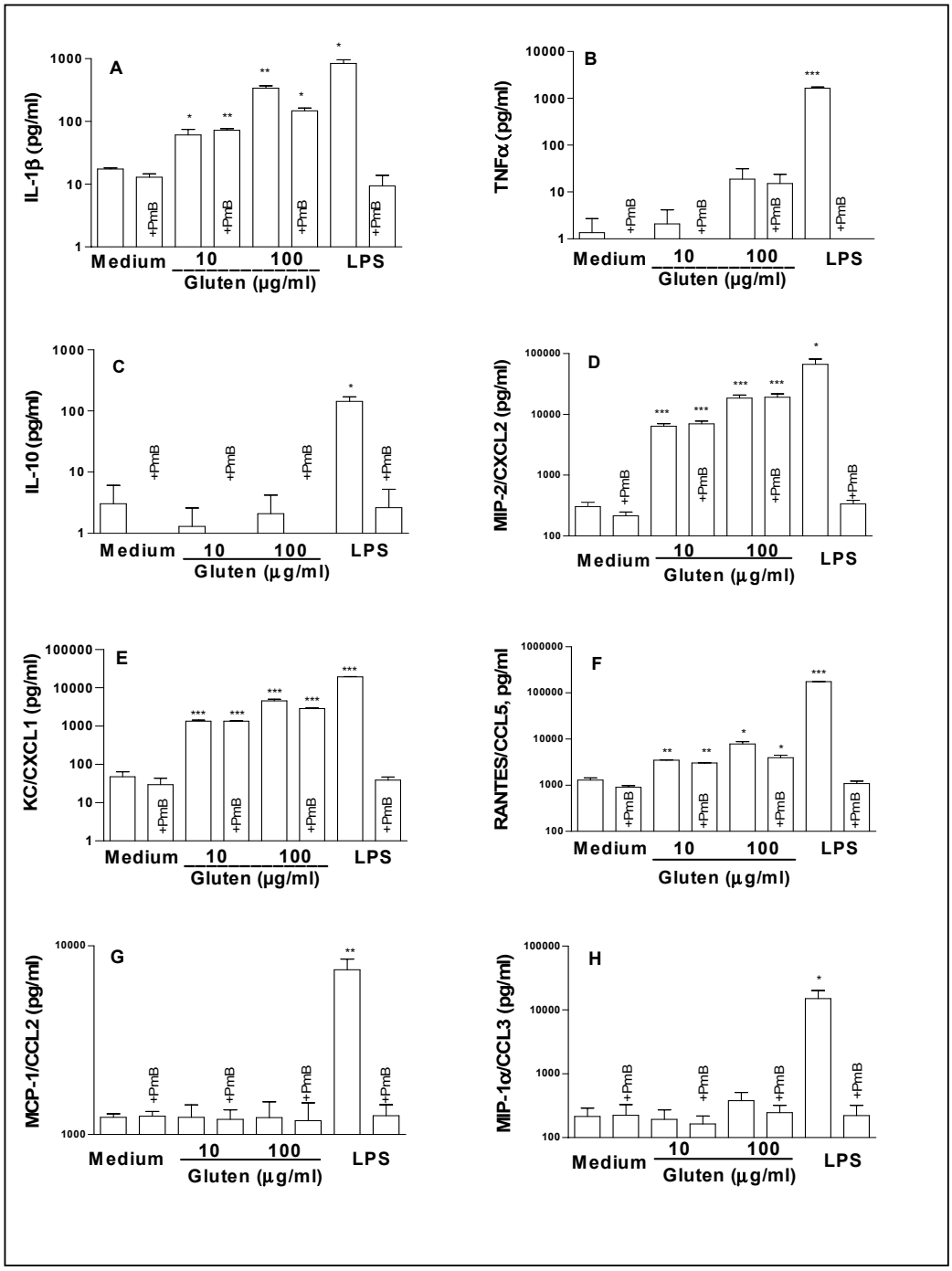

Fig. 3-8 Cytokine and chemokine secretion from BMDCs in response to wheat gluten or LPS. BMDCs of BALB/c mice were incubated for 20 h with 1, 10 or 100 µg/ml of wheat gluten or 10 ng/ml of LPS. Thereafter the cells were collected, centrifuged at 300 x g for 5 min and the concentrations of IL-1β (*A*), TNFα (*B*), IL-10 (*C*), MIP-2/CXCL2 (*D*), KC/CXCL1 (*E*), RANTES/CCL5 *(F)*, MCP-1/CCL2 (*G*) or MIP-1α/CCL3 *(H)* were determined in the supernatant by ELISA. The incubation of all the stimuli with 10 µg/ml of PmB (1 h, 4°C) was performed prior to their addition to BMDCs. The data represent mean values ± SEM of two to five independent experiments. Significant differences from medium control are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

was only induced by LPS (Fig. 3-8*C*). Interestingly, gluten induced the production of large amounts of chemokines macrophage inflammatory protein (MIP)-2/CXCL2 (Fig. 3-7*D*), keratinocyte-derived cytokine (KC)/CXCL1 (Fig. 3-8*E*) and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES)/CCL5 (Fig. 3-8*F*), while MIP-1α/CCL3 and monocyte chemoattractant protein (MCP-1)/CCL2 were not induced (Fig. 3-8,*G,H*). By contrast, LPS caused the secretion of all five chemokines from BMDCs (Fig. 3-8,*D-H*).

We have seen by using different approaches that LPS in wheat gluten digest does not participate in its maturation effects on BMDCs. In attempt to clarify this point in relation to wheat gluten-induced chemokine and cytokine secretion from BMDCs, we at first investigated the impact of LPS blocker PmB on it. PmB partially suppressed the production of IL-1β (Fig. 3-8*A*) and CXCL1 (Fig. 3-8*E*) in response to the highest dose of gluten ($p < 0.05$), but not for lower gluten concentrations or for CXCL2 or TNFα (Fig. 3-8,*A,B,D*). In contrast to gluten, LPS-induced secretion of all investigated cytokines and chemokines from BMDC was completely abolished by PmB (10 μg/ml) treatment (Fig. 3-8,*A-H*).

Secondly, we studied whether TLR4 as a main LPS receptor is necessary for gluten-stimulated cytokine and chemokine production.

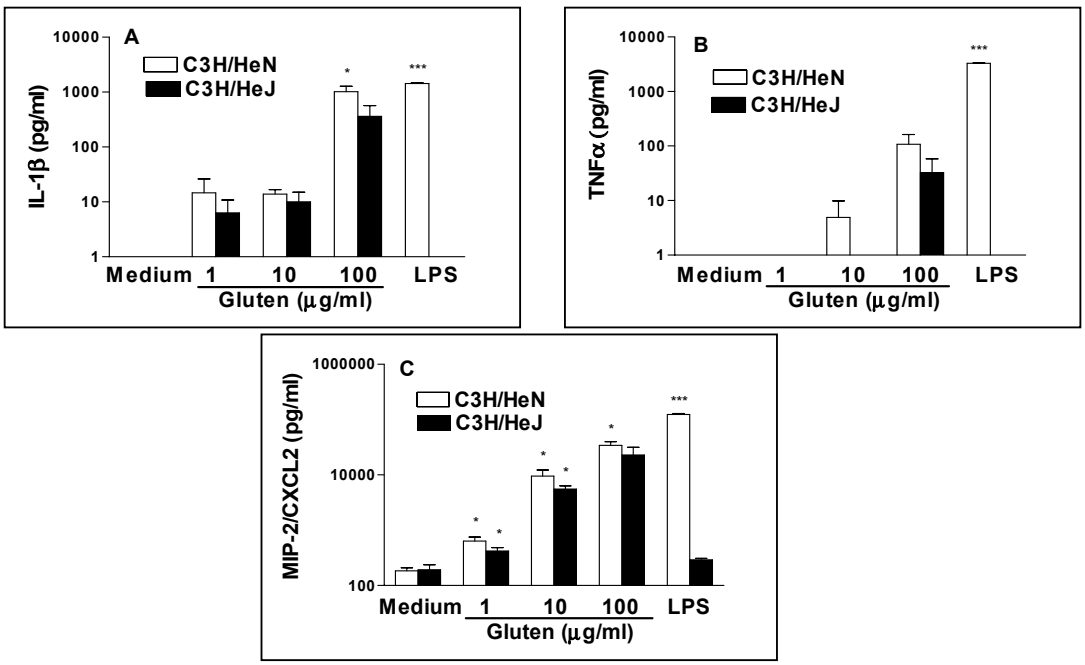

Fig. 3-9 Role of TLR4 in cytokine and chemokine production from BMDCs in response to gluten. BMDCs from C3H/HeN or C3H/HeJ mice were incubated with 1, 10 or 100 μg/ml of wheat gluten or 1 ng/ml LPS for 20 h. The content of IL-1β (*A*), TNFα (*B*) or MIP-2/CXCL2 (*C*) in supernatant was determined by ELISA. The data represent mean values ± SEM of two to three independent experiments. Significant differences from medium control are indicated as * $p < 0.05$, *** $p < 0.0001$, Student's unpaired *t* test.

BMDCs from control C3H/HeN and TLR4-defective C3H/HeJ mice were incubated with 1, 10 or 100 μg/ml gluten. Gluten up-regulated the release of IL-1β, TNFα and CXCL2 from BMDCs of both strains (Fig. 3.9). As seen in the BALB/c strain, there was good response for IL-1β and CXCL2 and only little TNFα production. LPS caused cytokine and chemokine secretion from BMDCs of C3H/HeN, but not from C3H/HeJ mice (Fig. 3-9). Again, BMDCs of the mutant C3H/HeJ strain showed consistently lower reactivity. We can conclude that defect of TLR4 receptor does not influence the signaling of wheat gluten digest in BMDCs.

3.1.1.4 Wheat gluten-stimulated chemokine production in BMDC is not secondary to IL-1β secretion

IL-1β was previously shown to increase the production of CXCL2 and CXCL1, the expression of cytokine and costimulatory receptors on immune cells and CD40L-mediated maturation of DC (Calkins C.M. et al., 2002; Ohtsuka Y. et al., 2001; Tailor A.A. et al., 1999; Iwakura Y., 2002; Dinarello C.A., 1998; Luft T. et al., 2002). We therefore addressed the question whether wheat gluten-induced increase in chemokine secretion from BMDCs was secondary to IL-1β release seen in our system. In response to the highest wheat gluten concentration used in our study the level of IL-1β secreted from DC was < 0.4 ng/ml (Fig. 3-8*A*). At doses of 1-100 ng/ml, rmIL-1β caused moderate maturation of BMDCs, and at 1 ng/ml of IL-1β the expression of maturation markers on BMDCs and CXCL2 level were not different from that of the control (Fig. 3-10, *A,C*). CXCL1 production at 1 ng/ml of IL-1β was 33% higher than in the control (110.7 ± 1.5 vs. 82.8 ± 6.4, $p < 0.05$, Fig. 3-10*B*). This increase was negligible as compared to the 5.4-, 12.9- and 35.1-fold enhancement after BMDC exposure to 10 ng/ml and 100 ng/ml of IL-1β and 10 μg/ml of wheat gluten, respectively.

Therefore IL-1β at a concentration less than 1 ng/ml cannot stimulate BMDCs, and, consequently, CXCL2 and CXCL1 production in BMDC after cell incubation with 10 μg/ml of wheat gluten is not secondary to IL-1β secretion from BMDC. In accordance with these data, antibodies against IL-1 receptor I (IL-1RI) did not interfere with wheat gluten-mediated expression of maturation markers on BMDCs (Fig. 3-11).

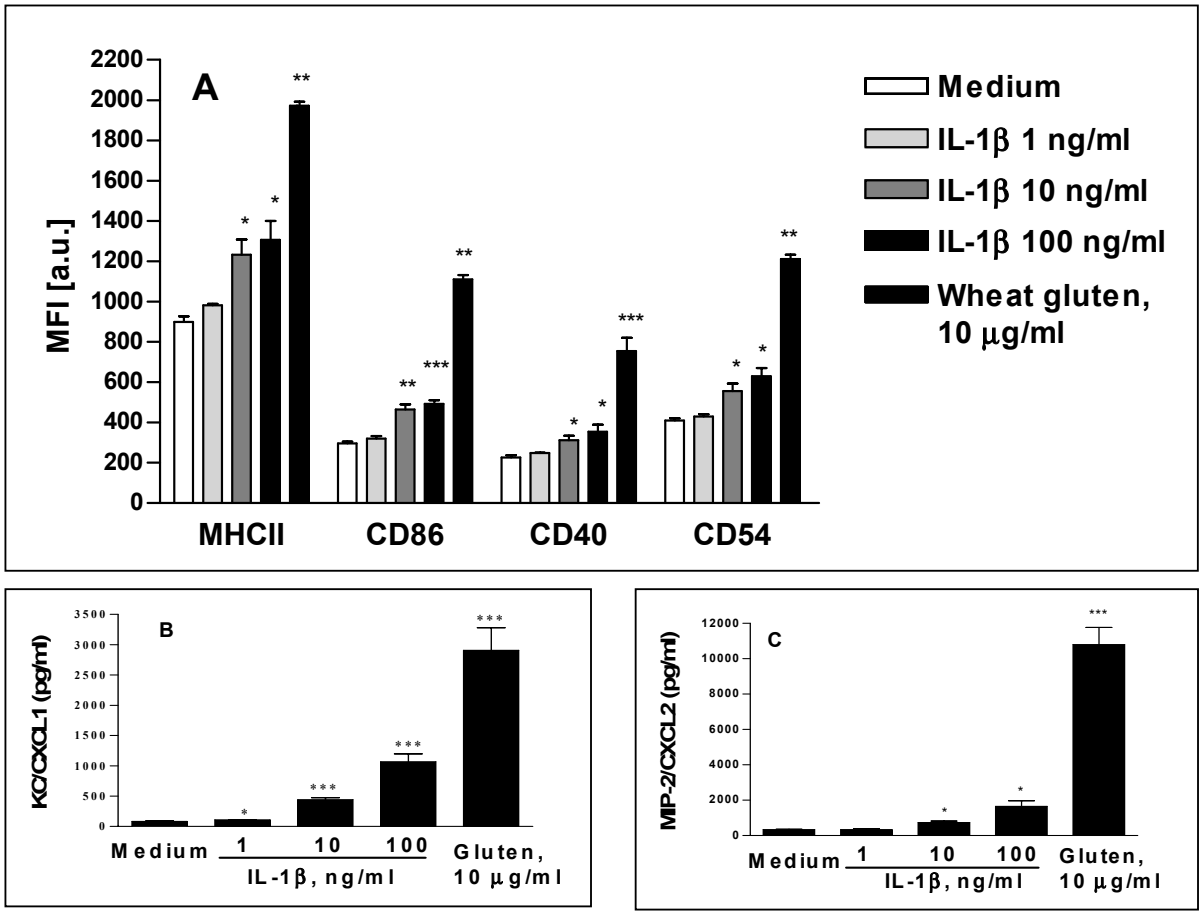

Fig. 3-10 Maturation of BMDCs and chemokine secretion in response to IL-1β. BMDCs of BALB/c mice were incubated for 20 h with 1, 10 and 100 ng/ml of IL-1β or 10 µg/ml of wheat gluten. Thereafter non-adherent cells were harvested, centrifuged at 300 x g for 5 min, stained for I-A^d, CD86, CD40 or CD54 and analyzed by FACS (*A*). The concentrations of KC/CXCL1 (*B*) and MIP-2/CXCL2 (*C)* were determined in the supernatant by ELISA. The data represent mean values ± SEM of two to five independent experiments. Significant differences from medium control are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

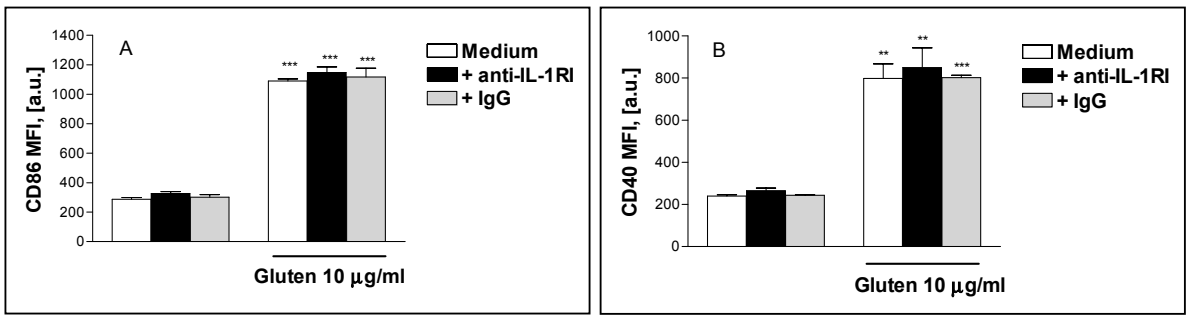

Fig. 3-11 The effect of anti-IL-1RI Ab on wheat gluten-induced BMDC maturation. BMDCs of BALB/c mice were incubated with 1 µg/ml of anti-IL-1RI Ab or 1 µg/ml of IgG for 15 min, followed by incubation with 10 µg/ml of wheat gluten for 20 h. Non-adherent cells were harvested, centrifuged at 300 x g for 5 min, stained for CD86 or CD40 and analyzed by FACS (*A,B*). The data represent mean values ± SEM of three independent experiments. Significant differences from respective controls are indicated as ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

3.1.1.5 Wheat gluten signals in BMDC through p38, extracellular signal-regulated kinases (ERK 1/2) and NFκB, but not c-Jun NH_2-terminal kinase (JNK)

ERK1/2, JNK and p38 represent a family of mitogen-activated protein kinases (MAPK), a members of an intracellular signalling cascade that are phosphorylated on both threonine and tyrosine in response to a wide variety of extracellular stimuli, including stress, cytokines, hormones etc. (Kyriakis, J.M. et al., 2001; Morrison, D.K. et al., 2003). NFκB is an important player in cellular inflammatory and immune responses, apoptosis and cell proliferation (Baeuerle, P.A. et al., 1994). Heterodimer NFκB composed of p65 and p50 subunits is sequestered in cytoplasm by an inhibitory proteins, IκBs, which are phosphorylated upon activation thus leading to the rapid degradation of the complex and the release of NFκB followed by its translocation to the nucleus for gene expression. During the phosphorylation and degradation of IκB p65 NFκB subunit is also phosphorylated. We investigated whether wheat gluten digest-induced BMDC stimulation signals through MAPK cascade and NFκB. LPS-treated BMDC were used as a control.

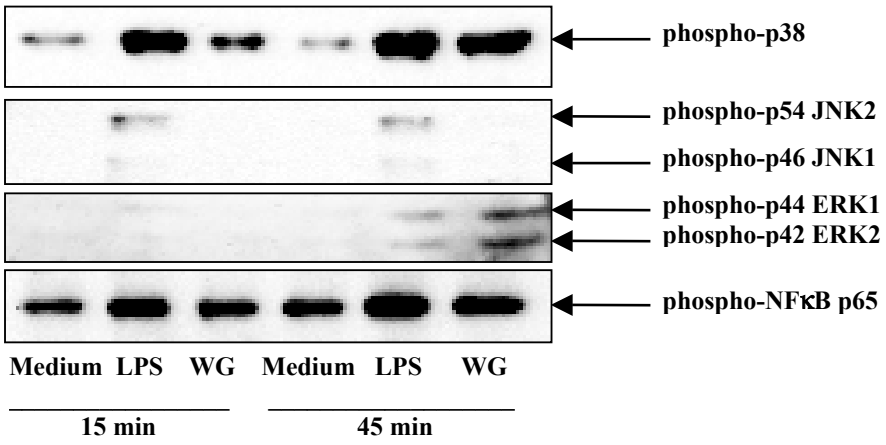

Fig. 3-12 Wheat gluten-induced signalling in BMDC. BMDCs of BALB/c mice were incubated with 10 µg/ml of wheat gluten digest or 10 ng/ml of LPS for 15 or 45 minutes. Thereafter cytoplasmic extracts of BMDCs were prepared and analyzed for the phosphorylated forms of p65-NFκB, p38, JNK 1/2, ERK 1/2 by Western blot.

Our results show that 10 µg/ml of wheat gluten activates p38 and NFκB already at 15 min and even more at 45 min after incubation, the same kinetic is seen for LPS at concentration 10 ng/ml, but with higher intensity. LPS induces low level of ERK 1/2 phosphorylation already 15 min after incubation with BMDC and moderate - at 45 min, in contrary to wheat gluten that

shows a prominent phosphorylation of ERK 1/2 45 min after BMDC stimulation, but the absence of any activation at 15 min. JNK1/2 in BMDCs were not activated by wheat gluten at investigated time points. In contrast, in BMDCs incubated with LPS for 15 and 45 min phospho-JNK2 is found at a moderate level and phospho-JNK1 – at a low level. In summary, wheat gluten digest stimulates BMDCs through activation of p38, NFκB and ERK 1/2, but not JNK 1/2. This pattern is different from one shown by LPS.

3.1.1.6 The active compound in wheat gluten preparation has a peptidic nature

To further investigate the chemical structure of the stimulatory compound of wheat gluten we digested it with proteinase K, a highly reactive serine endopeptidase with a broad specificity and the stability in a broad range of pH, buffer salts, detergents and temperature (Ebeling, W. et al., 1974; Petsch, D. et al., 1998). Proteinase K-treated wheat gluten completely lost the ability to induce the maturation of BMDC from BALB/c mice as it was estimated by the expression of surface molecules I-A^d, CD86, CD40 and CD54 (Fig. 3-13 *A-D*).

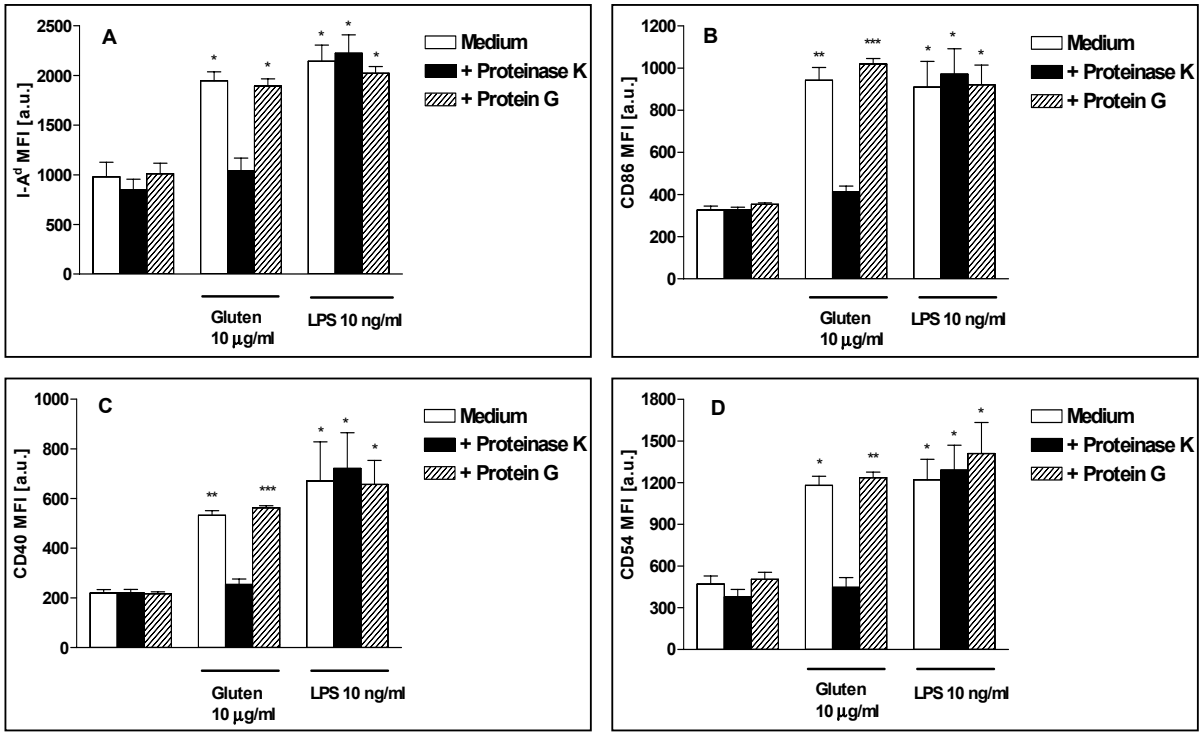

Fig. 3-13 The effect of proteinase K-digested wheat gluten on BMDC maturation. Wheat gluten or LPS were treated with 30 U/ml of proteinase K coupled to agarose beads and thereafter incubated with BMDCs of BALB/c mice for 20 h. Non-adherent cells were harvested, centrifuged at 300 x g for 5 min, stained for I-A^d, CD86, CD40 or CD54 and analyzed by FACS (*A-D*). The data represent mean values ± SEM of three independent experiments. Significant differences from medium control are indicated as $^{*}p < 0.05$, $^{**}p < 0.001$, Student's unpaired *t* test.

In contrast, proteinase K did not influence the stimulatory capacity of LPS on BMDCs. Protein G, which was used as a control to proteinase K, did not interfere with wheat gluten- or LPS-mediated maturation of BMDCs. Taken together, these data strongly suggest that the active compound of wheat gluten is of protein nature.

3.1.1.7 None of the investigated synthetic gliadin peptides reproduce stimulatory activity of wheat gluten α-chymotryptic digest on BMDC

Gluten peptides have various effects on immune system. Thus, many immunodominant T cell epitopes were found in α-gliadin, γ-gliadin and glutenin (de Ritis, G. et al., 1988; Molberg, Ø. et al., 1998; Quarsten, H. et al., 1999; Van de Wal, Y. et al., 1998, 1999; Arentz-Hansen, H. et al., 2000, 2002; Anderson, R.P. et al., 2000; Shan, L. et al., 2002; Vader, W. et al., 2002; Gianfrani, C. et al., 2003; Stepniak, D. et al., 2005), gliadin peptides stimulate murine peritoneal macrophages and human monocytes (Anderson, R.P. et al., 2000; Tučková, L. et al., 2002; Jelinkova, L. et al., 2004). Besides, nonimmunodominant peptide of α-gliadin induces the innate response and increase T cell recognition of dominant gliadin epitopes (Maiuri, L. et al., 2003).

To clarify the mechanism of gluten immunoreactivity on BMDCs in our study, we aimed to find the peptide from the crude α-chymotryptic wheat gluten digest that is responsible for observed gluten effects. From several immunoreactive gliadin peptides known from previous publications we chose for our study GP_{31-43} H2N-LGQQQPFPPQQPY-COOH (de Ritis, G. et al., 1988), $GDA7WHEAT_{242-253}$ ($GP_{242-253}$) H2N-FQQPQQQYPSSQ-COOH (Tučkova, L. et al., 2002), GP_{57-73} H2N-QLQPFPQPQLPYPQPQS-COOH (Anderson, R.P. et al., 2000) and GP_{56-88} H2N-LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF-COOH (Shan, L. et al., 2002), and ordered their synthesis with > 95 % purity to Eurogentec (Belgium). Upon receiving the peptides were diluted in sterile water for injections (aqua ad injectabilia DeltaSelect) to the concentration of 1 mg/ml, and aliquots were kept frozen at -20°C prior to their use in the experiments. LAL test was performed to check the presence of LPS in peptide samples. There was a trace amount of LPS in all the solutions: GP_{31-43} had 0.3 EU/mg of peptide (eq. to 3 pg LPS/mg of peptide), $GP_{242-253}$ - 0.03 EU/mg of peptide (eq. to 0.25 pg LPS/mg of peptide), GP_{57-73} - 0.014 EU/mg of peptide (eq. to 0.11 pg LPS/mg of peptide) and GP_{56-88} - 0.007 EU/mg of peptide (eq. to 0.2 pg LPS/mg of peptide). The synthetic gliadin peptide $GP_{242-253}$ induced a high production of TNFα, IL-10 and CCL5 from macrophages of C57BL/6 mice (Tučkova, L.

et al., 2002) and its preincubation with IFN-γ greatly increased the level of TNF-α. In attempt to reproduce this data we incubated wheat gluten, all investigated peptides or LPS with macrophages J774 that were prestimulated or not with IFNγ, and the production of TNFα was studied.

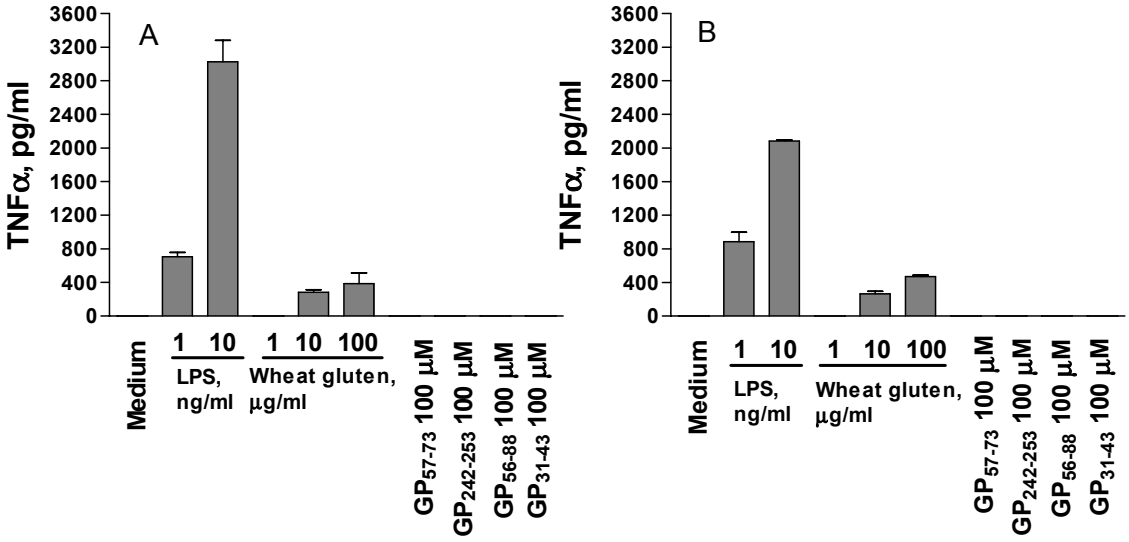

Fig. 3-14 TNFα production from macrophages J774 6 h after stimulation with wheat gluten, gliadin peptides or LPS. Macrophages J774 were incubated for 23 h with (A) or without (B) 25 U/ml of IFN-γ. Then 1 or 10 ng/ml of LPS, 1, 10 or 100 µg/ml of wheat gluten or 100 µM of gliadin peptides GP_{57-73} ,$GP_{242-253}$, GP_{56-88} or GP_{31-43} were added for another 6 h. Thereafter the cells were collected, centrifuged at 300 x g for 5 min and the concentrations of TNF-α was determined in the supernatant by ELISA. The data represent mean values ± SEM of three experiments.

According to our results, none of synthetic gliadin peptides, including $GP_{242-253}$, could induce TNFα synthesis from macrophages, although there was a clear dose-dependent TNFα production from macrophages after their incubation with wheat gluten or LPS (Fig. 3-14,*A,B*). Thereafter we investigated in vitro whether these peptides had any effect on the maturation of BMDCs. The statistical analysis of group data showed small, but significant effect of the peptide GP_{31-43} on CD40 and CD54 expression, although it was not dose-dependent in case of CD40 (Fig. 3-15,*G*,*H*). The peptide GP_{56-88} in concentrations 10µM and 100 µM stimulated the expression of CD86 and CD54 to the level higher than in the medium control, albeit the group statistic did not show the significance there (Fig. 3-15,*F*,*H*). The observed increase in the level of maturation markers on BMDCs in response to the exposure to GP_{56-88} and GP_{31-43} was very low and was not seen for all investigated maturation markers, as it was for wheat gluten preparation. That is why these gliadin peptides cannot be considered as being responsible for

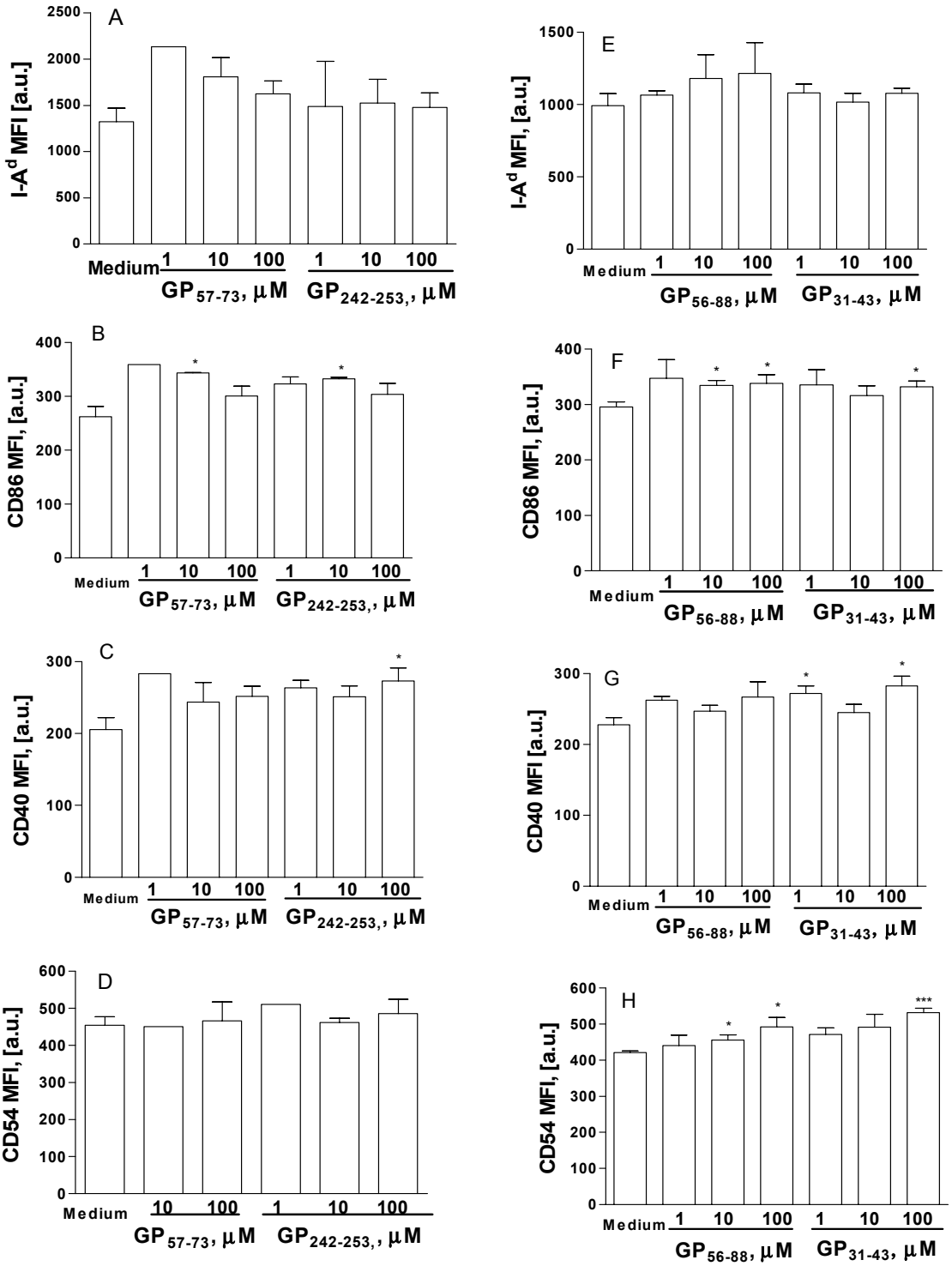

Fig. 3-15 The effect of gliadin peptides on BMDC maturation. BMDCs of BALB/c mice were incubated with 1, 10 or 100µM of gliadin peptides GP_{57-73} and $GP_{242-253}$ (*A-D*) or GP_{56-88} and GP_{31-43} (*E-H*) for 20 h. Non-adherent cells were harvested, centrifuged at 300 x g for 5 min, stained for I-A^d, CD86, CD40 or CD54 and analyzed by FACS. The data represent mean values ± SEM of one to five independent experiments. Significant differences from medium controls are indicated as $^{*}p < 0.05$, $^{***}p < 0.0001$, Student's unpaired *t* test. One-way ANOVA for GP_{31-43} CD40 (*G*) and CD54 (*H*) shows $p < 0.05$.

wheat gluten-induced BMDC maturation.

To have an additional support to this point of view, we studied the amount of CXCL2 and CXCL1, which are produced by BMDCs in response to the peptides GP_{56-88} and GP_{31-43}. As we saw previously, BMDCs produce a huge amount of CXCL2 and CXCL1 after exposure to wheat gluten (Fig. 3-8,*D,E*). In contrast, there was no difference between the level of chemokines in control medium and after the stimulation of BMDCs with the peptides (Fig. 3-16).

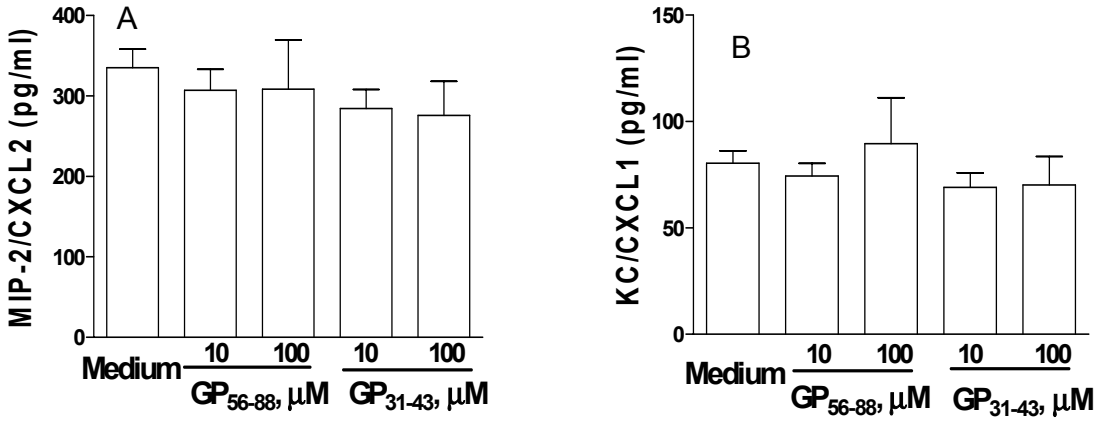

Fig. 3-16 Chemokine secretion from BMDCs in response to gliadin peptides GP_{56-88} and GP_{31-43}. BMDCs of BALB/c mice were incubated for 20 h with 10 or 100 µM of gliadin peptides GP_{56-88} and GP_{31-43}. Thereafter the cells were collected, centrifuged at 300 x g for 5 min and the concentrations of MIP-2/CXCL2 (*A*) and KC/CXCL1 (*B)* were determined in the supernatant by ELISA. The data represent mean values ± SEM of four to six independent experiments.

We conclude that none of four investigated synthetic gliadin peptides is responsible for the observed stimulatory effect of α-CT digest of wheat gluten on BMDCs.

3.1.1.8 PEP does not affect the stimulatory capacity of active wheat gluten peptide(s) on BMDC

Wheat gluten is a protein that is rich in proline (Pro) and glutamine (Gln) amino acids. After chymotryptic hydrolysis of wheat prolamines and glutenins followed by gel filtration, cation and anion exchange chromatography the resulted peptide fraction of wheat gliadins had Gln > 44, Pro > 28 and Phe > 7 mol-% and typical composition of the wheat glutenin peptides was Gln > 30, Pro > 15 and Gly > 8 mol-%, although some HMW peptide fractions from glutenin had Gln > 49 and Gly > 19 mol-% , Pro was on the third place (Wieser, H. et al., 1984). PEP is post-proline cleaving enzyme, which is generally cytosolic and found in vertebrates, plants and

Flavobacterium meningosepticum. Although this enzyme is not among pancreatic proteases or on the intestinal brush-border membrane, but when added to gliadin peptides, cleaves rapidly the T cell immunodominant epitopes in α-gliadin, although not so effectively in γ-gliadin and decreases the number of T cell immunostimulatory peptides in proteolyzed food-grade gluten (Shan, L. et al., 2002; Hausch, F. et al., 2002; Piper, J.L. et al., 2004; Marti, T. et al., 2005). We would like to investigate whether PEP could abrogate the capacity of wheat gluten chymotryptic digest to stimulate mouse BMDCs in our study. The specific activity of PEP from *Flavobacterium meningosepticum* (US Biological, USA) was 53 U/mg, and the enzyme solution was prepared in 0.1 M phosphate buffer, pH 7.0, as suggested by the company. The final PEP concentration in reaction mixture and incubation time with the substrate was calculated as described before (Shan, L. et al., 2002; Hausch, F. et al., 2002). According to our data PEP did not influence the stimulatory effect of α-chymotryptic digest of wheat gluten on mouse BMDC maturation (fig. 3-17).

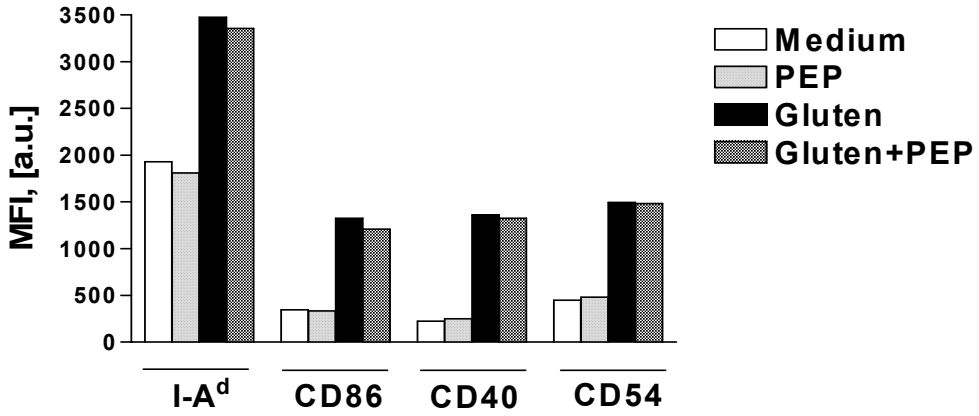

Fig. 3-17 The effect of PEP-digested wheat gluten on BMDC maturation. Wheat gluten (360 μg/ml) was incubated with PEP from *Flavobacterium meningoseptum* (500 mU/ml) for 5 h at 30°C followed by heat inactivation of PEP during 5 min at 95°C. After rapid cooling the mixture was added to BMDCs of BALB/c mice till final concentration of wheat gluten 10 μg/ml, and the cells were incubated with the stimuli for 20 h. Thereafter the cells were collected, centrifuged at 300 x g for 5 min, stained for I-A^d, CD86, CD40 or CD54 and analyzed by FACS. The figure represents one experiment.

3.1.2 Hsp60.

3.1.2.1 Hsp60 induces maturation of BMDC

Human recombinant Hsp60 for our experiments was kindly provided by Peptor, Rehovot, Israel. We aimed to find out whether Hsp60 could directly stimulate maturation of BMDC, and chose the concentration range 0.3-30 μg/ml. Before starting the study the amount of bacterial

LPS in Hsp60 preparation was determined by LAL assay. The endotoxin content appeared to be 0.05 EU (equivalent to 5 pg of LPS) per μg of protein. This means that at Hsp60 concentrations 0.3, 3 or 30 μg/ml corresponding amount of LPS in the media was rather low: 1.5, 15 or 150 pg of LPS. As a control stimuli 10 ng/ml of LPS were used.

Hsp60 induced dose-dependent maturation of BMDCs from BALB/c mice as shown from up-regulation of surface molecules I-A^d, CD86, CD40 or CD54 (Fig. 3.18,*A-D*). MFI of these maturation markers on the surface of BMDCs treated with 30 μg/ml Hsp60 were 1.7-, 4.2-, 6.4-, and 3.7-fold higher than for untreated control cells. These data were comparable with the level of surface expression of I-A^d, CD86, CD40 and CD54 on BMDCs after the stimulation with 10 ng/ml of LPS: their MFI were 1.8-, 4.5-, 7.5-, and 2.9-fold higher, respectively, than in control cells (Fig. 3-18,*A-D*). Already 0.3 μg/ml of Hsp60 was stimulatory for BMDCs, inducing not very high, but significant up-regulation of all investigated maturation markers (Fig. 3-18 *A-D,* Fig. 3-19).

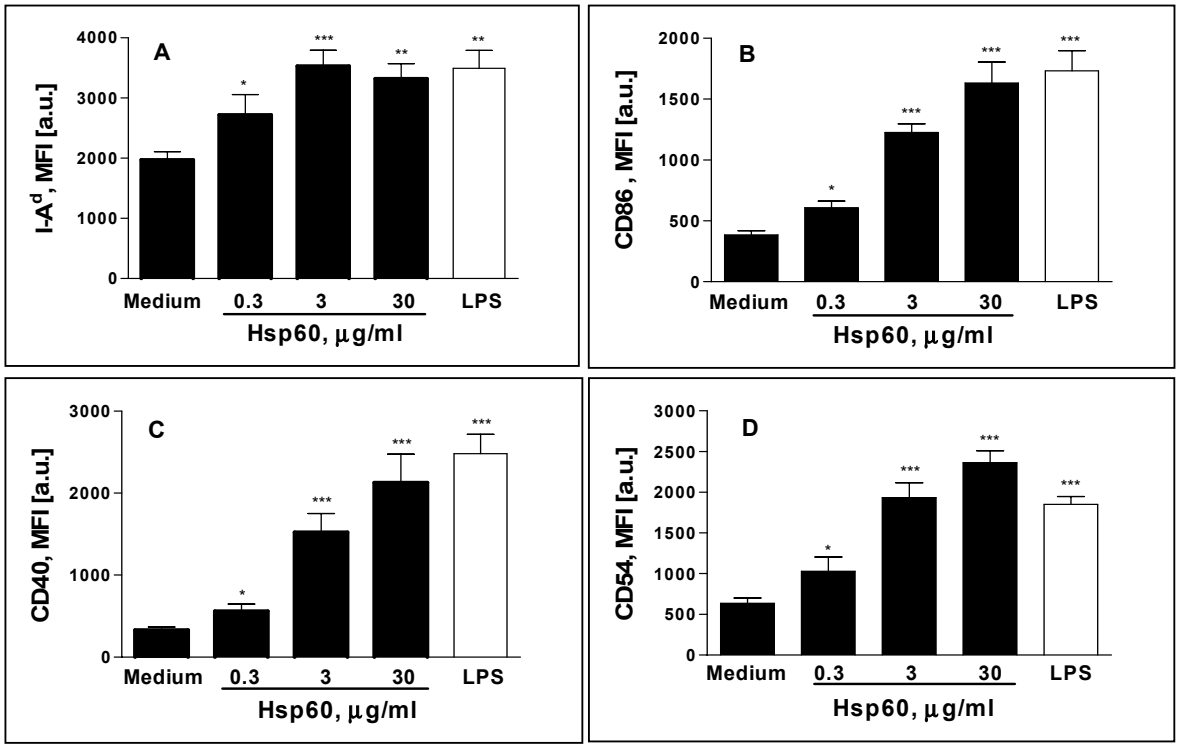

Fig. 3-18 Expression of surface molecules on BMDCs after treatment with Hsp60 or LPS. BMDCs from BALB/c mice were incubated for 20 h with 0.3, 3 or 30 μg/ml of Hsp60 or 10 ng/ml of LPS. Non-adherent cells were collected, stained for I-A^d (*A*), CD86 (*B*), CD40 (*C*) or CD54 (*D*), and analyzed by FACS. The data represent mean fluorescence intensities (MFI) ± SEM of three to eleven independent experiments (for CD54 – two to eight). MFI is expressed in arbitrary units, [a.u.]. Significant differences to medium are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test. One-way ANOVA for HSP60 data shows $p < 0.0001$ (*A-D*).

There were two subpopulations of untreated BMDCs: immature DCs (MHC II ^{low}) that were negative or slightly positive for CD86 or CD40and already mature DCs (MHC II ^{high}) that were also CD86^{high} and CD40^{high} (Fig. 3-19). During the maturation of DCs the up-regulation of surface expression of MHC II and co-stimulatory molecules occurs partly independently and in coordinated way, i.e. the cells with low or moderate level of MHC II express low level of co-stimulatory molecules and MHC II ^{high} cells express moderate or high level of co-stimulatory molecules (Fig. 3-19).

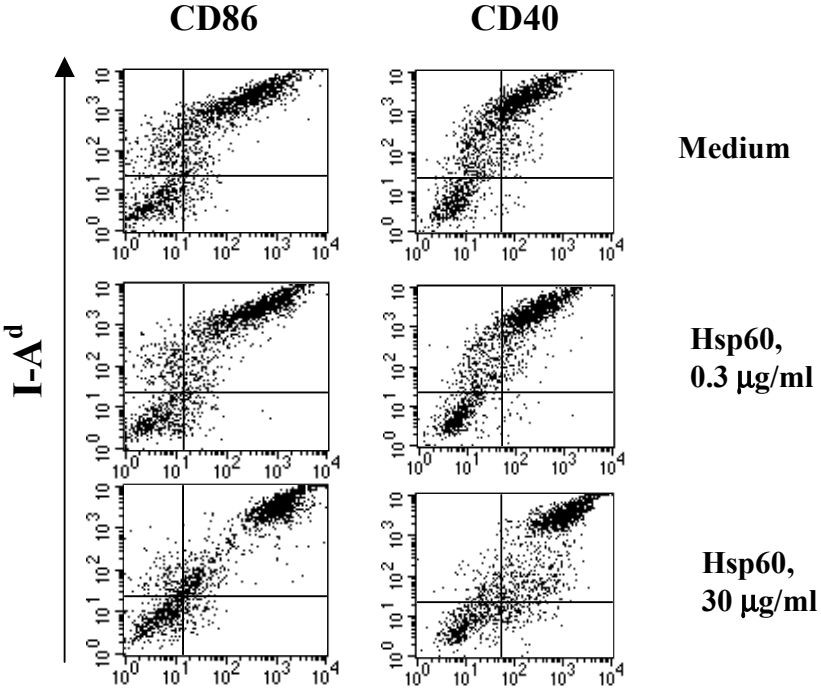

Fig. 3-19 Analysis of BMDC maturation after exposure to Hsp60. BMDCs from BALB/c mice were cultured in medium or medium supplemented with 0.3 or 30 μg/ml of Hsp60 for 20 h. After incubation, non-adherent cells were collected, stained for I-A^d, CD86 or CD40 and analyzed by FACS. Isotype controls were used to set quadrant lines (solid lines) in such a way, that the number of false positive cells was less than 5 % of all cells tested.

3.1.2.2 Hsp60 induces cytokine and chemokine production in BMDC

To investigate whether Hsp60 stimulates BMDCs to produce cytokines and chemokines in addition to its effect on maturation, cell supernatants after the incubation with the stimuli were tested for the presence of various cytokines and chemokines by ELISA. LPS in the concentration of 10 ng/ml was used as a control. Untreated BMDCs did not release cytokines TNFα, IL-10 and IL-12p70, produced in average 15 pg/ml of IL-1β and 155 pg/ml of IL-12p40, whereas mean chemokine level was around 600 pg/ml for CXCL2 and CCL2, 80 pg/ml

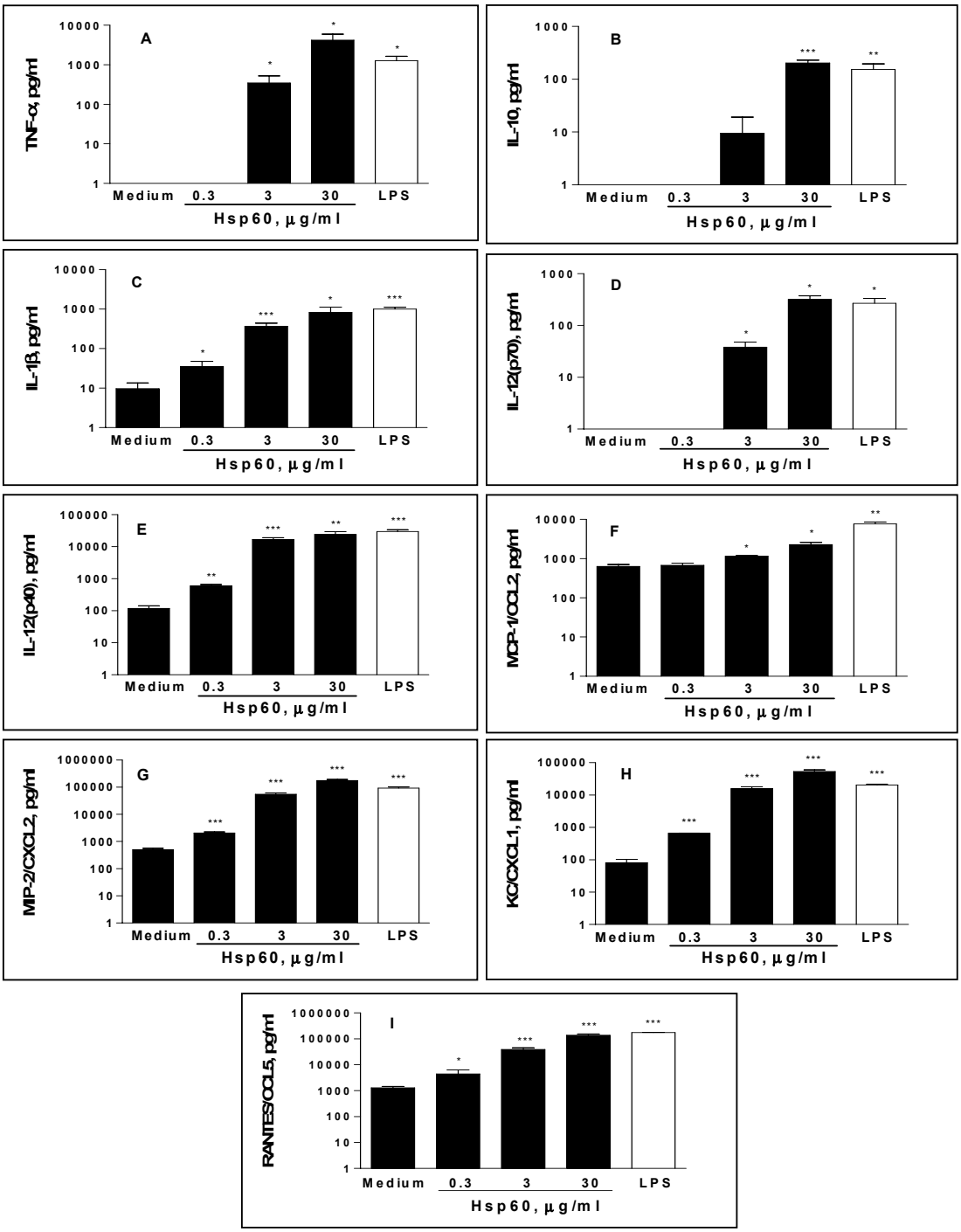

Fig. 3-20 Cytokine and chemokine secretion from BMDCs in response to Hsp60 or LPS. BMDCs of BALB/c mice were incubated for 20 h with 0.3, 3 or 30 µg/ml of Hsp60 or 10 ng/ml (for *D* – 1 µg/ml)of LPS. Thereafter the cells were collected, centrifuged at 300 x g for 5 min and the concentrations of TNFα (*A*), IL-10 (*B*), IL-1β (*C*), IL-12p70 (*D*), IL-12p40 (*E*), MCP-1/CCL2(F), MIP-2/CXCL2 (*G*), KC/CXCL1 (*H*), RANTES/CCL5 *(I)* were determined in the supernatant by ELISA. The data represent mean values ± SEM of two to eight independent experiments. Significant differences from medium control are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test. One-way ANOVA for HSP60 showed $p < 0.05$ (*A, C*), $p < 0.001$ (*D, F*) and $p < 0.0001$ (*B, E, G-I*).

for CXCL1 and 1300 pg/ml for CCL5 (Fig. 3-20). Hsp60 induced dose-dependent synthesis of all these cytokines and chemokines in BMDCs and already at concentration 0.3 μg/ml could stimulated the increase in production of IL-1β, IL-12p40, CXCL2, CXCL1 and CCL5, but not TNF-α, IL-10, IL-12p70 or CCL2 (fig. 3-20). At a concentration of 30 μg/ml Hsp60 BMDCs released the amounts of TNFα, IL-10, IL-1β, IL-12p40 and CCL5 that were comparable to quantities, induced by 10 ng/ml of LPS, and were as high as 4.2, 0.2, 0.8, 24.0 and 140.0 ng/ml, respectively. The level of CCL2 in the culture medium after incubation of BMDCs with 30 μg/ml of Hsp60 was 3.4 times lower then that after the stimulation with 10 ng/ml of LPS and reached 2.3 ng/ml. In contrast, the production of CXCL2 and CXCL1 by BMDCs in response to 30 μg/ml of Hsp60 (175.0 ng/ml of CXCL2 and 53.0 ng/ml of CXCL1) was 2.6 and 1.9 times higher, respectively, then in response to 10 ng/ml of LPS. It is worth therefore to mention that the level of the expression of co-stimulatory molecules on BMDCs after the stimulation with 30 μg/ml of Hsp60 was comparable with that after incubation of BMDs with 10 ng/ml of LPS, whereas the amount of chemokines induced by these stimuli in BMDCs was different.

3.1.2.3 HSP60-induced maturation of BMDC is partly due to LPS

HSPs are highly conserved molecular chaperones, which participate in correct folding of newly synthesized proteins and correct assembly of protein subunits. It was recently shown that Hsp60 specifically binds LPS and mediates its stimulatory effect on macrophages (Habich, C., et al., 2005). Specific LPS binding site on the human Hsp60 molecule, aa 354-365, is different from the epitope aa 481-500 responsible for the binding to the surface of macrophages (Habich, C., et al., 2004; Habich, C., et al., 2005). We would like to investigate if LPS is involved in the stimulatory effects of human Hsp60 on murine BMDC seen in our study. The same approaches as in wheat gluten experiments were used: the determination of LPS contamination by LAL assay, blockage of LPS with PmB and digestion of Hsp60 with proteinase K.

LAL assay showed that LPS content of our Hsp60 preparation was 0.05 EU (equivalent to 5 pg of LPS) per μg of protein. It means that 10 μg/ml of Hsp60 in our study contain 50 pg of LPS. Endotoxin in concentration 100 pg/ml stimulates the increase of the surface expression of MHC class II molecule I-A^d, CD86 and CD40 (Fig. 3-21, A-C). The level of the expression of I-A^d and CD86 on BMDCs after the incubation with 100 pg/ml of LPS can be compared with that after BMDC stimulation with 10 μg/ml of Hsp60 (Fig. 3-21, A, B). The maturation of BMDCs,

induced by this amount of LPS, can be completely inhibited by PmB (Fig. 3-21, D).

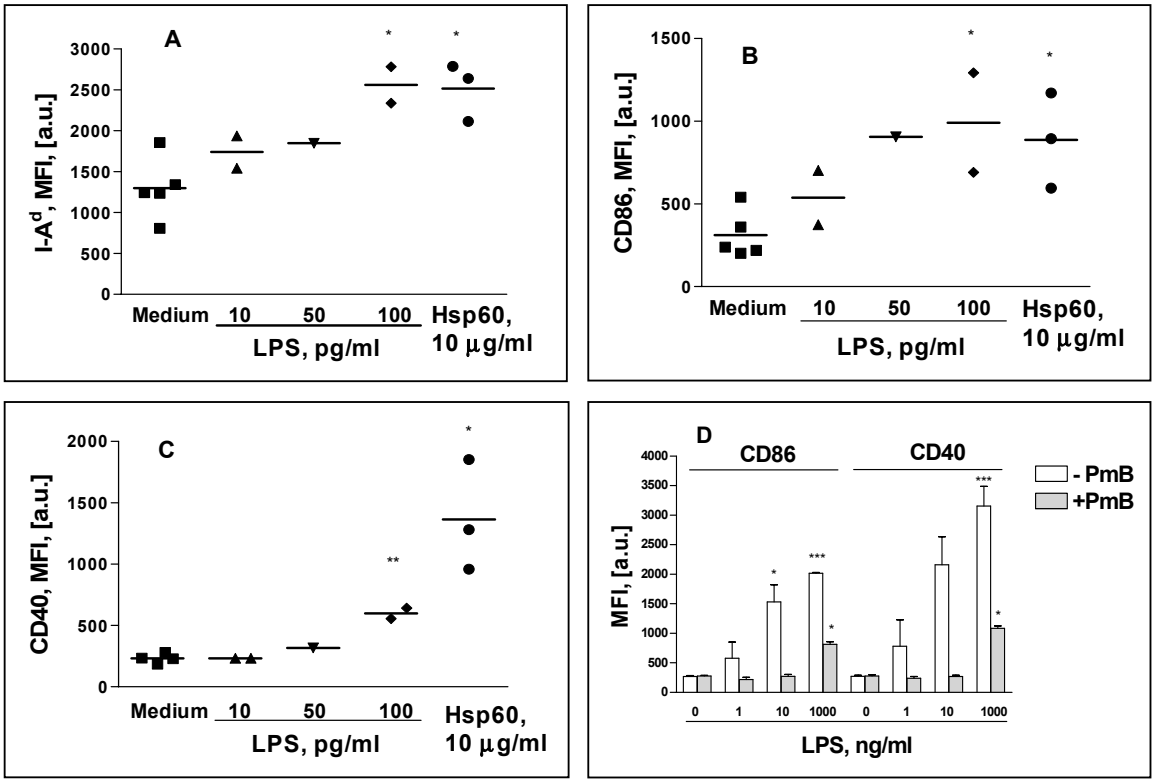

Fig. 3-21 LPS-stimulated maturation of BMDCs and its inhibition by PmB. LPS (10, 50 and 100 pg/ml) (A, B and C) or LPS (1, 10 or 1000 ng/ml) incubated with 10 µg/ml PmB (1 h, 4°C) (D) were added to BMDCs from BALB/c mice for 20 h. Thereafter non-adherent cells were harvested, stained for I-A^d, CD86 or CD40 and analyzed by FACS. The data represent MFI ± SEM of one to five independent experiments. Significant differences to the respective control are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

It is possible that some LPS is bound to Hsp60 and therefore could not be determined by LAL assay, but could be blocked by PmB in case of free access. So the next study aimed to find out, whether treatment of Hsp60 (or LPS as a control) with PmB, prior to the addition to BMDCs can influence the effect of Hsp60 on BMDC maturation. Preincubation of 10 ng/ml LPS with 10 µg/ml of PmB 1 h at 4 °C completely abolished its effect on maturation of BMDC from BALB/c mice (Fig. 3-22). The same treatment of 10 µg/ml of Hsp60 had no impact on the surface level of I-A^d and CD86 and partly inhibited Hsp60-stimulated expression of CD40 (fig. 3-22). PmB inhibited (Hsp60 + LPS)-induced expression of all investigated maturation markers on BMDCs to the level caused by Hsp60 alone (Fig. 3-22). The treatment of BMDCs with 10 µg/ml of Hsp60 significantly stimulated the production of TNFα compared to the control (46.30 ± 7.95 vs. 14.97 ± 5.54 pg/ml, triplicate culture, $p < 0.05$). Preincubation of Hsp60 with 10 µg/ml of PmB for 1 h at 4°C inhibited TNFα release from BMDC to 20.10 ± 1.78 pg/ml.

This amount of TNFα was still higher than TNFα production by BMDCs in the presence of PmB alone (8.67 ± 0.94 pg/ml, $p < 0.05$).

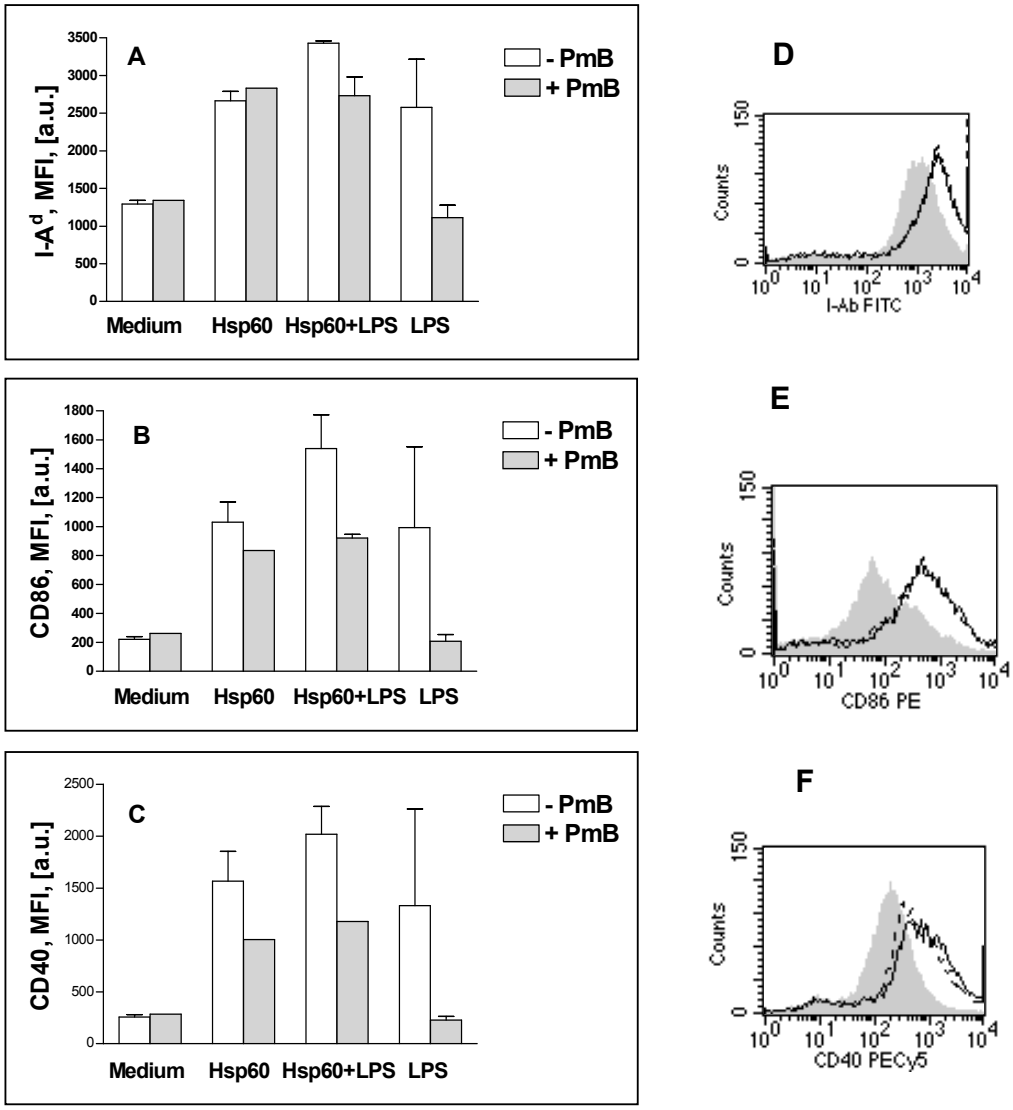

Fig. 3-22 The effect of PmB on Hsp60- or LPS-stimulated maturation of BMDCs. Hsp60 (10 µg/ml) or LPS (10 ng/ml) were incubated with 10 µg/ml PmB (1 h, 4°C) prior to the addition to BMDCs from BALB/c mice for another 20 h. Thereafter non-adherent cells were harvested, stained for I-A^d (*A, D*), CD86 (*B, E*) or CD40 (*C, F*) and analyzed by FACS. The data represent MFI ± SEM of one to two independent experiments. The expression of surface molecules on untreated BMDCs is shown as a shaded grey area, on HSP60-stimulated BMDCs as solid line and on (Hsp60+PmB)-treated BMDCs as a broken line (*D-F)*.

Another approach to prove the specificity of the observed effects of Hsp60 on BMDCs was to investigate whether Hsp60 digested with highly reactive proteinase K retains its ability to stimulate BMDCs.

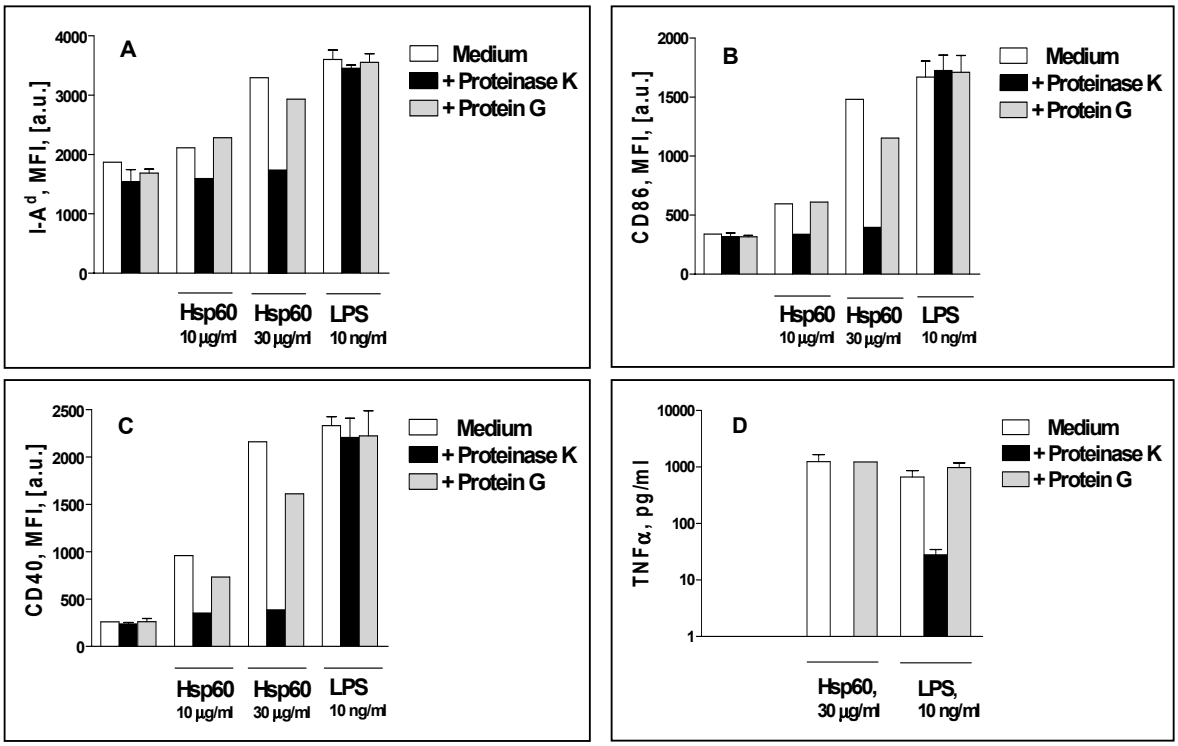

Fig. 3-23 The effect of digestion of Hsp60 by proteinase K on BMDC maturation. Proteinase K- or protein G-agarose were incubated overnight in 10 % ethanol in PBS at +4°C, then washed 3 times with RPMI w/o FCS and antibiotics, centrifuged 30 c at 500 g, RPMI was added to the pellet. Hsp60 or LPS were incubated 3.5 h at 37°C in thermoshaker with proteinase K at final concentration 30 U/mg of the stimuli (or with protein G as a control), centrifuged 1min at 8 000 g, filtered through 0.2 µm Supor Acrodisk 32 syringe filter and added to the BMDCs for another 20 h. Non-adherent cells were harvested, centrifuged at 300 g for 5 min, stained for I-A^d (*A*), CD86 (*B*) or CD40 (*C*) and analyzed by FACS. The amount of TNF-α in the supernatant was determined by ELISA (*D*). The data represent one to two independent experiments (*A*-*C*) or duplicates (*D*).

Proteinase K successfully recovers up to 100 % of endotoxin that is masked in protein-endotoxin complexes for its determination in LAL assay (Petsch, D., et al., 1998). Therefore, the use of proteinase K in our study would help to detect also a bound LPS. Hsp60 was digested with proteinase K, coupled to agarose beads, to be able to get rid of the enzyme by centrifugation after the digestion. In contrast to LPS, Hsp60-induced maturation of BMDCs was completely abrogated by treatment of Hsp60 with proteinase K (Fig. 3-23, *A*-*C*). Protein G, coupled to agarose beads, did not influence the expression of maturation markers on BMDCs stimulated by LPS and 10 µg/ml of Hsp60, but caused a small decrease of cell maturation after

incubation of BMDCs with 30 µg/ml Hsp60 (Fig. 3-23, *A*-*C*). Hsp60 stimulated the production of TNF α in BMDsC, which was completely abolished by the preincubation of Hsp60 with poteinase K, but not with protein G (Fig. 3-23*D*). The integrity of Hsp60 molecule is therefore absolutely necessary for the observed effect of Hsp60 on BMDC maturation and TNFα secretion.

3.1.2.4 The involvement of TLR4 in Hsp60 signaling in BMDC

To address the question whether TLR4 is involved in Hsp60 signaling in BMDCs, the effect of Hsp60 on the maturation of BMDCs from C3H/HeN mice in comparison with TLR4-mutant C3H/HeJ mice (Poltorak A. et al, 1998) was investigated.

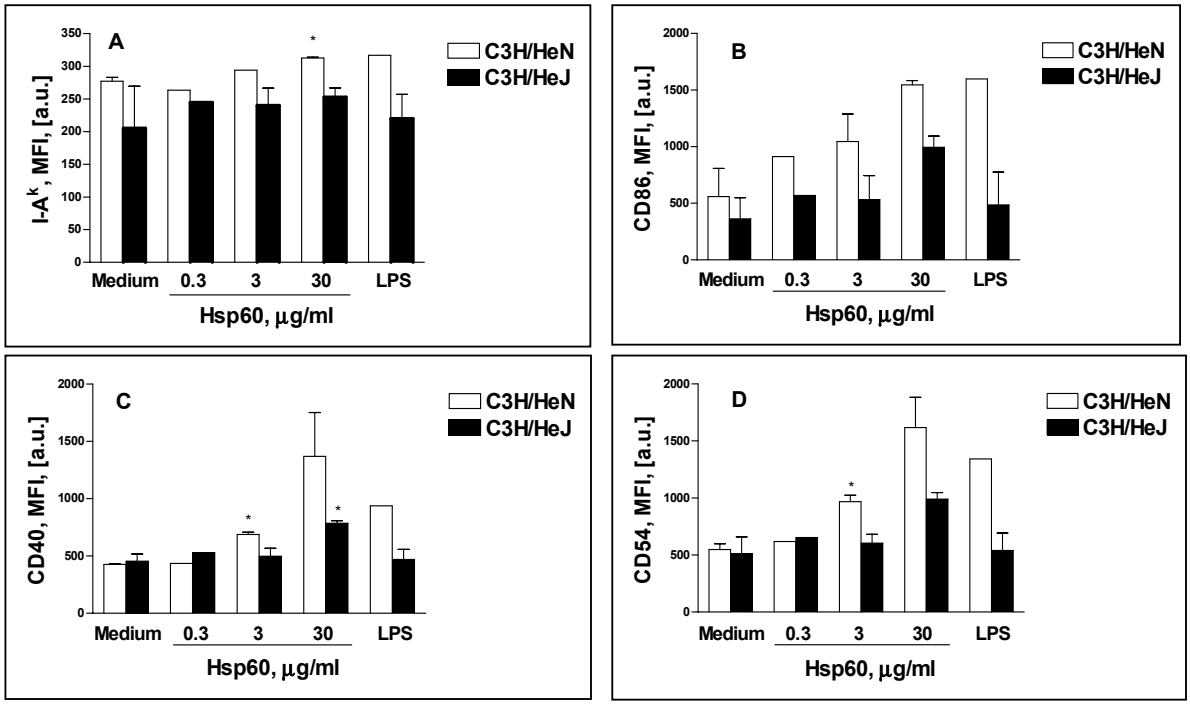

Fig. 3-24 Dose-dependency of Hsp60-induced BMDC maturation in C3H/HeN versus C3H/HeJ mice. BMDCs were incubated with 0.3, 3 or 30 µg/ml of Hsp60 or 10 ng/ml LPS for 20 h. Thereafter non-adherent cells were harvested, stained for I-A^k (*A*), CD86 (*B*), CD40 (*C*) or CD54 (*D*) and analyzed by FACS. The data represent mean MFI ± SEM of one to two independent experiments. Significant differences to the medium control of corresponding strains are indicated as * $p < 0.05$, Student's unpaired *t* test.

LPS that mainly signals through TLR4 (Akira S., 2001) was used as control. LPS stimulated the maturation of BMDCs from C3H/HeN mice as indicated by the expression of MHC class molecule I-A^k, CD86, CD40 or CD54 (Fig. 3-24). This stimulatory effect of LPS was completely abrogated in BMDCs from TLR4-defective C3H/HeJ mice. In contrast, Hsp60

caused dose-dependent increase in the expression of all investigated maturation markers on BMDCs from both C3H/HeN and C3H/HeJ mice. Because background expression of I-A^k, CD86, less of CD54, but not CD40, was consistently higher on BMDCs from C3H/HeN mice as compared to TLR4-defective mice, overall maturation effect of Hsp60 on BMDCs from C3H/HeJ mice was not as strong as for BMDCs from C3H/HeN mice (Fig. 3-24).

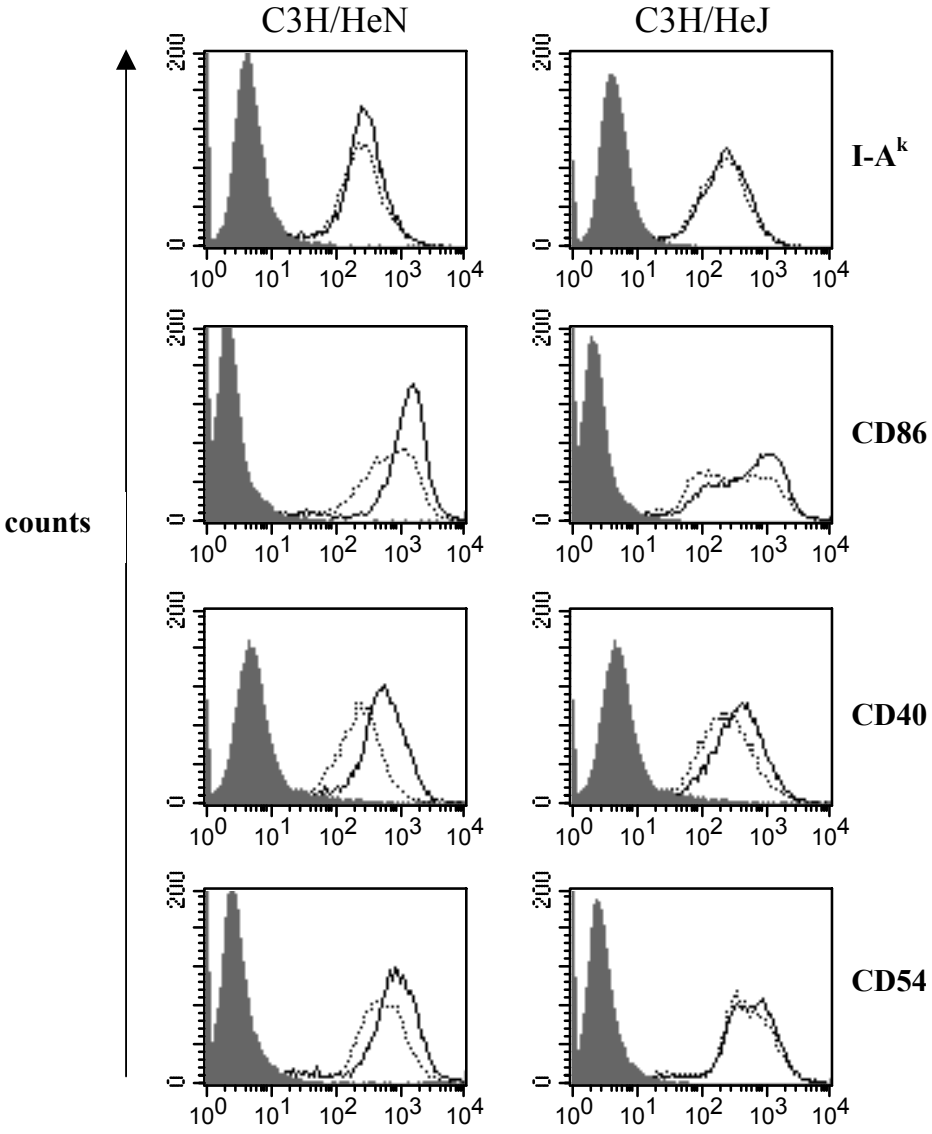

Fig. 3-25 Involvement of TLR4 in the maturation of BMDC induced by Hsp60. BMDCs of C3H/HeN and C3H/HeJ were incubated with 3 µg/ml of Hsp60 for 20 h. Thereafter nonadherent cells were harvested and stained for I-A^k, CD86, CD40 or CD54 and analyzed by FACS. Isotype control staining is shown as grey shaded area. Broken line represent control untreated cells, solid line – Hsp60-treated cells.

To further analyze whether TLR4 is involved in Hsp60 signaling in BMDCs, we compared the relative inhibition of the expression of maturation markers on BMDCs of mutant mice versus normal mice. At 3 μg/ml and 30 μg/ml of Hsp60 the level of inhibition of CD40 and CD54 expression was much higher than it could be expected from the strain differences. One representative experiment shows that after the incubation with 3 μg/ml of Hsp60 BMDCs of TLR4-mutant C3H/HeJ mice did not increase the expression of MHC class II molecule I-A^k and CD54, and only weakly up-regulated surface level of CD86 and CD40 in contrast to BMDCs from C3H/HeN mice, which were well-responsive to this concentration of Hsp60 as assessed by the enhanced expression of all maturation markers (Fig. 3-25). We therefore conclude that TLR4 is partly involved in Hsp60-induced BMDC maturation. To check this point of view, the determination of cytokine release from BMDCs of both strains in response to Hsp60 or LPS was performed. Only 30 μg/ml of Hsp60 could induce the synthesis of TNFα and IL-10 in BMDCs of C3H/HeN mice (Fig. 3-26). Hsp60- and LPS-induced production of IL-10 in BMDCs of C3H/HeN mice was completely abolished in BMDCs of TLR4-mutant mice, whereas Hsp60-stimulated TNFα secretion from BMDCs of control mice was not abrogated in BMDCs of TLR4-defective mice, in contrary to LPS-induced TNFα release.

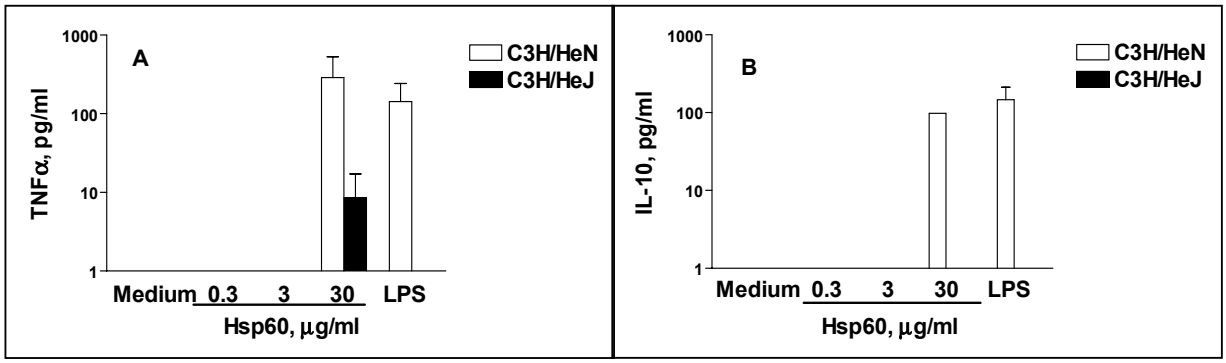

Fig. 3-26 Cytokine production from BMDC of C3H/HeN and TLR4-mutant C3H/HeJ mice in response to HSP60. BMDCs were incubated with 0.3, 3 or 30 μg/ml of HSP60 or 10 ng/ml LPS for 20 h. The content of TNFα (*A*) or IL-10 (*B*) was determined by ELISA. The data represent mean values ± SEM of one to two independent experiments.

This data provide an additional support to the suggestion that TLR4 is partly involved in Hsp60 signaling in BMDCs.

3.1.2.5 MAPK signalling in BMDC after stimulation with Hsp60

The intracellular signalling through MAPK cascade and NFκB is an early event in activation of the cells induced by different stimuli. We aimed to clarify whether activation of BMDCs by Hsp60 is accompanied by the activation of these pathways, i.e. phosphorylation of the cascade members. Cytoplasmic extracts of BMDCs 15 and 45 min after cell stimulation with 3 μg/ml of Hsp60 or non-stimulated BMDC were prepared, separated by SDS-PAGE and further investigated by Western blot for the presence of the phosphorylated forms of IκB, p38, JNK, ERK 1/2 or raf-1. LPS-treated BMDC were used as a control.

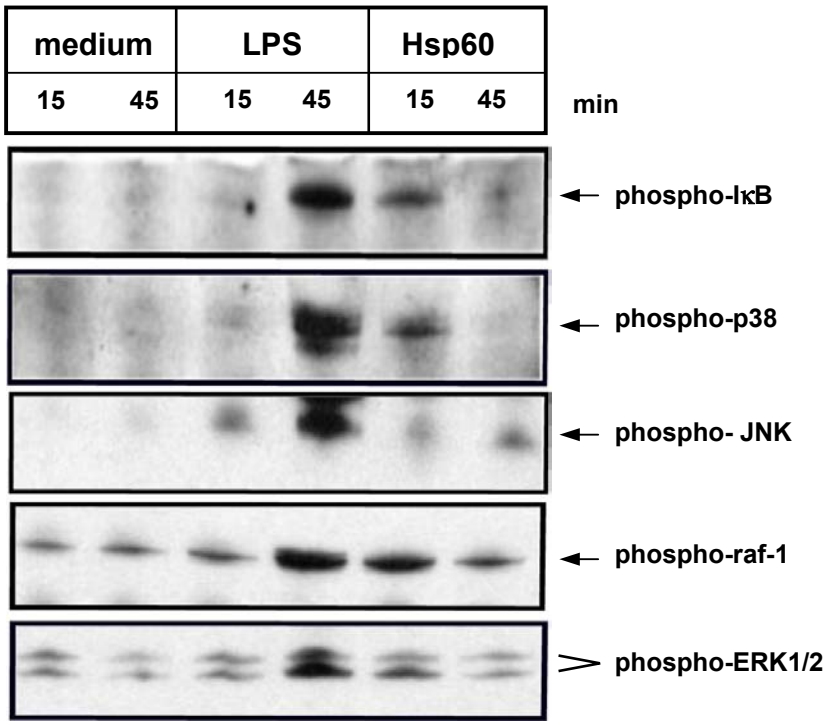

Fig. 3-27 Hsp60-induced signalling in BMDC. BMDCs were incubated with 3 μg/ml of HSP60 or 10 ng/ml LPS for 15 or 45 minutes. Thereafter cytoplasmyc extracts of BMDCs were prepared and analyzed for the phosphorylated forms of IκB, p38, JNK, ERK 1/2 or raf-1 by Western blot.

Untreated BMDCs did not have detectable amount of phospho-p38, JNK or IκB, although phospho-raf-1 and phospho-ERK 1/2 were present at a certain basal level (Fig. 3-26). BMDC stimulation with 3 μg/ml of Hsp60 for 15 min induced rapid phosphorylation of p38, raf-1, ERK 2, JNK (at a low level) and IκB, but not ERK1. At 45 min after BMDC incubation with Hsp60 the amount of phospho-p38 and phospho-ERK2 was the same as in unstimulated control cells, phospho-IκB and phospho-raf-1 were present at a minimal level, and there was a slight

increase in phospho-JNK (Fig. 3-26). In contrast to Hsp60, 10 ng/ml of LPS induced significant phosphorylation of all investigated kinases and IκB after 45 min, but not after 15 min of stimulation, when phospho-p38, phospho-ERK 1/2 and phospho-IκB were at the control level, and phospho-JNK and phospho–raf-1 were only slightly increased (Fig. 3-26). In summary, Hsp60 activates the members of MAPK cascade and NFκB pathway in BMDCs with kinetic quite different from that of LPS signalling.

3.2 In vivo study of the effect of dietary wheat gluten on gut immunity

The aim of this study was to investigate the level of some cytokines and chemokines in the upper intestine (duodenum) of BBdp, BBc and WF rats fed WG, NTP-2000 (Rao, G.N., 1996) or HC diet during 120 days after birth, namely on days 10, 30, 45, 70, 95 and 120. The first two diets contain wheat (NTP-2000) and wheat gluten (WG) as a main component and are diabetogenic for BB rats, whereas HC diet is diabetes-retardant (MacFarlane, A.J. et al., 2003). The highest incidence of diabetes in the Ottawa colony of BBdp rats is 65.3 ± 14.9 % (total of 169 rats) for the animals fed a non-purified NTP-2000 diet, and this level remains constant over the past six years (MacFarlane, A.J. et al., 2003; Graham, S. et al., 2004). When two isocaloric and isonitrogenous semi-purified diets, WG and HC, were compared, the former one (WG diet) was diabetogenic and gave the similar incidence of diabetes in BBdp rat colony as NTP-2000 diet (50.6 ± 11.1 %, total 282 rats), whereas HC diet was highly protective from diabetes development in BBdp rats (18.8 ± 10.6 %, total 322 rats, $p < 0.001$) (MacFarlane, A.J. et al., 2003). Diabetes in BBdp rats in the Ottawa colony was diagnosed when fasting blood glucose was > 11.1 mmol/l, and the overt disease developed from ~ day 60 till ~ day 120 (MacFarlane, A.J. et al., 2003). For the current experiment only asymptomatic rats were selected in order to avoid probable interference of diabetic state with the investigated parameters. Based on their important role in establishing of oral tolerance, we have chosen for our study IFN-γ, IL-10, CCL2 and CXCL2 as the cytokines/chemokines of interest.

3.2.1 The level of IFN-γ in duodenum of BB rats fed the WG, NTP-2000 or HC diets

In this study the amount of IFNγ was determined by ELISA in the upper small intestine of BBdp, BBc and WF rats fed the diabetes-promoting WG or NTP-2000 diet or with diabetes-retardant HC diet. The animals were weaned at day 23 of age. To analyse better this big plot of

data we expressed them at first as the kinetic of the effect of three different diets on IFNγ level in the duodenum of the rats of the same strain (Fig. 3-28) and, secondly, as the kinetic of IFNγ level in the duodenum of rats of three different strains fed the same diet (Fig. 3-29). In BBdp rats intestinal IFNγ was not influenced by the type of diet, except a decrease in animals fed the NTP-2000 vs. the HC diet at day 95 (Fig. 3-28, *A, A1*). Only BBdp rats fed the HC diet had an

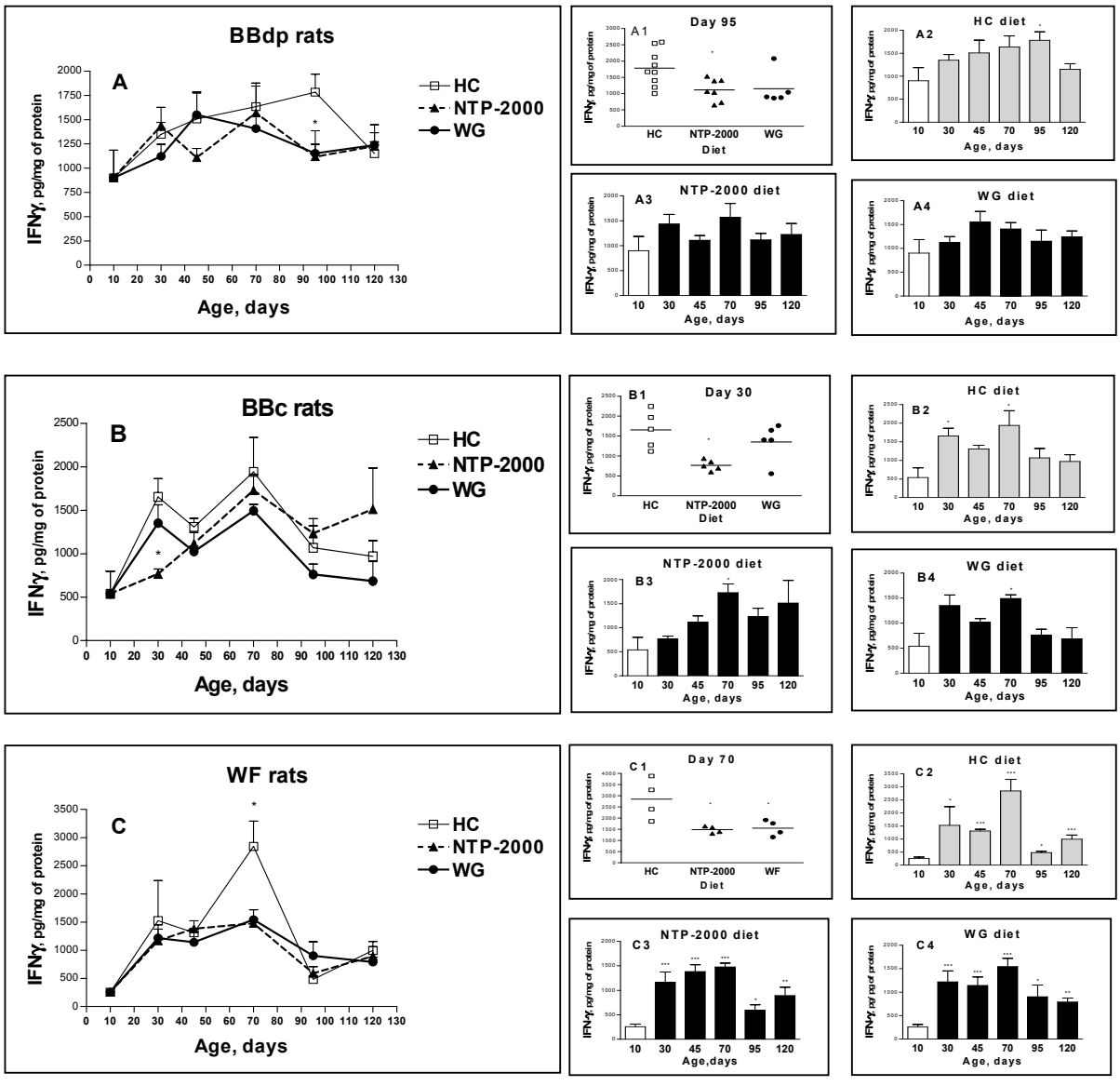

Fig. 3-28 IFNγ content in the gut duodenum of rats fed the diabetes-promoting or diabetes-protective diet. Part I. BBdp (*A*), BBc(*B*) or WF (*C*) rats were fed the diabetes-promoting WG or NTP-2000 diet , or the diabetes-retardant HC diet. The gut duodenum was isolated and frozen at days 10, 30, 45, 70, 95 and 120 after birth. Thereafter the homogenates were prepared, and the levels of IFNγ by ELISA and protein by Bio-Rad assay were measured. The data are expressed as mean values ± SEM, n = 4-12. Significant differences between the animals of the same age and strain fed the different diets vs. HC group (*A1-C1*) or the animals of the same strain but of different age fed with the same diet vs. 10 days-old rats (*A2-A4, B2-B4, C2-C4*) are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

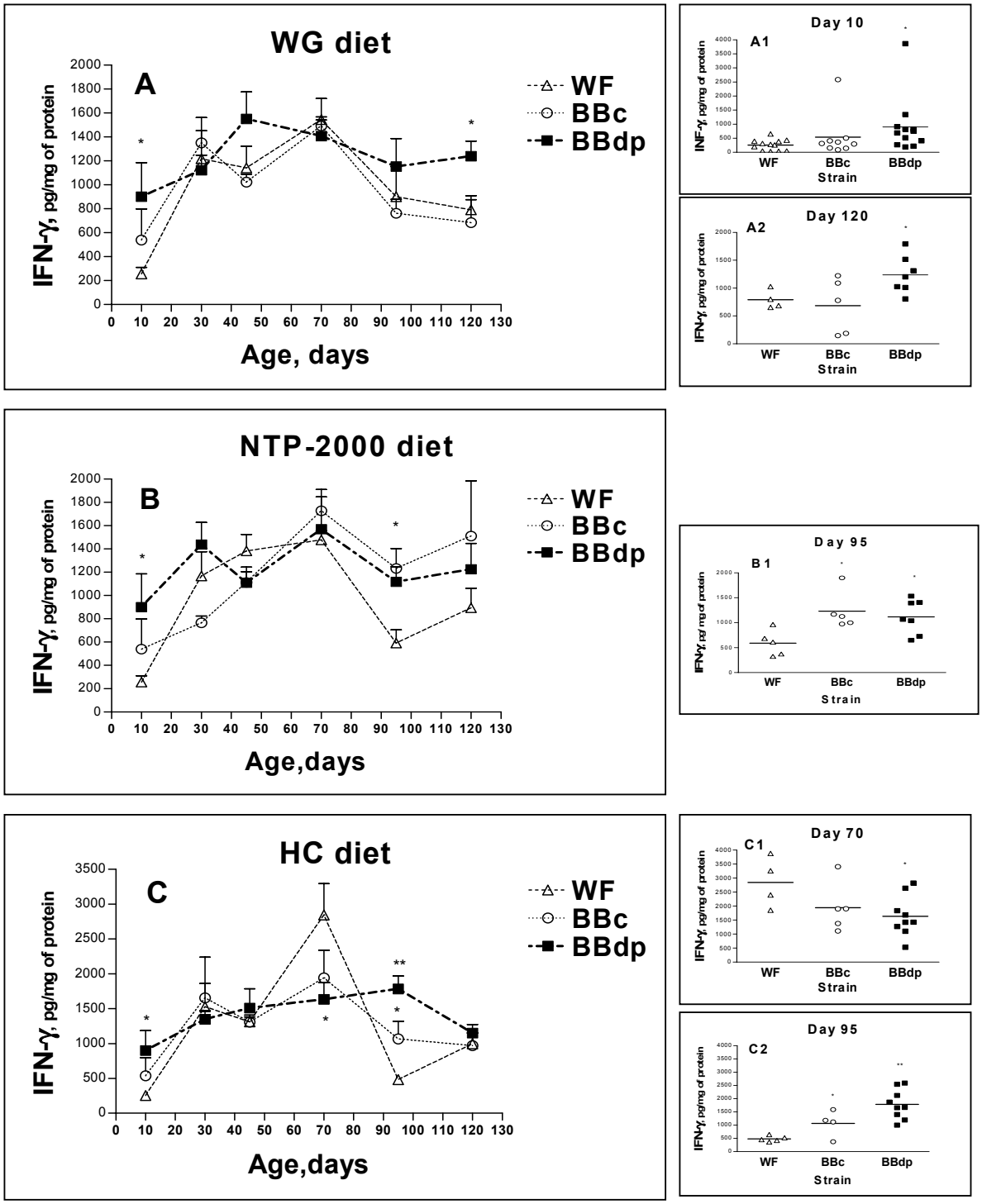

Fig. 3-29 IFNγ content in the gut duodenum of rats fed the diabetes-promoting or diabetes-protective diet. Part II. BBdp, BBc or WF rats were fed the diabetes-promoting WG (*A*) or NTP-2000 diet (*B*), or with diabetes-retardant HC diet (*C*). The gut duodenum was isolated and frozen at days 10, 30, 45, 70, 95 and 120 after birth. Thereafter the homogenates were prepared, and the levels of IFNγ by ELISA and protein by Bio-Rad assay were measured. The data are expressed as mean values ± SEM, n = 4-12. Significant differences between the different strains of rats of the same age vs. WF group (*A1, A2, B1,C1, C2*) are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

increase in duodenal IFNγ at day 95 of age as compared to 10 days-old animals, whereas this was not the case for the rats fed the NTP-2000 or WG diet (Fig. 3-28, *A2-A4*). The level of IFNγ in upper intestine of BBc rats was also not influenced by the type of the diet, except a decreased amount of cytokine in animals fed the NTP-2000 diet as compared to those fed the HC diet at day 30 (Fig. 3-28, *B,B1*). In contrast to BBdp rats, BBc rats fed not only the HC, but also the NTP-2000 or WG diet had at day 70 after birth higher IFN-γ level in duodenum then 10 days-old animals, for HC diet there was an additional enhancement at day 30 (Fig. 3-28,*B2-B4*). As for BBdp and BBc rats, the amount of IFNγ in duodenum of WF rats was also not affected by the type of the diet, except a significant increase in the gut of the animals fed with HC vs. those fed the NTP-2000 or WG at day 70 after birth (Fig. 3-28,*C,C1*). But in contrast to BB rats the level of duodenal IFNγ in WF rats independently of the diet was higher in all investigated groups after weaning as compared to10 days-old rats (Fig.3-28, *C2-C4*).

As the second approach we expressed these data as strain differences in the animals fed the same diet and got additional information (Fig. 3-29). Thus, at day 10 after birth the concentration of IFNγ in the duodenum of BBdp rats was higher than in WF rats (900.2 ± 286.0 vs. 255.3 ± 56.35 pg of IFNγ/mg of protein, n = 12, fig. 3-29,*A-C,A1*). BBdp rats weaned on WG diet had an increased level of intestinal IFNγ as compared to WF rats at age 120 days (Fig. 3-29,*A,A2*). BBdp and BBc rats fed the NTP-2000 and HC showed an elevated amount of IFNγ in comparison to WF rats at age 95 days (Fig. 3-29, *B,B1,C,C2*). But at age 70 days WF rats fed the HC diet had higher amount of IFNγ as compared to BBdp rats (Fig. 3-29,*C,C1*).

3.2.2 The level of IL-10 and of the ratio IFNγ/IL-10 in gut duodenum of BB rats fed the WG, NTP-2000 or HC diets

Concentration of IL-10 was measued by ELISA in the same homogenates of rat duodenum that were used for IFN-γ determination. In BBdp, BBc and WF rats of all investigated ages fed diabetes-retardant HC diet the mean values of IL-10 in gut duodenum were higher as compared to those fed diabetogenic NTP-2000 or WG diets (Fig. 3-30,*A-C*). This tendency gained statistical significance for 30- and 45-days-old BBdp and BBc rats, and for 70- and 120-days-old WF rats (Fig. 3-30,*A-C,A1-C1,A2-C2*). Moreover, curve of duodenal IL-10 level during investigated period of life was very similar for BBc or WF rats fed the diabetogenic NTP-2000 or WG diets (Fig. 3-30,*A-C*). When the amount of IL-10 was compared in the animal groups of

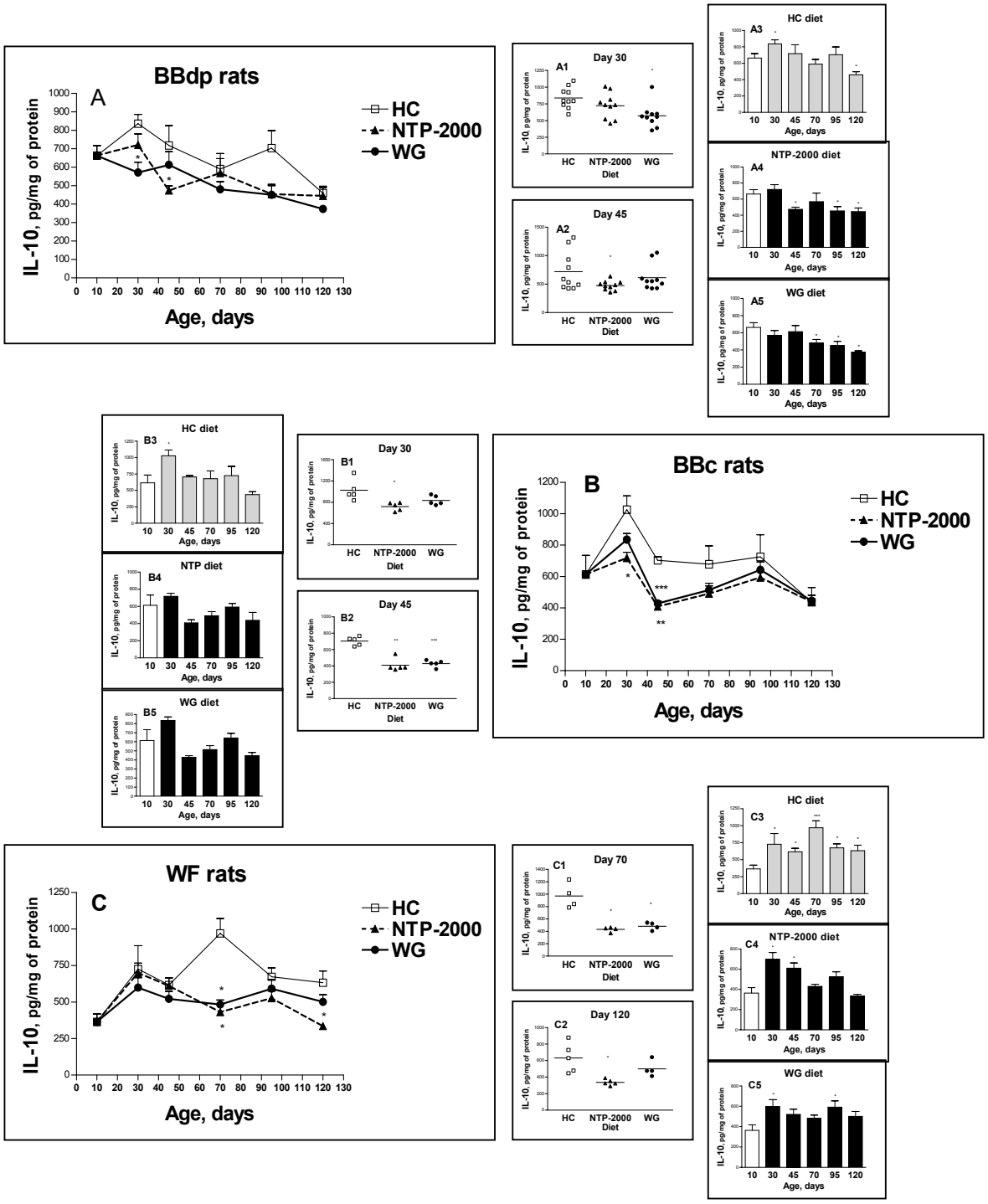

Fig. 3-30 IL-10 content in the gut duodenum of rats fed the diabetes-promoting or diabetes-protective diet. Part I. BBdp (*A*), BBc(*B*) or WF (*C*) rats were fed the diabetes-promoting WG or NTP-2000 diet , or the diabetes-retardant HC diet. The gut duodenum was isolated and frozen at days 10, 30, 45, 70, 95 and 120 after birth. Thereafter the levels of IL-10 by ELISA and protein by Bio-Rad assay were measured in duodenum honogenates. The data are expressed as mean values ± SEM, n = 4-12. Significant differences between the animals of the same age and strain fed the different diets vs. HC group (*A-C*, *A1-C1, A2-C2*) or the animals of the same strain but of different age fed with the same diet vs. 10 days-old rats (*A3-A5, B3-B5, C3-5*) are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

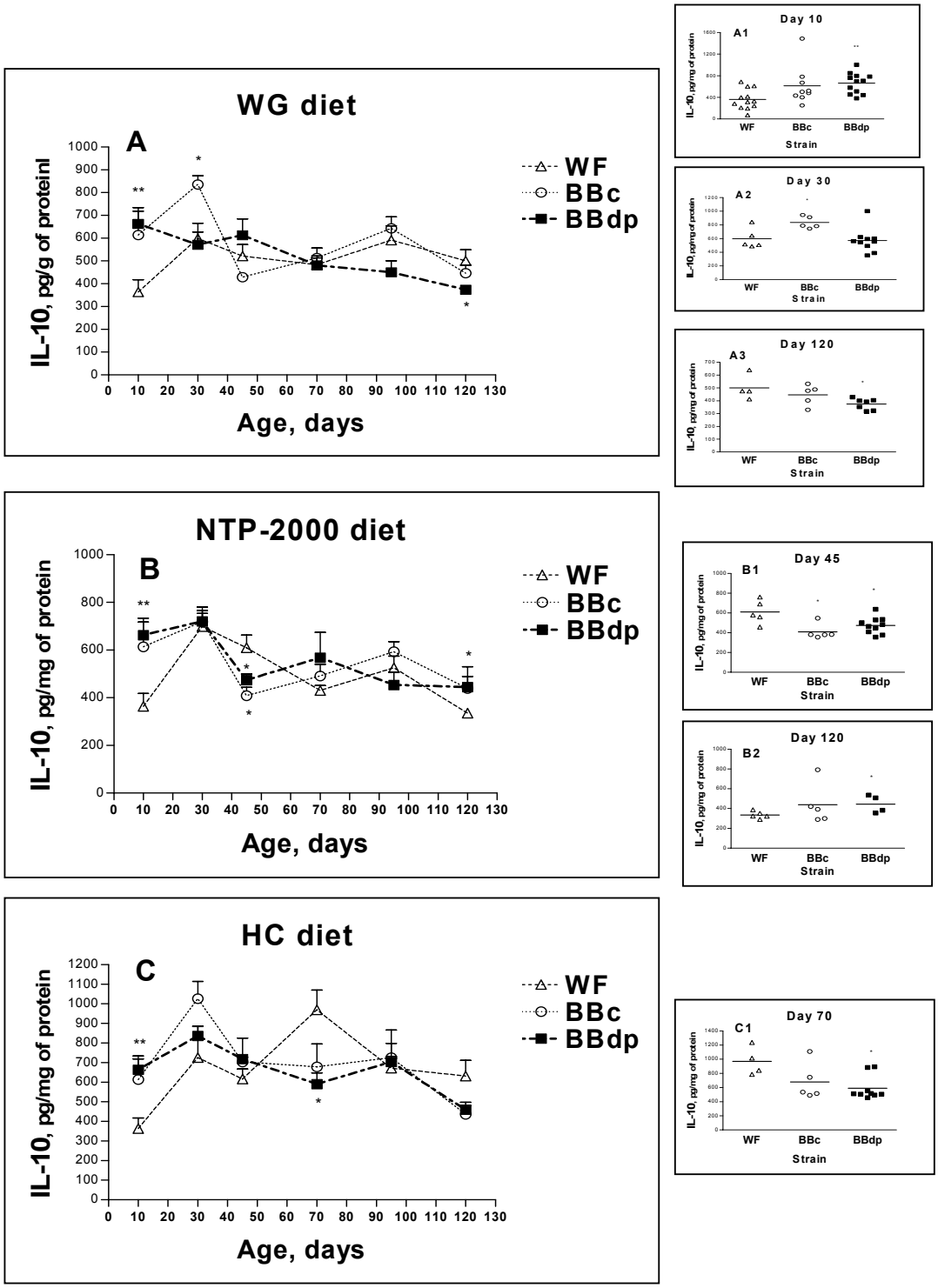

Fig. 3-31 IL-10 content in the gut duodenum of rats fed the diabetes-promoting or diabetes-protective diet. Part II. BBdp, BBc or WF rats were fed the diabetes-promoting WG (*A*) or NTP-2000 diet (*B*), or with diabetes-retardant HC diet (*C*). The gut duodenum was isolated and frozen at days 10, 30, 45, 70, 95 and 120 after birth. Thereafter the levels of IL-10 by ELISA and protein by Bio-Rad assay were measured in duodenal homogenates. The data are expressed as mean values ± SEM, n = 4-12. Significant differences between the different strains of rats of the same age fed the same diet vs. WF group (*A1-A3, B1,B2, C1*) are indicated as * $p < 0.05$, ** $p < 0.001$, Student's unpaired *t* test.

the same strain fed the same diet, WF rats weaned on all three diets, and BBdp and BBc rats weaned on HC diet had an elevated amount of intestinal IL-10 at day 30 after birth in comparison with day 10 (Fig. 3-30,*A-C,A3-C3*). Otherwise BBdp and BBc rats fed all investigated diets had a decrease in duodenal IL-10 level up to day 120 as compared to 30-days-old animals, and BBdp rats independently of diet had significantly lower content of IL-10 in the duodenum at day 120 as compared to day 10 ($p < 0,05$, Fig. 3-30*,A3-A5, B3-B5*). This was not the case for WF rats fed HC or WG diet, but NTP-2000-fed animals had also a decrease of IL-10 level after day 30 up to day 120, but at day 120 duodenal IL-10 was not lower than at day 10 (Fig. 3-30,*C3-C5*). The comparison of IL-10 content in the gut of the rats of different strains allowed to find out the feature similar to one found for IFN-γ: 10-days-old BBdp rats had higher level of IL-10 in gut duodenum in comparison to WF rats (663.2 ± 54.9 vs. 363.7 ± 54.2 pg of IL-10/mg of protein, $n = 12$, $p < 0.001$, Student's unpaired t test, Fig. 3-31,*A-C,A1*). Otherwise WF, BBc and Bbdp rats fed the same diet produced very similar amount of IL-10 in the gut, although some differences could get statistical significance (Fig. 3-31,*A-C,A2,A3,B1,B2,C1*).

After the Th1 cytokine IFN-γ and Th2 cytokine IL-10 were measured, we calculated the ratio IFN-γ to IL-10 in order to better analyse gut immunity after feeding the different diets. Diet did not influence IFN-γ/IL-10 ratio in BB dp, BBc and WF rats during the whole investigated period (Fig. 3-32,*A-C*). The animals of all three strains had strikingly similar continuous increase of IFN-γ/IL-10 ratio from 10 days to 70 days after birth (Fig. 3-32,*A-C*). All investigated differences were observed after day 70. Thus, BBdp rats, in contrast to BBc and WF rats, did not have a big decrease of the ratio at day 95, which fell to the level of 10-days-old-animals in HC-fed WF rats (Fig. 3-32). 95-days-old BBdp rats kept the level of IFN-γ/IL-10 ratio of day 70, and had a higher ratio values in comparison with 95-days-old WF and BBc rats independently of the diet (Fig. 3-33,*A-C,A1,B2,C1*). At day 120 there was an elevation of the duodenal ratio of BBc and WF rats for all three diets, but its level was lower than at day 70 (Fig. 3-32). BBdp rats fed WG, NTP-2000 or HC diet had unchanged ratio values at day 120 as compared to days 70 or 95 (Fig.3-32,*A, A1-A3*). According to our previous data, the amount of IFN-γ and IL-10 in duodenum is higher in 10-days-old BBdp rats as compared to WF rats, but not to BBc rats. Although the mean ratio of IFN-γ to IL-10 in the gut at day 10 was also higher

in BBdp rats than in BBc or WF rats, this tendency did not gain the statistical significance (Fig. 3-33,*A-C*).

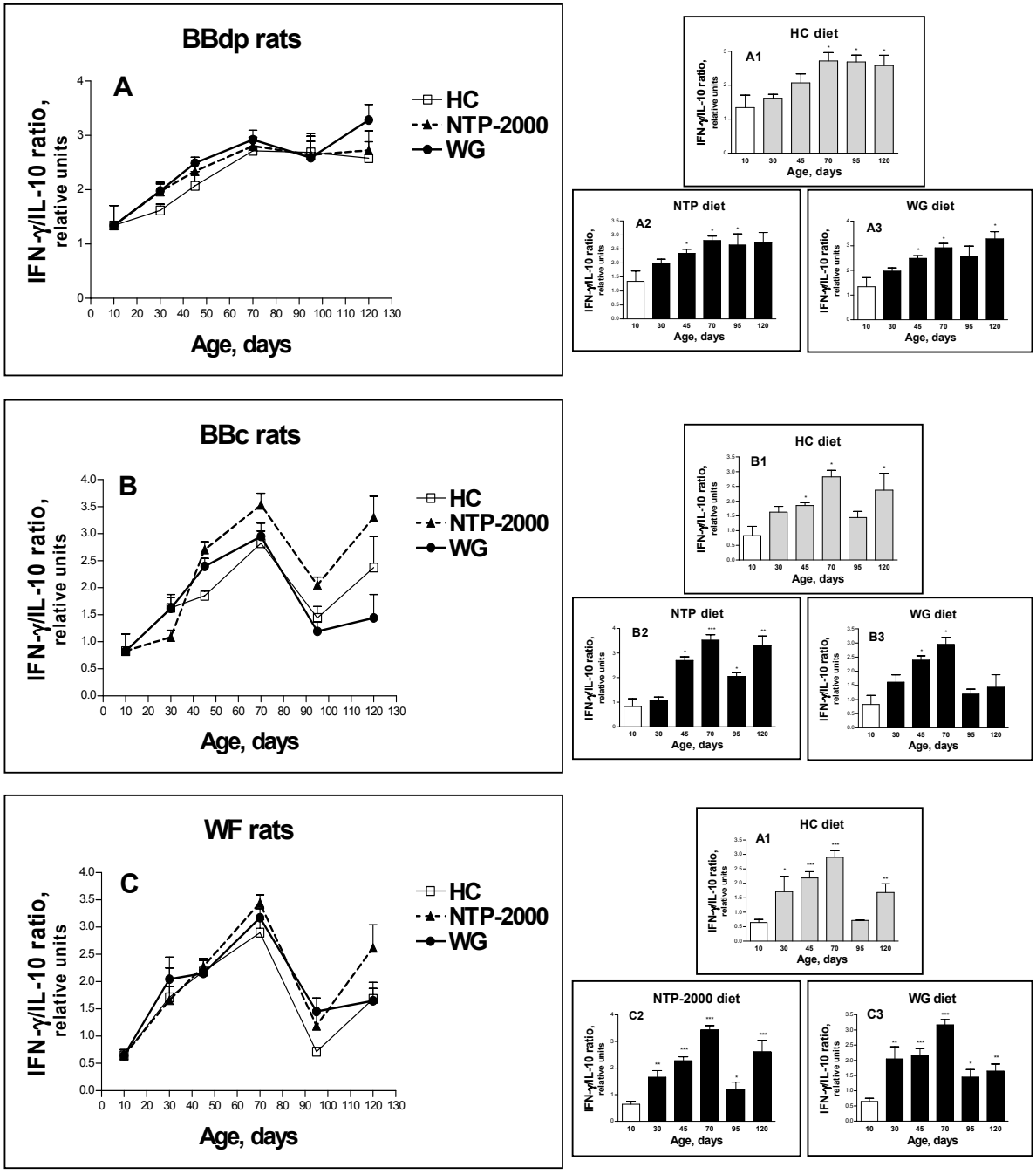

Fig. 3-32 FNγ/IL-10 ratio in the gut duodenum of rats fed WG, NTP-2000 or HC diet. Part I. IFNγ/IL-10 ratio was calculated from the measured IFNγ and IL-10 values in gut duodenum of BBdp (*A*), BBc(*B*) or WF (*C*) rats fed the diabetes-promoting WG or NTP-2000 diet , or the diabetes-retardant HC diet. The data are expressed as mean values ± SEM, n = 4-12. Significant differences between the animals of different age of the same strain fed with the same diet vs. 10 days-old rats (*A1-A3, B1-B3, C1-C3*) are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

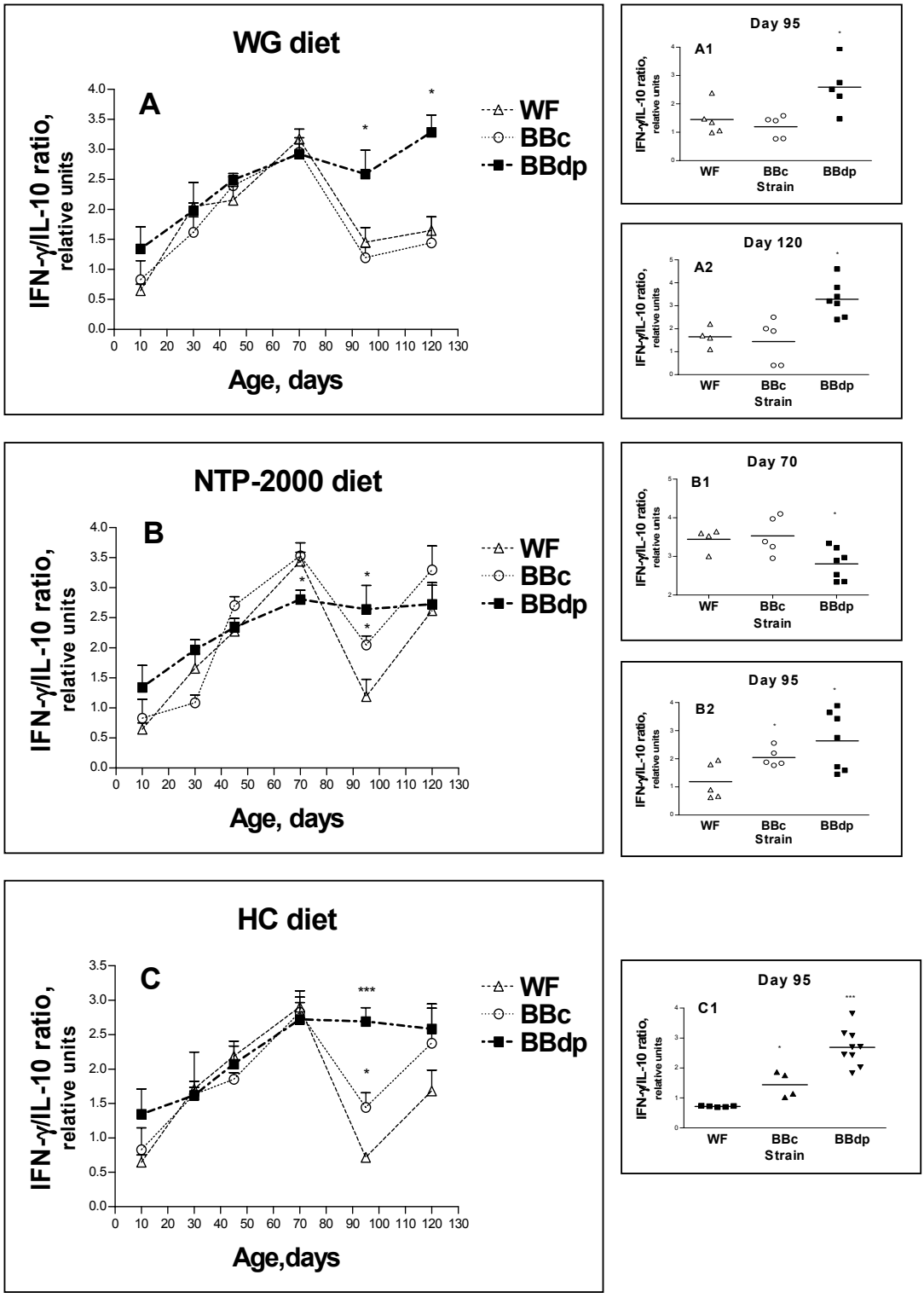

Fig. 3-33 IFNγ/IL-10 ratio in the gut duodenum of rats fed WG, NTP-2000 or HC diet. Part II. IFNγ/IL-10 ratio was calculated from the measured IFNγ and IL-10 values in gut duodenum of BBdp, BBc or WF rats fed the diabetes-promoting WG (*A*) or NTP-2000 (*B*) diets, or the diabetes-retardant HC diet (*C*). The data are expressed as mean values ± SEM, n = 4-12. Significant differences between the animals of different strains, but of the same age fed with the same diet vs. 10 days-old rats (*A, A2, B1, B2, C1*) are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

3.2.3 MCP-1 content in gut duodenum of BB rats fed the WG, NTP-2000 or HC diets

In the gut duodenum of BBdp rats fed WG, NTP-2000 or HC diet during all investigated period of life, i.e. between days 10 and 120 after birth, there were no major differences of the amount of MCP-1/CCL2 (Fig. 3-34,*A,A2-A4*). This held true when the animals of different age fed the same diet (Fig. 3-34,*A2-A4*) or the groups of rats of the same age fed the different diet (Fig. 3-34,*A*) were compared to each other. Although the mean intestinal CCL2 level was higher in BBdp rats fed HC diet vs. WG diet for all investigated ages and vs. NTP-2000 diet after day 30 till day 95, this feature could not reach the statistical significance (Fig. 3-34,*A*). Only on day 45 there was a decrease in CCL2 level in the duodenum of BBdp rats fed NTP-2000 diet in comparison with those fed HC diet (1407.0 ± 74.2 vs. 2186.0 ± 321.5 pg of CCL2/mg of protein, n=10, $p < 0.05$, Fig. 3-34,*A1*). BBc rats fed HC diet had higher amount of CCL2 in duodenum as compared to the animals fed NTP-2000 or WG (day 30, Fig. 3-34,*B,B1*) or only NTP-2000 (day 45, Fig. 3-34,*B,B2*). But from day 95 this difference disappears (Fig. 3-34*B*). It is noteworthy that the kinetic of duodenal CCL2 content for both diabetes-promoting diets was very much alike in BBc rats (Fig. 3-34*B*). BBc rats kept on the same diet had an increased mean CCL2 values in each investigated age group in comparison to 10-old rats, but this was not statistically significant (Fig. 3-34,*B3-B5*). WF rats fed WG or NTP-2000 diet showed, as in BBc rats, very similar kinetic of intestinal CCL2 content during an investigated period (Fig. 3-34*C*). But, differently from BBc rats, the increase in CCL2 duodenal content after weaning in WF rats was significantly higher in each age group in comparison to 10-days-old rats for all the diets (Fig. 3-34,*C,C3-C5*). WF rats fed HC diet had always higher mean CCL2 levels as compared to the animals fed WG or NTP-2000 diet, and this was statistically significant at days 70 and 120 of age (day 70: 2759.0 ± 310.4 vs. 1403.0 ± 42.1 or 1269.0 ± 87.0 pg of CCL2/mg of protein, $n = 4$, $p < 0.05$, and day 120: 1399.0 ± 116.2 vs. 1039.0 ± 29.5 or 877.2 ± 64.7 pg of CCL2/mg of protein, $n = 4\text{-}5$, $p < 0.05$; Fig. 3-34,*C,C1,C2*). As for the strain differences, WF and BBc rats, independently of the diet, had similar kinetic of duodenal CCL2 values as compared to BBdp, and this feature was more evident for the animals fed diabetes-promoting WG or NTP-2000 diet (Fig. 3-35). BBdp rats fed HC diet had decreased content of CCL2 in duodenum than WF rats on days 30 and 70 (Fig. 3-35, *C,C1, C2*). The amount of duodenal CCL2 in BBdp rats fed WG diet was lower in comparison with BBc rats at days 95 and 120 of age (day 95: 1305.0 ± 168.3 vs. 1845.0 ± 156.1 pg of CCL2/mg of protein,

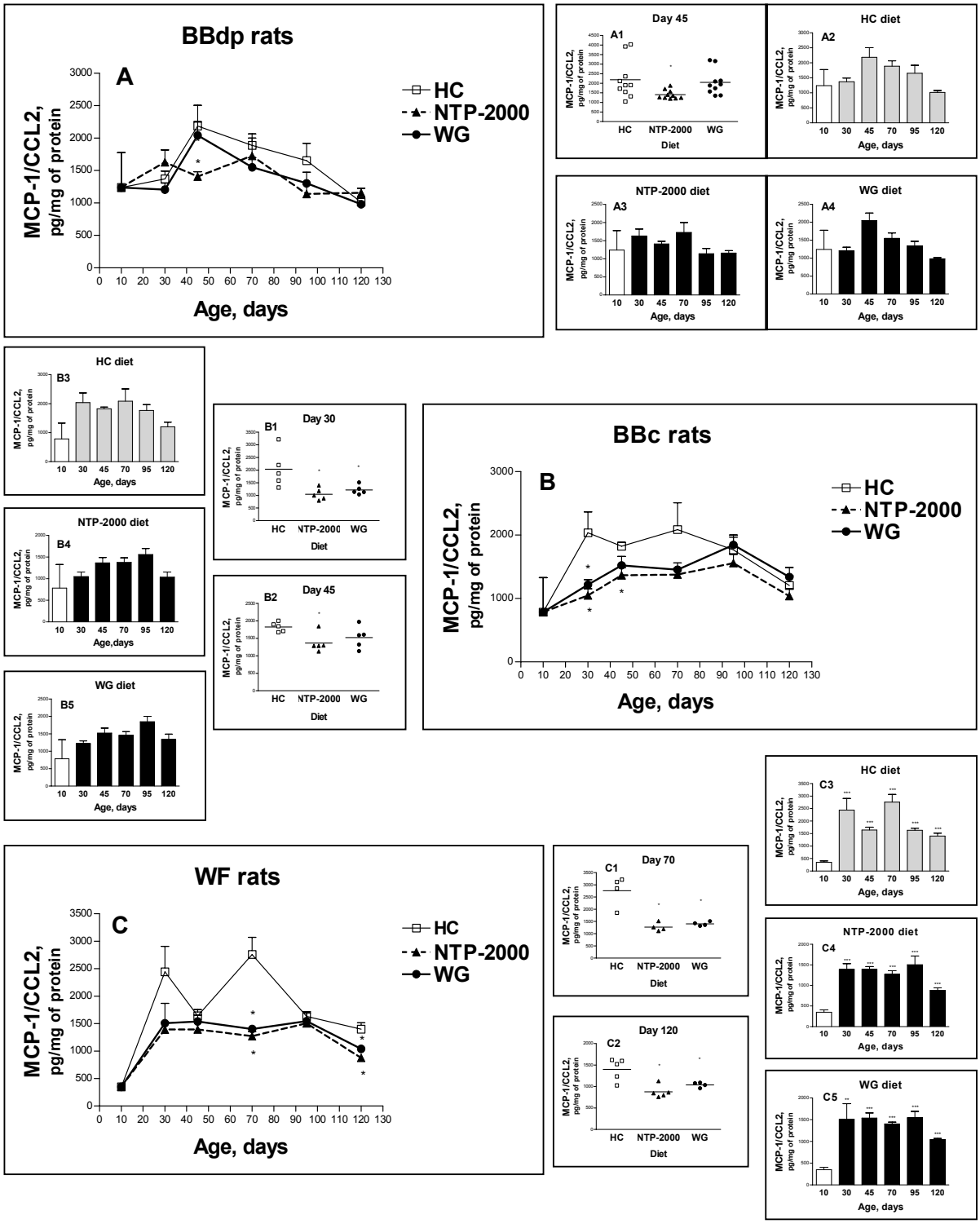

Fig. 3-34 MCP-1/CCL2 content in the gut duodenum of rats fed WG, NTP-2000 or HC diet. Part I. The amount of MCP-1/CCL2 was measured in gut duodenum of BBdp (*A*), BBc (*B*) or WF (*C*) rats fed the diabetes-promoting WG or NTP-2000 diet , or the diabetes-retardant HC diet. The data are expressed as mean values ± SEM, n = 4-12. Significant differences between the animals of the same age and strain fed the different diet and of different age of the same strain fed with the same diet vs. 10 days-old rats (*A1-A3, B1-B3, C1-C3*) are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

n = 5, p < 0.05, and day 120: 981.8 ± 34.6 vs. 1338.0 ± 151.7 pg of CCL2/mg of protein, n = 5-7, p < 0.05, Fig. 3.35*A*).

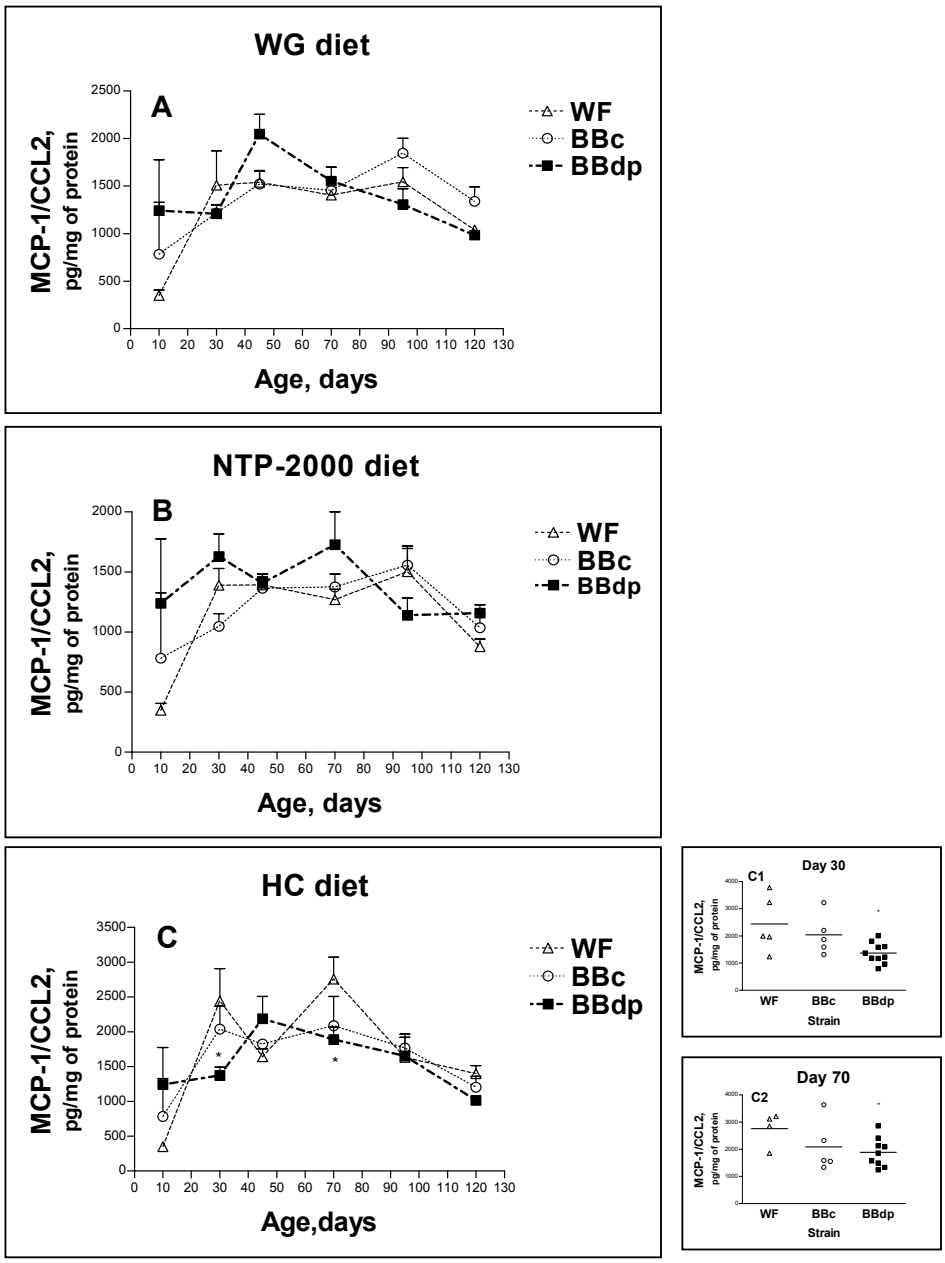

Fig. 3-35 MCP-1/CCL2 content in the gut duodenum of rats fed WG, NTP-2000 or HC diet. Part II. The amount of MCP-1/CCL2 was measured in gut duodenum of BBdp, BBc or WF rats fed the diabetes-promoting WG (*A*) or NTP-2000 diet (*B*), or the diabetes-retardant HC diet (*C*). The data are expressed as mean values ± SEM, n = 4-12. Significant differences between the animals of different strain and of the same age fed the same diet vs. WF rats *(C1, C2*) are indicated as * $p < 0.05$, Student's unpaired *t* test.

3.2.4 MIP-2 level in duodenum of BB rats fed the WG, NTP-2000 or HC diets

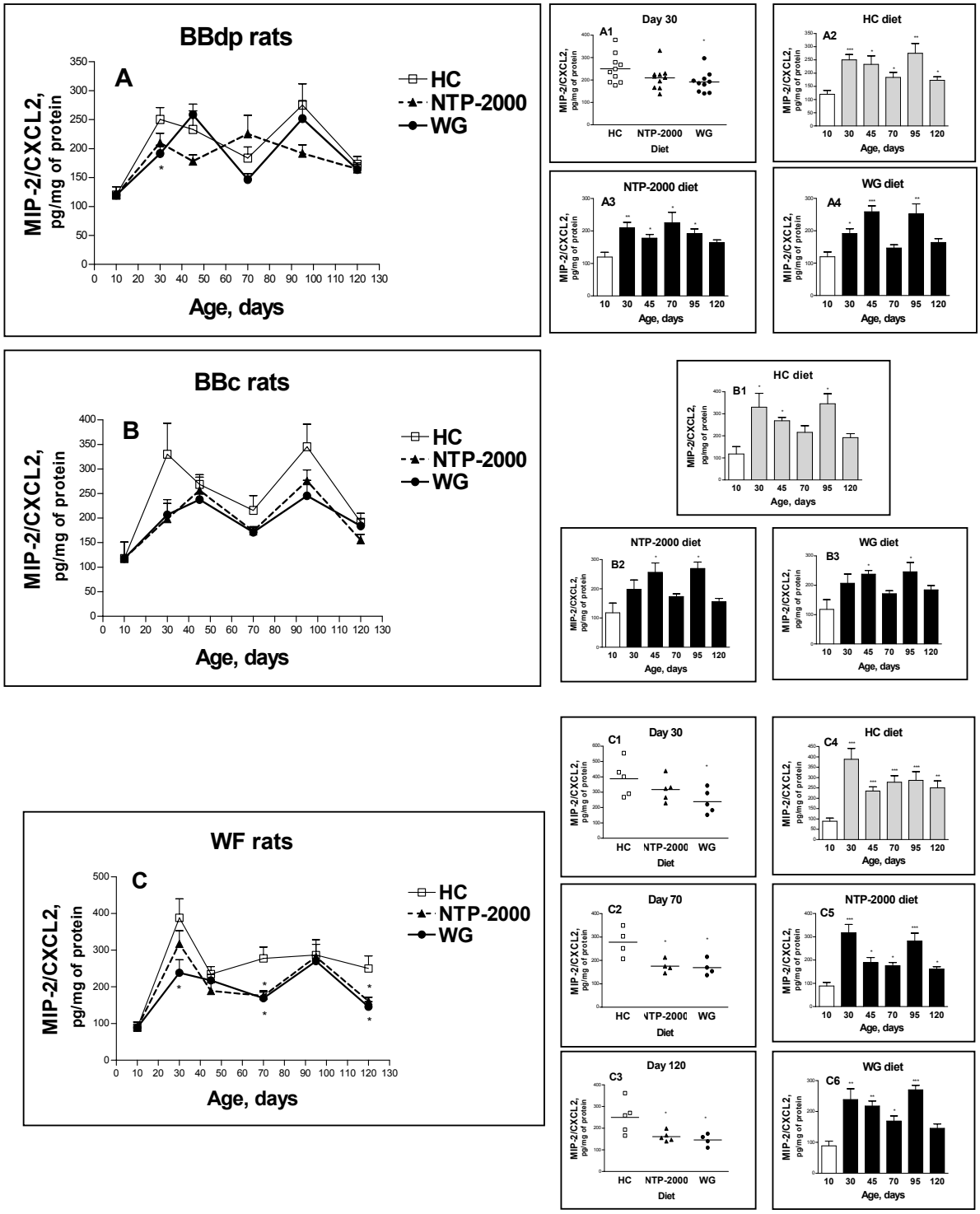

Fig. 3-36 MIP-2/CXCL2 content in the gut duodenum of rats fed WG, NTP-2000 or HC diet. Part I. The amount of MIP-2/CXCL2 was measured in gut duodenum of BBdp (*A*), BBc(*B*) or WF (*C*) rats fed the diabetes-promoting WG or NTP-2000 diet , or the diabetes-retardant HC diet. The data are expressed as mean values ± SEM, n = 4-12. Significant differences between the animals of the same age and strain fed the different diet and of different age of the same strain fed with the same diet vs. 10 days-old rats (*A1-A3, B1-B3, C1-C3*) are indicated as $^{*}\ p < 0.05$, $^{**}\ p < 0.001$, $^{***}\ p < 0.0001$, Student's unpaired *t* test.

The duodenal levels of MIP-2/CXCL2 in BBdp rats did not significantly varied between the groups of animals weaned onto different diets (Fig. 3-36*A*). The only exception was a moderate decrease of CXCL2 in 30-days-old rats fed WG diet in comparison with those fed HC diet (191.4 ± 14.7 vs. 250.5 ± 20.3 pg/mg of protein, n = 10, $p < 0.05$, Fig. 3-36*A1*). But after weaning, independently of the type of diet, the level of CXCL2 became much higher and kept to be so, with some fluctuations, till the end of the experiment at day 120, although for BBdp rats fed the diabetes promoting diet this increase at day 120 was not statistically significant (Fig. 3-36, *A,A2-A4*). The mean CXCL2 content in duodenum of BBc and WF rats, similarly to CCL2, was higher in all investigated age groups for HC diet in comparison with NTP-2000 and WG diets (Fig. 3-36,*B*,*C*). Although this increase could not gain the statistical significance in BBc rats, in WF rats this enhancement was proved at days 30, 70 and 120 after birth (Fig. 3-36,*C,C1-C3*). As it was previously seen for IL-10 and CCL2, the level of CXCL2 in the duodenum of BBc and WF, fed the diabetes-promoting NTP-2000 and WG diets was very similar during all the investigated period (Fig. 3-36,*B*,*C*). This was in contrast to BBdp rats, where such a feature was not found. Irrespectively of the diet, BBc rats had an elevated amount of duodenal CXCL2 after weaning, especially at 30, 45 and 95 days of age (Fig. 3-36,*B,B1-B3*). This enhancement reached its maximum at day 95, being for HC, NTP-2000 or WG diets 2.9-, 2.3- or 2.1-fold higher, respectively, as compared to day 10 ($p < 0.05$, Fig. 3-36,*B1-B3*). WF rats had a sharp increase of CXCL2 concentration in duodenum after weaning, at day 30, when for HC, NTP-2000 or WG diets the CXCL2 level was 4.4-, 3.6- or 2.7-fold higher, respectively, than at day 10 (Fig. 3-36, *C4-C6*). As strain differences are concerned, at day 10 all the animals had similar content of MIP-2 in duodenum of small intestine (Fig. 3-37, *A-C*). For the rats fed WG diet, irrespectively of strain, the curves for chemokine level between 30 and 120 days of age were very much alike, with the enhancements at days 45 and 95 and decreases at days 70 and 120 (Fig. 3-37*A*). The animals fed NTP-2000 diet were not very different in their duodenal amount of CXCL2, but nevertheless at days 30 and 95 BBdp rats had lower concentration of CXCL2 in comparison with WF rats (Fig. 3-37,*B,B1,B2*). The mean CXCL2 level in the duodenum of BBdp rats fed HC diet was lower between days 30 and 120 as compared to BBc or WF rats (Fig. 3-37*C*). This could gain the statistical significance at days 30 and 70 (day 30: 250.5 ± 20.3 pg of CXCL2/mg of protein in BBdp rats, n = 10, vs. 388.4 ± 51.8 pg of CXCL2/mg of protein in WF rats, n = 5, $p < 0,05$; day 70: 183.5 ± 19.3 pg of CXCL2/mg of

protein in BBdp rats, n = 9, vs. 277.8 ± 31.0 pg of CXCL2/mg of protein in WF rats, n = 4, $p < 0,05$, Fig. 3-37,*C,C1,C2*).

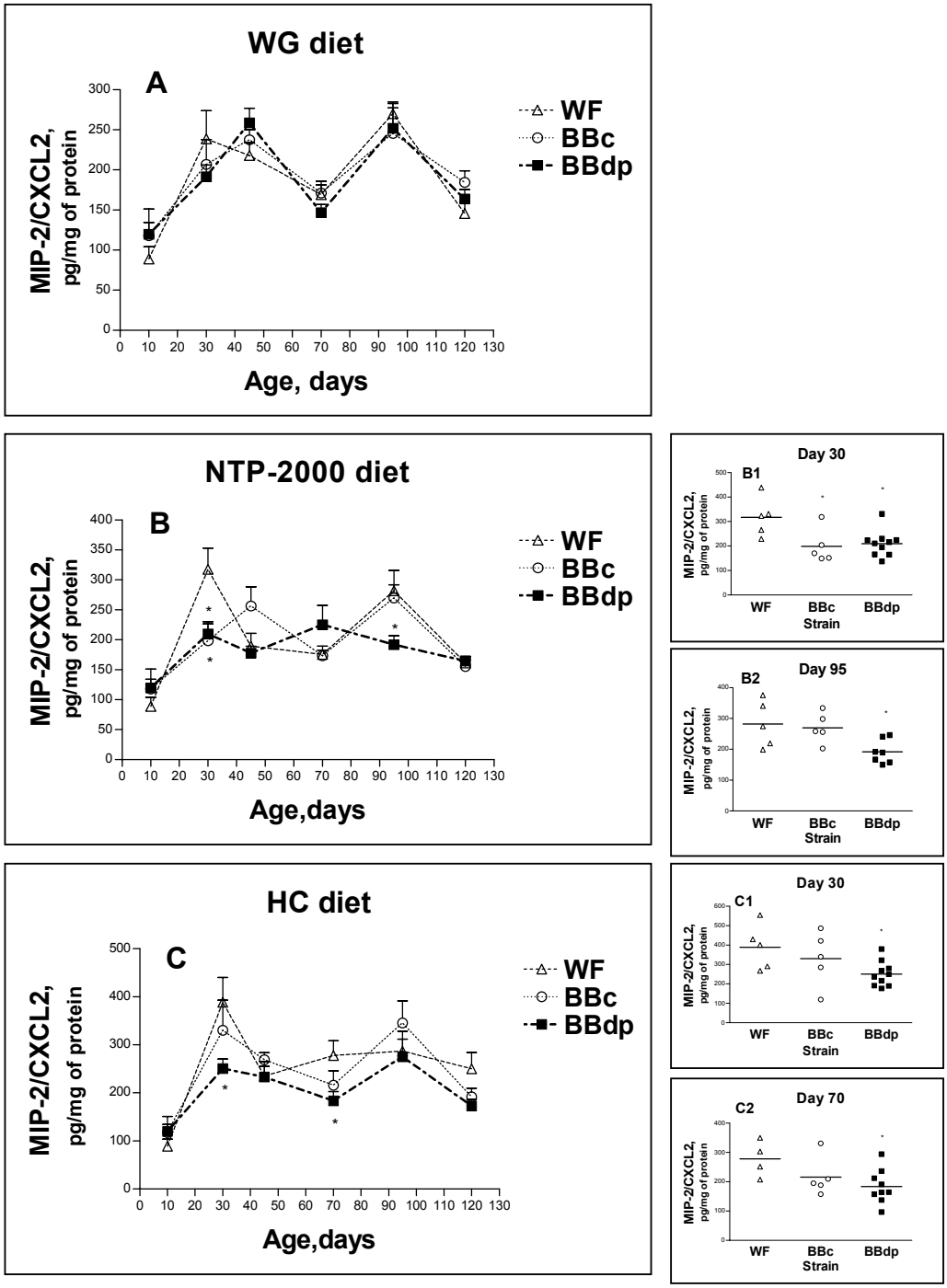

Fig. 3-37 MIP-2/CXCL2 content in the gut duodenum of rats fed WG, NTP-2000 or HC diet. Part II. The amount of MIP-2/CXCL2 was measured in gut duodenum of BBdp, BBc or WF rats fed the diabetes-promoting WG (*A*) or NTP-2000 diet (*B*), or the diabetes-retardant HC diet (*C*). The data are expressed as mean values ± SEM, n = 4-12. Significant differences between the animals of different strain and of the same age fed the same diet vs. WF rats *(C1, C2*) are indicated as * $p < 0.05$, Student's unpaired *t* test.

3.2.5 IFNγ, IL-10 and MCP-1 levels in duodenum of small intestine of BB rats before and right after weaning

After summarizing the data from the above-described experiments on BB rats we found some characteristic features. First of all, already before weaning 10-days-old BBdp rats had higher IFNγ, IL-10 and IFNγ/IL-10 ratio, and the tendency to the enhanced CCL2 level in duodenum as compared to WF rats. Secondly, the weaning, irrespective of the diet, significantly increased all the investigated immune markers in WF rats. In BBc rats there was a rise of IFNγ, IFNγ/IL-10 ratio and CXCL2 and in BBdp rats only IFNγ/IL-10 ratio and CXCL2 were elevated after weaning, IL-10 level was even decreased. That is why we decided to focus our attention on the time around the weaning and perform the additional experiment, where BBdp, BBc and WF rats of 10 and 23 days of age would be investigated after they were weaned onto HC, NTP-2000 and WG diet at day 22.

BBdp rats at 10 days of age had significantly higher level of IFNγ, IFNγ/IL-10 ratio and CCL2 in the duodenum of small intestine in comparison to WF rats and for IFNγ and CCL2 also vs. BBc rats, IL-10 content did not differ (Fig. 3-38,*A-D,A1-D1*). Ten days-old BBc rats had only an increased amount of duodenal CCL2 as compared to WF rats (Fig. 3-38 *C1*). The next day after weaning WF and BBc rats showed a rise of IFNγ, IFNγ/IL-10 ratio and CCL2 in the upper gut, irrespective of the diet, although for BBc rats fed the HC diet the increase of IFNγ was not statistically significant (Fig. 3-38 *A,C,D*).The level of IL-10 in duodenum of WF and BBc rats tended to be lower after weaning in comparison to 10 days-old animals, but only for BBc rats weaned onto NTP-2000 diet this appeared to be significant (Fig. 3-38*B*). BBdp rats at day 23, irrespective of the diet, had a very sharp decrease of IL-10 and CCL2, and increase of IFNγ/IL-10 ratio in the duodenum, the amount of IFNγ was not changed (Fig. 3-38 *A-D*).

The new data on the cytokine and chemokine levels in gut duodenum at day 10 were very much alike and supportive to those obtained in the previous experiment. After the new data were adjusted to an old ones using positive control, it became possible to analyze the combined data set. In the upper small intestine of 10 days-old BBdp rats the amounts of IFNγ, IFNγ/IL-10 ratio and CCL2 were significantly higher than those in WF and BBc (Fig. 3-39,*A,C,D*). BBdp rats had in duodenum 1784.0 ± 301.4 pg of IFNγ/mg of protein, 4261.0 ± 856.4 pg of CCL2/mg of protein and IFNγ/IL-10 ratio 1.44 ± 0.20 relative units in comparison with 443.6 ± 82.7 pg of

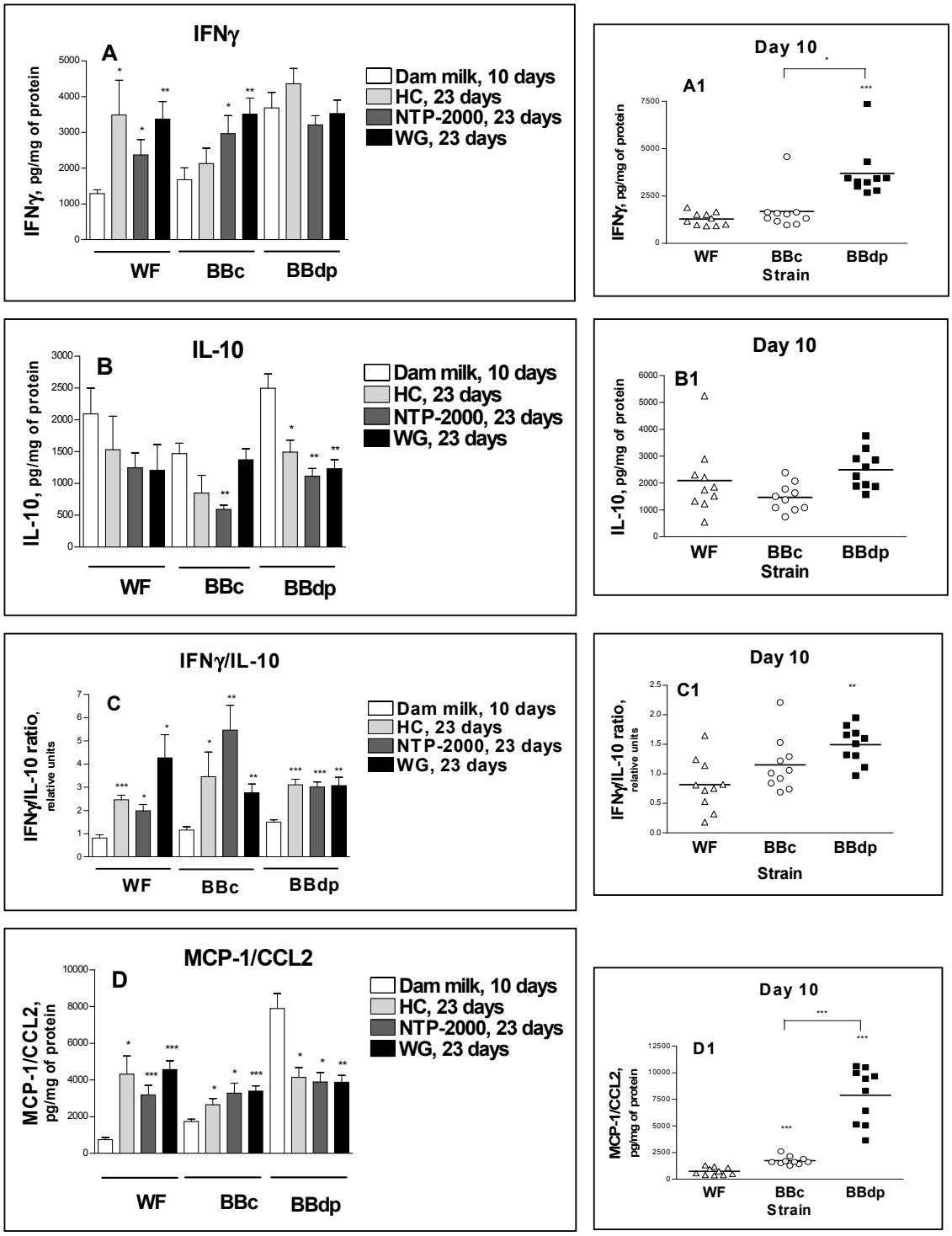

Fig. 3-38 IFNγ, IL-10, IFNγ/IL-10 ratio and MCP-1/CCL2 content in duodenum of BB rats before and after weaning. The concentrations of IFNγ (*A*), IL-10 (*B*) and MCP-1/CCL2 (*D*) were measured in gut duodenum of BBdp, BBc or WF rats at 10 and 23 days of age after they were weaned at 22 days of age onto the diabetes-promoting WG or NTP-2000 diet, or the diabetes-retardant HC diet. IFNγ/IL-10 ratio was calculated from known values for IFNγ and IL-10. The data are expressed as mean values ± SEM, n = 7-10. Significant differences between the animals of the same strain vs. 10 days-old rats (*A-D*) and vs. WF rats if not specified additionally (*A1-D1*) are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

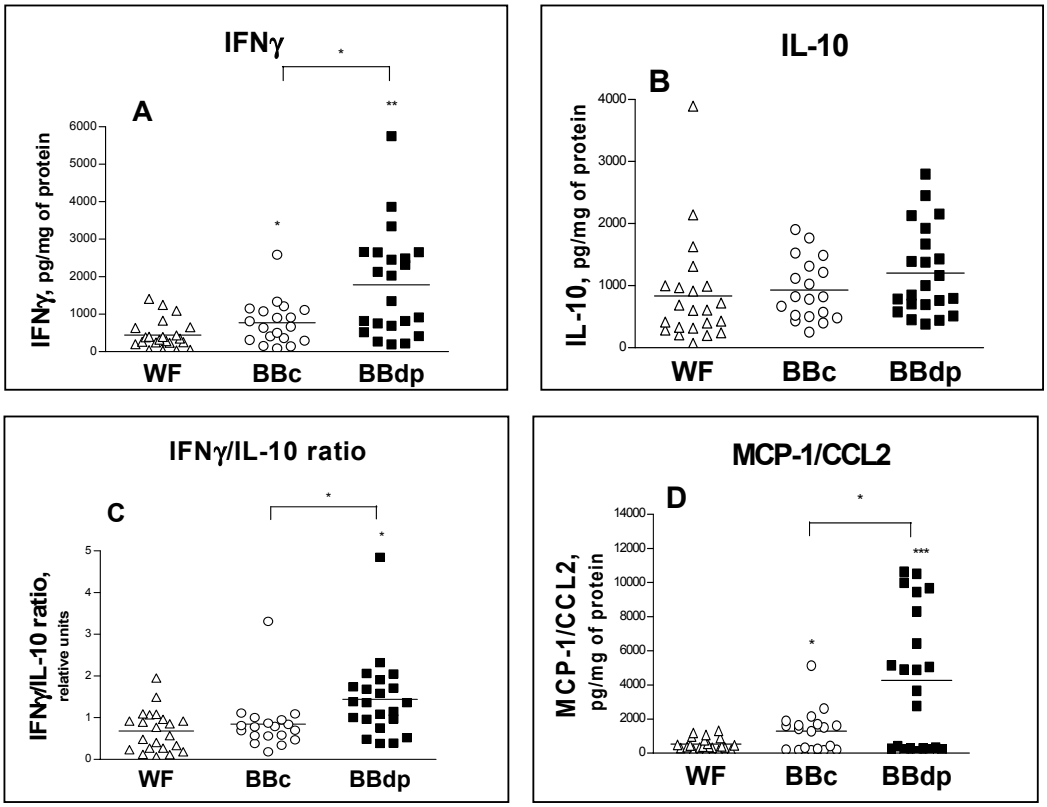

Fig. 3-39 Cytokine and chemokine level in duodenum of 10 days-old BB rats. The concentrations of IFNγ (*A*), IL-10 (*B*) and MCP-1/CCL2 (*D*) were measured in gut duodenum of BBdp, BBc or WF rats at 10 days of age. IFNγ/IL-10 ratio (*C*) was calculated from known values for IFNγ and IL-10. The data are expressed as mean values ± SEM, n = 19-22. Significant differences to WF rats if not specified additionally are indicated as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, Student's unpaired *t* test.

IFNγ/mg of protein, 530.6 ± 72.9 of CCL2/mg of protein and IFNγ/IL-10 ratio 0.68 ± 0.11 relative units, respectively, in WF rats. The duodenal level of IL-10 did not differ between the strains (Fig. 3-39*B*). All the investigated cytokines/chemokine in the animals of each strain were in a good correlation with each other. Thus, in the duodenum of BBdp rats the level of IFNγ was highly correlated to IL-10, CCL2 content and IFNγ/IL-10 ratio ($p < 0.0001$), the same was for IL-10 vs. IFNγ/IL-10 ratio ($p < 0.0001$) and for CCL2 vs. IFNγ/IL-10 ratio ($p < 0.01$). In BBc rats there were also a good correlations of IFNγ level with IL-10, CCL2 content and IFNγ/IL-10 ratio ($p < 0.0001$, $p < 0.01$ and $p < 0.001$, respectively) and of IFNγ/IL-10 ratio with the amount of CCL2 ($p < 0.0001$). In WF rats the correlations between IFNγ and IL-10, IFNγ/IL-10 ratio and CCL2 ($p < 0.05$, $p < 0.0001$ and $p < 0.0001$, respectively), IL-10 and CCL2 ($p < 0.001$), IFNγ/IL-10 ratio and CCL2 ($p< 0.05$) were also significant. It is therefore evident that the immune activity in the upper small intestine of BBdp rats is significantly higher than that of WF and BBc rats.

4 DISCUSSION

This study clearly demonstrated that two dietary antigens, associated with the pathogenesis of T1DM, wheat gluten or Hsp60, activate the innate immune system in specific manner that follows classical ligand-receptor interaction.

The addition of α-CT digest of wheat gluten to immature BMDC induced their maturation that was characterized by the increased surface expression of MHC class II and co-stimulatory molecules CD86 (B7.2), CD 40 and CD54 (ICAM-1). Up-regulation of the surface density of MHC class II and co-stimulatory molecules occurred in parallel on the same cell, which was indicative of a physiologically regulated response. Increased expression of MHC class II and co-stimulatory molecules CD86, CD40 and CD54 was dependent on the concentration of wheat gluten present, over the range of 1-100 μg/ml. At the highest gluten dose DC response was similar to that seen in response to LPS at concentration 10 ng/ml, which underscores the biological potency of wheat gluten. Because of the comparability with the effects of LPS it seemed mandatory to exclude the contribution of a possible endotoxin contamination of wheat gluten preparation. Several lines of evidence excluded an involvement of endotoxin. For one, experiments were repeated in the presence of an excess of antibiotic peptide PmB, a potent LPS antagonist (Storm D.R. et al., 1977; Morrison D.C. et al., 1976), with virtually the same outcome for gluten, while the effects of LPS were completely blocked. In a second approach we analyzed DCs from the LPS non-responder strain C3H/HeJ (Poltorak, A., et al., 1998). DCs from this strain were not affected by LPS, but preserved the ability to mature when exposed to wheat gluten. A third argument is that wheat gluten preparation, digested with highly reactive serine endopeptidase, proteinase K, completely lost the ability to stimulate DCs, in contrast to LPS. Finally, the secretory response of DCs to gluten was strikingly different from that to LPS.

Upon exposure to wheat gluten, DCs produced both cytokines and chemokines. The production was dose-dependent in that 100 μg/ml of gluten induced higher levels of secreted molecules in the supernatant than 10 μg/ml of gluten. The highest secretory responses were seen for the two chemokines CXCL2 and CXCL1, whereas the two chemokines CCL3 and CCL2 were not induced, in contrast to LPS-treated DC cultures. Wheat gluten stimulated a dose-dependent secretion of CCL5 from DCs, although the

observed increase of its production in response to gluten was not so prominent as for CXCL2 and CXCL1 and lower as compared to LPS stimulation. Again in contrast to LPS, wheat gluten secreted only minimal amounts of TNFα, lower amounts of IL-1β and failed to induce IL-10 secretion. It is noteworthy that wheat gluten in our study induced neither two strongly pro-inflammatory mediators TNFα and CCL3 (Tracey, K.J., 2002; Lokati, M., t al., 2002; Luther, S.A., and J.G. Cyster, 2001) nor the potent anti-inflammatory cytokine IL-10 (Singh, B., et al., 2001; Von Herrath, M.G., and L.C. Harrison, 2003). The induction of high CXCL2, CXCL1 and CCL5 and low IL-1β secretion can not easily be classified as pro- or anti-inflammatory. Rather, these immune mediators are expected to prepare the immune system for better reactivity, by attracting leukocytes and by increasing the reactive state of immune cells. The murine chemokines CXCL2 and CXCL1, which are homologous to human CXCL8 and share the receptor CXCR1 and CXCR2, are the main attractants of polymorphonuclear neutrophils in mice (Bozic, C.R., et al., 1994). Chemokine-induced migration of neutrophils across the intestinal epithelium increases its permeability and might thereby enhance Ag influx (Nash, S.J., et al., 1987). Interestingly, intracellular Ags from necrotic cells induce CXCL2 and CXCL1 production in macrophages, fibroblasts and DC (Li, M., et al., 2001). Importantly, CXCL2 is necessary for the induction of peripheral tolerance, it is selectively up-regulated in tolerance-conferring monocytes/macrophages and attracts NKT cells to the spleen that is absolutely required for the generation of Ag-specific negative Tr cells that convey peripheral tolerance (Faunce, D.E., et al., 2001). The observed in our study high CCL5 production in response to wheat gluten deserves an attention. CCL5 is chemotactic for T cells, eosinophils, basophils, NK cells, monocytes, macrophages and DCs and recruites leukocytes into inflammatory sites (Lillard, J.W., et al., 2001; Dwinell, M.B., et al., 2003). This chemokine might play an essential role in regulation of mucosal immunity. The level of CCL5 is increased in the gut after Ag feeding (DePaolo, R.W., et al., 2004). CCL5 enhances mucosal and systemic humoral Ag-specific antibody responses (Lillard, J.W., et al., 2001). One of the receptors for CCL5, CCR5, is critical for the induction of high dose oral tolerance, and it was suggested that the lack of oral tolerance seen in $CCR5^{-/-}$ mice is related to CCL5 regulation of CCL2 expression by macrophages in the PPs and MLN (DePaolo, R.W., et

al., 2004). In our study CCL2 was not secreted by DC in response to wheat gluten. But, according to above-mentioned consideration, CCL5 originated from DC after gluten stimulation can induce CCL2 production from macrophages thereby increasing the level of such an important regulatory molecule for oral tolerance. Indeed, CCL2 was previously shown to suppress IL-12 and increase TGF-β expression in GALT-derived APCs after Ag feeding (Karpus, W.J., and N.W. Lukas, 1996; Karpus, W.J., et al., 1998; DePaolo, R.W. et al., 2003). In addition, CCL5 specifically induces rapid expression of chemokines CXCL2, CXCL1, CCL3 and CCL4 and cytokines TNFα and IL-6 from immature murine BMDC, and none of the known CCL5 receptors, expressed on DC, CCR1, CCR3 or CCD5, were exclusively necessary for CXCL2, CXCL1 or CCL3 expression suggesting the presence of a novel receptor (Fischer, F.R., et al., 2001).

Altogether, the spectrum of chemokines identified as being responsive to wheat gluten challenge in our *in vitro* system, i.e. CXCL2, CXCL1 and CCL5, is in a good correlation with available *in vivo* data on inflammatory intestinal diseases. Thus, the increased expression of CXCL2 and CXCL1 homolog IL-8 in biopsies of the patients with active inflammatory bowel disease (IBD) correlated with the severity of inflammation, and there was an enhanced CXCR1 expression on macrophages and lymphocytes in the colon of patients with active ulcerative colitis (Mazzucchelli, L., et al., 1994; Daig, R. et al., 1996; Williams, E.J., et al., 2000; Ajuebor, M.N., and M.G. Swain, 2002). The protein levels of CCL5 and CCL2 were elevated in the mucosa of patients with IBD (McCormack, G., et al., 2001). In active IBD CCL5 mRNA expression was increased in IEL and the subepithelial LP of patients' rectal biopsies, while CCL2 mRNA expression was higher in endothelial cells and smooth muscle cells (Mazzucchelli, L., et al., 1996). CCL5 may play an important role in the pathogenesis of food-mediated gastrointestinal allergy, mRNA level of this chemokine significantly correlated with eosinophil infiltration to the intestinal LP and with mucosal eosinophil cationic protein concentration in ovalbumin (OVA)-sensitized BALB/c mice in response to OVA challenge (Lee, J.B., et al., 2004). A borderline association of CCL5 promoter genetic variants with predisposition to CD was recently observed (Rueda, B., et al., 2005).

Hence, wheat gluten appears to prepare the immune system for easier response, by causing maturation of APCs, by attracting further leukocytes and by increasing their

reactive state. In the presence of an appropriate genetic predisposition this may be expected to increase the risk of adverse immune reactions, to wheat gluten itself or to other antigens presented. Although not detectable in our experimental system, a pro-inflammatory activity of wheat gluten cannot be excluded. It has been reported that the treatment of human monocyte-derived DC with gliadin digest induced a high production of TNFα, in contrast to our data (Palová-Jelínková, L., et al., 2005). This discrepancy can be explained by the use of different cell types (human peripheral blood mononuclear cells-derived DC vs. murine BMDC) and culture conditions, i.e. the addition of IFNγ to stimulate human monocyte-derived DC maturation. Indeed, IFN-γ in the culture media greatly potentiated TNFα secretion from peritoneal macrophages induced by digested gluten or gliadin (Tučková, L., et al., 2000). Also in contrast to peptic gliadin digest used on human DC, we stimulated DC with α-CT digest of wheat gluten that can have an additional stimulatory potential. From the other hand, the absence of IL-10 production by DC in response to wheat gluten digest seen in our study was supported by the observation on human monocyte-derived DC, where 10 μg/ml of gliadin digest did not cause IL-10 secretion, while 100 μg/ml could stimulate it to a minimal level (Palová-Jelínková, L., et al., 2005). It is therefore difficult to explain a very high spontaneous as well as gliadin-inducible IL-10 production by murine peritoneal macrophages seen in another study (Tučková, L., et al., 2002). None of other Th2 cytokines (IL-4, IL-5 or IL-13), but also no Th1 cytokines IFNγ, IL-2 or IL-12 were produced in response to gliadin digest by human DC, there were also no expression of IL-15, IL-1α or IL-1β, and a strong increase of IL-6 (Palová-Jelínková, L., et al., 2005). Similar to our results, gliadin fragments induced the production of CCL5 and chemokines IL-8 and GROα, the human homologs of muirne CXCL1 and CXCL2, but in contrast to our data, also CCL2 was up-regulated (Palová-Jelínková, L., et al., 2005).

Studies on the stimulation of innate immunity by wheat gluten represent a novel concept. Previous works focused on T cell stimulatory capacity of gliadin and glutenin peptides (Lundin, K.E.A. et al., 1993; van de Wal, Y., 1996; van de Wal, 1999; Quarsten, H., et al., 1999; Anderson, R.P. et al., 2000; Arentz-Hansen, H., S.N. McAdam et al., 2000; Arentz-Hansen, H., et al., 2002, Shan, L., et al., 2002; Vader, W. et al., 2002; Molberg,

Ø., 2003; Gianfrani, C. et al., 2003). There were some data suggesting or showing the involvement of innate immunity in the development of gluten-sensitive enteropathy (Maiuri, L., et al., 1996; Anderson, R.P. et al., 2000; Maiuri, L., et al., 2003). Another studies investigated the direct effect of gluten and gliadin fragments on the activation of monocytes or macrophages *in vitro* (Tučková, L., et al., 2000, 2002; Maiuri, M.C., et al., 2003; Jelínková, L., et al., 2004). Our work and the recent results of *Palová-Jelínková, L., et al., 2005,* are the only available so far data describing the effects of gluten or gliadin fragments on murine or human DC.

The finding that wheat gluten effects are dose-dependent and quite selective in terms of the induced cytokine/chemokine pattern argues against a non-specific "irritation" of DC. Rather, we assume the involvement of a single or a limited number of receptor types, such as scavenger receptors or other surface proteins. TLR family is the best characterized class of PRRs that detect conserved microbial molecular patterns, PAMPs. They are critical PRR, because they can induce effector Th1 or CTL responses (Iwasaki, A., and R. Medzhitov, 2004). Our study excluded the involvement of TLR2 and TLR4 in gluten digest-mediated stimulation of murine DC. In support of our results quite recent investigation demonstrated that gliadin-mediated stimulation of murine macrophages was neither TLR2- nor TLR4-dependent, the involvement of Myeloid Differentiation Factor 88 (MyD88)-associated PRR in gliadin signaling was suggested (Thomas, K.E., et al., 2006). It is nevertheless possible that other TLRs participate in gluten signaling in DC. To date 10 members of TLR family have been identified in human and 13 in mice, and it is likely that the other members will be discovered in the nearest future. Indeed, if we take into consideration that TLR3, TLR7/8 and TLR9 that recognize viral components, are exclusively localized to endosomal compartments, but not on the cell surface, that TLR2 and TLR4 according to our study are not involved in gluten-mediated effects on DC, and that TLR2 functions together with TLR1 or TLR6, the number of candidate TLRs as wheat gluten sensors becomes much reduced (Kawai, T., and S. Akira, 2006). It is well known that different DC subsets express distinct sets of TLRs (Iwasaki, A., and R. Medzhitov, 2004). In mice, all splenic DC subsets express TLRs 1, 2, 4, 6, 8 and 9, pDCs do not express TLR3, $CD8\alpha^+$ DCs lack TLR5 and TLR7, and $CD11c^+CD11b^+$ DCs derived from bone-marrow precursors in the presence of GM-CSF, as in our case, have

high expression of TLR4 (Iwasaki, A., and R. Medzhitov, 2004). Recently discovered subset of interferon-producing killer DCs (IKDCs) was found to be specifically activated by TLR9 ligands (Chan, C.W., et al., 2006; Taieb, J., et al., 2006). TLR signaling results in the up-regulation of co-stimulatory molecules on DCs, production of cytokines and chemokines, and all TLRs use intracellular signaling pathways, mostly mediated by MAP kinases, i.e. JNK, p38 and ERK and culminating in the activation of NFκB and activating protein-1 (AP-1) (Kawai, T., and S. Akira, 2006). Many TLR ligands activate these MAP kinases in similar kinetics. In our study wheat gluten digest-induced $CD11c^+CD11b^+$ DCs activation was accompanied by activation of MAP kinase signaling. There was a dose-dependent phosphorylation of p38 and p65 subunit of NFκB, at 45 min more than at 15 min after stimulation. Two ERK kinases, 1 and 2, were simultaneously and equally phosphorylated at 45 min, but not at 15 min after challenge with wheat gluten digest. JNK 1 and JNK2 were not activated at these time points by wheat gluten. From these results p38 can be considered as a main pathway for gluten signaling on murine $CD11c^+CD11b^+$ DCs.

Although, in contrast to our study, all three MAP kinases were phosphorylated after treatment of human monocyte-derived DC with gliadin digest, the inhibition of MAP kinase p38, but not ERK, and transcription factor NFκB activation had a significant effect on gliadin-induced DC maturation, supporting the importance of p38 and NFκB signaling for DC maturation in response to different stimuli and our finding (Palová-Jelínková, L., et al., 2005; Arrighi, J.F., et al., 2001; Rescigno, M., et al., 1998). Moreover, p38 MAPK pathway participates in NFκB activation (Vanden Berghe, W.V., et al., 1998; Carter, A.B., et al., 1999). Transcription factors of NFκB family regulate expression of many genes, involved in immune response, including pro-inflammatory cytokines (Baeuerle, P.A., et al., 1994; 1997; 1998). NFκB was activated in LP mononuclear cells and epithelial cells of inflamed intestinal mucosa, but not in non-inflamed tissue, and the degree of activation correlated with the level of mucosal inflammation (Rogler, G., et al., 1998). The participation of NFκB in gliadin-induced IL-8 and TNFα production from human monocytes and in gliadin-mediated increase of iNOS gene expression and NO

production in mouse monocytes/macrophages was shown earlier (Maiuri, M.C., et al., 2003; Jelínková, L., et al., 2004).

As for p38, another study revealed that non-immunodominant gliadin peptide p31-43, which is known to induce small intestinal damage in CD, induced phosphorylation of p38 MAP kinase in CD83+ and CD68+ cells (probably, in DC and monocytes/macrophages, respectively) in biopsy samples from patients with treated celiac disease, and inhibition of p38 MAP kinase phosphorylation with SB203580 prevented the innate immune response (Maiuri L., et al., 2003). Moreover, inhibition of p38 MAP kinase pathway was also effective in controlling enterocyte apoptosis driven by gliadin peptide p31-43 and, generally, signalling through different p38 isoforms was specifically required for the differentiation and renewal of human intestinal enterocytes (Maiuri L., et al., 2003; Vachon, P.H., et al., 2002).

Using BMDC from TLR4-defective and TLR2-knockout mice in comparison with control mice without seeing a big difference in the stimulatory capacity of wheat gluten digest, we largely excluded a possible involvement of LPS or the majority of bacterial ligands in this process, supporting the specificity of gluten effects. Our data show that the active compound in wheat gluten α-CT digest has a peptidic nature, because the treatment of wheat gluten with proteinase K completely abrogated its ability to induce DC maturation, in contrast to LPS. In search of the relevant epitopes of wheat gluten we have tested a series of gliadin-derived small peptides, both immunodominant and non-immunodominant, for their ability to mimic the DC stimulating activity of wheat gluten digest, but have not identified an active peptide. If indeed a PRR mediates the effects of wheat gluten, the responsible epitope may be larger than a small peptide. In attempt to determine the nature of an active compound, we tried to digest it by post-proline cleaving enzyme PEP. PEP from *Flavobacterium meningosepticum* (500-540 mU/ml) incubated with gliadin peptides for 2-3 h could completely degrade them (Shan, L., et al., 2002; Matusiak-Budnik, T., et al., 2005). We used the same concentration of PEP and longer time of exposure (5 h), but there was no effect of PEP on wheat gluten digest-driven DC maturation, although PEP is known for its capacity not only to cleave rapidly proline-rich immunodominant gliadin epitopes, but also to decrease the number of T cell

immunostimulatory peptides in proteolyzed food-grade wheat gluten, especially in combination with the enzymes of the intestinal brush border, i.e. aminopeptidase N, dipeptidyl peptidase IV, and dipeptidyl carboxypeptidase I, known to further process peptide fragments generated by PEP (Shan, L., et al., 2002; Hausch, F., et al., 2002; Piper, J.L., et al., 2004; Marti, T., et al., 2005). Our data could have several explanations. First of all, that active wheat gluten fragment does not contain Pro-Xaa-Pro tripeptides – the motifs, preferentially used by PEP (Bordusa, F., and H.D. Jakubke, 1998). Secondly, it is possible, that PEP alone without additional gastric, pancreatic and brush border membrane enzymes could not degrade an active wheat gluten compound, especially if it is a low molecular mass protein. And, finally, the active wheat gluten fragment could not be rich in proline and glutamine residues and not belong to gliadins or glutenins. Indeed, it was found that wheat storage globulin, Glb1, a normal trace component in wheat gluten, was particularly antigenic in human diabetic patients and diabetic animals, and this immunoreactivity closely correlated with autoimmune aggression against pancreatic β cells (MacFarlane, A.J., et al., 2003). In addition, sequence homologies were found between Glb1 and tight junction protein 2, a member of protein complex that controls permeability of intestinal epithelium (MacFarlane, A.J., et al., 2003). Cross-reactivity between these proteins might be the cause of increased permeability of gut mucosa seen in BBdp rats and patients with T1DM leading to the break of oral tolerance (Meddings, J.B., et al., 1999; Carratu, R., et al., 1999).

Protein Hsp60 was another antigenic molecule investigated for its potential innate immune reactivity in our study. Human Hsp60 induced maturation of murine BMDC that was characterized by up-regulation of surface expression of MHC class II and co-stimulatory molecules CD86, CD40 and CD54. This effect was dose-dependent in the concentration range 0.3-30 μg/ml. Already 0.3 μg/ml of rhHsp60 was stimulatory for BMDC, and the level of BMDC maturation induced by 30 μg/ml of rhHsp60 was comparable with that of 10 ng/ml of LPS. The maturation of BMDC induced by rhHsp60 was accompanied by dose-dependent increase in cytokine and chemokine production. Thus, rhHsp60 at concentration 0.3 μg/ml could stimulate the increase in IL-1β, IL-12p40, CXCL2, CXCL1 and CCL5 secretion, but not TNFα, IL-10, IL-12p70 or CCL2.

At 30 μg/ml of Hsp60 the amount of released cytokines IL-10, IL-1β, IL-12 was similar to that induced by LPS at concentration 10 ng/ml, only the mean TNFα value was higher, but this did not gain a statistical significance. The level of chemokines produced in response to 30 μg/ml of Hsp60 was significantly different as compared to LPS, i.e. higher secretion of CXCL2 and CXCL1 and lower CCL2 production, although the highest CCL5 level induced by Hsp60 almost reached the value obtained after LPS treatment. Hsp60 induced a minor production of anti-inflammatory cytokine IL-10 by BMDC, in comparison to Th1 cytokines. Murine BMDC were stimulated by human Hsp60 to mature versus Th1-promoting phenotype with the production of pro-inflammatory cytokines TNFα, IL-12 and IL-1β. IL-12 is necessary for Hsp60-mediated release of IFNγ (Moré, S.H., et al., 2001). Similar to our data, rhHsp60 activated BMDC from C57BL/6 mice to secrete TNFα, IL-1, but, in contrary, not IL-12 (Moré, S.H., et al., 2001). Also in accordance with our results, rhHsp60 and rhHsp72 induced TNFα and IL-12p40 release from human monocyte-derived DC, but, contradictory, failed to produce IL-12p70 or IL-10 (Bethke, K., et al., 2002). Really, it was previously demonstrated that human Hsp60 caused pro-inflammatory TNFα production from murine bone marrow-derived macrophages thus supporting the concept that autologous Hsp60 is a danger signal for the innate immune system (Retzlaff, C., et al., 1994; Kol, A., et al., 1998; Kol, A., et al., 1999; Chen, W., et al., 1999; Ohashi, K., et al., 2000).

We show that TLR4 is partly involved in human Hsp60-induced maturation and cytokine production in BMDC. This finding well correlates with the data that TLR2- and TLR4-defective murine BMDC express significantly decreased TNFα response to chlamydial Hsp60, moreover, the level of inhibition of TNFα production in BMDC from TLR4-non-responsive mice is similar to that in our study (Vabulas, R.M., et al., 2001). Both TLRs, TLR2 and TLR4, are therefore involved in autologous Hsp60 recognition in BMDC, and it was suggested that Hsp60 can engage multiple members of TLR family (Vabulas, R.M., et al., 2001). Hsp60 signalling in monocytes and macrophages was shown previously to be TLR2-, TLR4- and MyD88-dependent (Ohashi, K., et al., 2000; Vabulas, R.M., et al., 2001; Bulut, Y., et al., 2002). This was not supported by another study, where participation of TLR2, TLR4 and MyD88 in *H.pylori* Hsp60-mediated IL-6

production by murine macrophages could not be determined (Gobert, A.P., et al., 2004). In addition, CD14 and CD11b/CD18 receptors were suggested as participants in Hsp60 signalling in human macrophages (Kol, A., et al., 2000; Long, K.H., et al., 2003). Hsp60 mediated the binding of *Hitsoplasma capsulatum* to CD18 receptor on human macrophages, but not to DC (Long, K.H., et al., 2003). It was shown that for binding to murine macrophages rhHsp60 used specific receptor, distinct from receptors for Hsp70, Hsp90 or gp96 (Habich, C., et al., 2002). Moreover, CD14-TLR4 receptor complex was not involved in Hsp60 binding, but was necessary for downstream signalling and cytokine production (Habich, C., et al., 2002).

Interestingly, the binding of Hsp60 to macrophages strictly depends on the origin of Hsp60 (eukaryotic or prokaryotic) and on the type of innate immune cells, because different regions of Hsp60 molecule were involved in binding to primary macrophages and to macrophages of the J774A.1 line, and eukaryotic and prokaryotic Hsp60 proteins used different binding sites on primary macrophages (Habich, C., et al., 2003; Habich, C., et al., 2004; Habich, C., et al., 2006). It can be therefore suggested that such specificity of Hsp60 binding can be also seen for the other cells of innate immune system, i.e. DC or B cells. Indeed, Hsp60 has a multivalent structure, it is a multimeric protein composed of 2x seven identical subunits that symmetrically assembled together to form the most effective structure for capture the proteins (Bukau, B., and A.L. Horwich, 1998). From the other hand, it was also shown that both Hsp60 and Hsp70 bind to common receptor(s) on human monocyte-derived DC, which do not express any detectable CD14 or TLR4 at their surface, and that epidermal and $CD34^+$-derived Langerhans cells do not bind these heat shock proteins at all (Lipsker, D., et al., 2002). Importantly, binding and internalization of Hsp60 by APC via receptor-mediated endocytosis is necessary for signalling (Vabulas, R.M., et al., 2001).

Our study did not aim to find BMDC receptor responsible for binding of rhHsp60, but we investigated signalling pathways. It appeared that rhHsp60-mediated activation of BMDC lead to the phosphorylation of MAP kinases p38, ERK 1/2 and JNK and the activation of transcription factor NFκB pathway. In contrast to LPS-induced signalling in BMDC, when a higher level of phosphorylation for p38, ERK1/2, JNK, and IκB was observed at

45 min after stimulation, Hsp60-mediated phosphorylation of p38, ERK2 and IκB was seen at 15 min after treatment, whereas at 45 min did not differ from control, ERK1 was not activated after rhHsp60 stimulation of DC. Very moderate, in comparison with LPS, phosphorylation of JNK was observed at 45 min after BMDC stimulation with Hsp60. These data clearly show the involvement of MAP kinase cascades and NFκB pathway in rhHsp60-induced BMDC activation and that LPS-mediated signalling in BMDC differ from that of rhHsp60. To our knowledge, these results give the only available so far information about MAPK and NFκB pathways in DC after Hsp60 treatment.

Activation of MAP kinase p38 and NFκB pathway is essential for DC maturation, i.e. expression of co-stimulatory molecules, in response to LPS and cytokines (Rescigno, M., et al., 1998; Ardeshna, K.M., et al., 2000; Arrighi, J.-F., et al., 2001; Neumann, M., et al., 2000). It is therefore likely that it also occurs in response to Hsp60, especially if one considers a similar kinetic of activation of p38 and NFκB pathways as seen in our study. The p38 MAPK pathway participates in NFκB activation, and, moreover, p38 may regulate the recruitment of NF-κB to selected inflammatory stimulus-induced cytokine and chemokine genes by phosphorylation of histone H3 in their promoters (Vanden Berghe, W.V., et al., 1998; Carter, A.B., et al., 1999; Saccani, S., et al., 2002). In addition, p38 signalling may be involved in cytokine production by DC. Thus, p38 mediated the expression of cytokines IL-1α, IL-1β, TNFα after stimulation of human blood-derived DC with LPS or TNFα, it was also required for IL-12 production by macrophages and DC in (MAP) kinase kinase 3 (Mkk3)-deficient mice (Arrighi, J.-F., et al., 2001; Lu, H.T., et al., 1999). Another MAP kinase that was activated in DC after Hsp60 stimulation in our study, ERK, might be involved in DC survival, as it was shown for murine splenic DC during LPS treatment, and in DC differentiation and generation (Rescigno, M., et al., 1998; Xie, J., et al., 2003; Xie, J., et al., 2005). Interestingly, phosphorylation/activation of p38 MAPK leads to the inhibition of ERK and it possibly occurs through direct contact between two MAP kinases (Zhang, H., et al., 2001). Raf/MEK/ERK and p38 MAP kinase pathways have an opposing effect on DC differentiation and survival (Xie, J., et al., 2005). Signalling through p38 MAPK mediated maturation or apoptosis in monocyte-derived DC, depending on the dosage of

ultraviolet B radiation (Nakagawa, S., et al., 2004). Differential regulation of monocyte-derived human DC maturation by ERK and p38 MAPK signalling might play role in T cell activation towards Th1 or Th2 subsets (Puig-Kroger, A., et al., 2001).

Human Hsp60 induced maturation and pro-inflammatory response of murine BMDC in our study. The presence of detectable amount of LPS (50 EU/mg of protein) in rhHsp60 preparation and the similarity of cytokine and chemokine pattern produced by DC after stimulation with rhHsp60 or LPS raised the concern about the specificity of the observed effect of rhHsp60. PAMPs, such as lipoproteins and CpG DNA, which are not recognized in LAL assay, may also activate APC (Lee, H.K., et al., 2002; Hemmi, H., et al., 2000). Indeed, being molecular chaperone, Hsp60 may be a putative carrier of microbial- or necrosis-associated lipophilic compounds. It was demonstrated that highly purified rhHsp60 did not affect any of 96 common cytokine genes and could not induce TNFα and IL-1β synthesis in murine nacrophages (Gao, B., and M.-F. Tsan, 2004). When contaminating LPS in recombinant human or murine Hsp60 preparation was depleted below 10 EU/mg of protein, Hsp60 could not induce TNFα release from murine macrophages (Osterloh, A., et al., 2004). At last, rhHsp60 can specifically bind LPS (Habich, C., et al., 2005). We therefore used several approaches to determine possible involvement of LPS in observed stimulatory effect of rhHsp60 on murine BMDC. Antibiotic peptide PmB, which normally binds free LPS, did not significantly influence rhHsp60-mediated BMDC maturation, while completely prevented LPS-triggered expression of co-stimulatory molecules on BMDC. PmB could inhibit TNFα secretion, triggered by rhHsp60. Proteinase K that effectively digests protein HSP60 and can successfully recover up to 100 % of protein-bound LPS blocked DC maturation after rhHSP60 challenge without any effect on LPS-mediated DC maturation. In contrast to *E.coli* LPS signalling in DC, which has an absolute requirement for TLR4, rhHsp60-induced maturation and cytokine production from DC was partly TLR4-independent. In addition, the kinetics of activation of MAP kinases and NFκB was different after rhHsp60 and LPS stimulation. We can conclude that the integrity of Hsp60 molecule is absolutely necessary for the induction of BMDC maturation and TNFα production as it was shown in the experiment with proteinase K. If LPS is present in the rhHsp60

preparation, rhHsp60 can tightly bind it, and the region aa354-365 on Hsp60, responsible for the contact with LPS, is different from the region aa481-500, responsible for specific binding of rhHsp60 to cell surface of murine macrophages (Habich, C., 2002; Habich, C., et al., 2005). It is therefore possible that Hsp60 can deliver LPS or other microbial molecules to the surface of innate immune cells. Indeed, CD14, TLR4 and TLR2 receptors that are involved in bacterial LPS and lipoprotein recognition, were also implicated in Hsp60 signalling in APC (Wright, S.D., et al., 1990; Poltorak, A., et al., 1998; Lee, H.K., et al., 2002; Kol, A., et al., 2000; Vabulas, R.M., et al., 2001). However, Hsp60 binds to the surface receptor other than CD14 or TLR4 on human monocyte-derived DC (Lipsker, D., et al., 2002).

Binding and internalization of Hsp60 by APC via receptor-mediated endocytosis is necessary for signalling, and, probably, different surface molecules are involved in binding and signal transduction (Vabulas, R.M., et al., 2001; Habich, C., et al., 2002). It could be hypothesized that signal, delivered by Hsp60 on APC, partly depends on chaperoned molecule, and Hsp60 can be a sensor of the presence of bacterial compounds in microenvironment that can drive specific pro-inflammatory cytokine response from innate immune cells. It does not mean that Hsp60 itself is not immunogenic. Normally intracellular protein, Hsp60 present on the cell surface is a danger signal for immune system. It was shown that murine Hsp60 expressed as a transmembrane protein on the surface of two different eukaryotic cell lines increased the T cell activation in the absence of LPS or PAMPs *in vitro* (Osterloh, A., et al., 2004). Low endotoxin recombinant human and murine Hsp60 (1.6 EU/mg of protein and <3EU/mg of protein, respectively) lost the ability to induce TNFα release from murine macrophages as compared to rhHsp60 with 22 EU/mg of protein and rmHsp60 with 48 EU/mg of protein, respectively, but retained the capacity to stimulate increase in INFγ production of T cells (Osterloh, A., et al., 2004). It can be therefore suggested that maturational signal, delivered by Hsp60 to DC, can be independent from pathway leading to cytokine and chemokine synthesis.

This study gives an additional support to the hypothesis that autologous Hsp60 *per se* elicits danger signal to the innate immune system, e.g. when released extracellularly in vivo from necrotic cells. However, in the blood the contact between Hsp60 and LPS may

be normal physiological characteristics, because autologous Hsp60 is present in the circulation of healthy individuals at microgram level, and LPS level in portal venous blood between gut and liver is in the range of 0.1-1.0 ng/ml (Pockley, A.G., et al., 2000; Knolle, P.A., et al., 1999). Indeed, after the clearance of endotoxin in the liver only a minimal amount can reach systemic circulation under physiological conditions (Freudenberg, M.A., et al., 1982). But during disease development the situation may be changed. Autologous Hsp60 or its peptides can elicit such different effects, as induction of inflammatory disease and protection against it (Stanford, M.R., et al., 1994; Hu, W. et al., 1998; Scheckelhoff, M., and G.S. Deepe, Jr., 2002; Elias, D., et al., 1991; Raz, I., et al., 2001).

It is conceivable that Hsp60 represents LPS receptor. For instance, the protection from tularaemia afforded by Hsp60 from *F.tularensis* was effective only in the presence of LPS (Hartley, M.G., et al., 2004). Hsp60 appears to bind readily bacterial products from the microenvironment. Hence, "contamination" with LPS may reflect a physiological situation. Autologous Hsp60-mediated Th1-skewed innate immune response would help in protection from cancer and infectious diseases, whereas can also lead to the development of autoimmune diseases. Autologous Hsp60 could induce tolerance in human monocytes to repeated Hsp60 and LPS stimulation (Kilmartin, B., and D.J. Reen, 2004). Because Hsps are highly evolutionary conserved molecules, human and bacterial Hsp60 are highly homologous and cause immunological cross-reactivity. The association between certain microbial infections and autoimmune diseases allowed their treatment by mucosal vaccination (Weiner, H.L., 2004; Holmgren, J., and C. Czerkinsky, 2005; Hansson, G.K., 2002). Due to "bystander suppression" regulatory cells induced by fed antigen can suppress immune responses stimulated by a different antigen, as long as the fed antigen is present in the anatomic vicinity (Faria, A.M.C., and Weiner H.L., 1999). This strategy has been used to protect against autoimmunity. Because of cytokine-mediated bystander suppression oral antigen-specific therapy can be effective in autoimmune diseases in case of unknown antigen or multiple autoantigens. Mucosal administration of bacterial Hsp60/65 or its peptide alone or together with immunomodulating agents gives promising results in the treatment of autoimmune diseases such as T1DM, arthritis, atherosclerosis, Behcet's disease (Bellmann, K., 1997;

Wendling, U., 1998; Brugman, S., et al., 2004; Prakken B.J., et al., 1997; Maron, R., et al., 2002; Harats, D., et al., 2002; Stanford, M., et al., 2004). Although mucosally induced tolerance seems to be effective in struggling against autoimmunity, sometimes oral administration of antigens not affects autoimmune diseases in animals and humans or even leads to its worsening (Bellmann K., et al., 1998; Chaillous, L., et al., 2000; Postlethwaite, A.E., 2001; Wiendl, H., and R. Hohfeld, 2002; Hänninen, A., 2000; Weiner, H.L., 2004).

Diet influences the development of T1DM, and wheat gluten can be a risk factor for T1DM induction in humans (Scott, F.W., et al., 1989; Akeblom, H.K., and M. Knip, 1998; Scott, F.W., 1996; Ventura, A., 1999). We therefore studied the effect of diabetes-promoting wheat gluten-based NTP-2000 or WG diets and diabetes-retardant HC diet on the immunity of upper small intestine of diabetes-prone rats in comparison with control animals. The level of investigated immune markers, Th1 cytokine IFNγ, Th2 cytokine IL-10 and chemokines CCL2 and CXCL2, was not significantly different in the duodenum of rats after feeding WG or NTP-2000 diets vs. HC diet. However, there was a clear tendency, which sometimes could gain a statistical significance, towards decreased levels of IL-10, CCL2 and CXCL2 in the duodenum of BBc and WF rats fed NTP-2000 or WG diets in comparison with those fed HC diet. For BBdp rats this feature was not seen for CXCL2 and was not so evident for IL-10 and CCL2. Weaning, irrespectively of diet type, was accompanied by immune activation in the duodenum of BBdp, BBc and WF rats, i.e. the ratio IFNγ/IL-10 was gradually elevated up to day 70 of age. Moreover, already at 10 days of age BBdp rats had higher IFNγ/IL-10 ratio than BBc or WF. In addition, significant increases of IFNγ, IL-10, CCL2 and CXCL2 were observed in the duodenum of WF rats after weaning. Differently to WF rats, BBc rats did not exhibit an enhancement of IL-10 and the elevation of CCL2 did not gain statistical significance. BBdp rats had statistically significant increase of IFNγ duodenal level only in response to HC diet, IL-10 production was even decreased and CCL2 unchanged, as compared to neonates fed with dam milk. These strain differences may be partly explained by already much higher IFNγ or CCL2 production in the duodenum of 10 days-old BBdp rats as compared to BBc or even more to WF, so introduction to food did not significantly

increase these parameters. Previous study of *Bellmann, K., et al., 1998,* showed a higher IFNγ/IL-10 mRNA ratio in the small intestine of 50 days-old BBdp rats fed wheat-containing NIH-07 diet as compared to WF rats, but we could not see higher IFNγ/IL-10 protein ratio in the duodenum of BBdp rats fed NTP-2000 diet vs. WF rats at 30, 45 or 70 days of age. However, it was the case for BBdp rats at 23 and 95 days of age in comparison to WF. The animals fed WG, or HC diet did not differ in duodenal IFNγ/IL-10 protein ratio up to day 70. Our data therefore demonstrate that although BBdp neonates had increased IFNγ/IL-10 ratio in small intestine at 10 days of age and, independently of the diet, at day 95 and for WG diet also at day 120, there were no major differences in this parameter between strains and diets after weaning till day 70. It was shown that wheat-based NTP-2000 diet augmented mRNA level of IFNγ, IL-10, TNFα, TGFβ and iNOS in small intestine of NOD mice, but not in gut-associated PP (Flohé, S.B., et al., 2003). The oral exposure of BBdp neonates to diabetes-promoting food antigens led to the downregulation of IFNγ mRNA in the gut duodenum 1 day later (Scott, F.W., et al., 2002).

Our inability to find significant differences in the immune response of upper small intestine of BBdp rats to diabetes-promoting vs. diabetes-retardant food, especially in case of IFNγ and IFNγ/IL-10 ratio, can be due to several reasons. First of all, the hypothesis that gut immune system plays a primary role in the development of T1DM may be not true, although it was shown that enteropathy in BBdp rats precedes the onset of insulitis and is clearly recognized at 30 days of age (Graham, S., et al., 2004). But, in accordance with our results, introduction to wheat gluten-containing diabetes-promoting NTP-2000 or WG diet vs. diabetes-protective HC diet did not make any difference in the development of gut damage in BBdp rats, suggesting that enteropathy and diabetes are driven by different mechanisms, or they are independent consequences of *Iddm1/lyp/Ian4* mutation responsible for lymphopenia (Jacob, H., et al., 1992; Hornum, L., et al., 2002; Graham, S., et al., 2004). Indeed, lymphopenia is present in BBdp rats, but not in BBc rats that also do not develop enteropathy, and thymectomised BBdp rats, which do not have insulitis or clinical diabetes showed enteropathy (Mordes, J.P., et al., 2001; Graham, S., et al., 2004).

If nevertheless the dysfunction of gut immune system predispose to the development of β cell destruction, it might be possible that in response to diabetes-promoting food the small intestine produces a new cell population(s) or expands already existed one(s) responsible for the autoagression of β cells. This cell population may be so small that it is not possible to catch the difference in its cytokine/chemokine production in our crude preparation of duodenal homogenate or that we did not choose a right immune marker, although IFNγ seems to be one of the most important cytokines.

It was recently shown that MLN of BBdp rats in pre-insulitis period specifically respond to wheat gluten antigens by proliferation of IFNγ-producing $CD3^+CD4^+$ Th1 cells associated with a high level of DC and a low number of $CD4^+CD25^+$ T cells, moreover Th1 bias exists in MLN of BBdp rats already 1 week after weaning (Chakir, H., et al., 2005). MLN is an important inductive site in the gut. MLN lymphocytes from 3 weeks-old NOD mice were previously shown to transfer diabetes, and MLN lymphocytes traffic through the pancreas (Hänninen, A., et al., 1998; Jaakkola, I., et al., 2003; Whalen, B.J., et al., 1999). However, in isolated MLN from NOD mice fed wheat-containing diet gliadin stimulation did not induce increased T cell proliferation or INFγ transcription, although these animals had higher incidence of diabetes in comparison with the animals fed gluten-free diet (Maurano, F., et al., 2005). This discrepancy can be explained by the use of MLN from 43 weeks-old mice with already established diabetes vs. young, 45 days-old BBdp rats in the study by *Chakir, H., et al., 2005*. Also in contrary to these two studies, diabetes-protective HC diet given to BBdp rats from weaning to day 60 of age caused an increased IFNγ and decreased IL-10 production from MLN cells (Visser, J., et al., 2003).

According to our data, BBdp rats had strong Th1 bias in upper small intestine already at 10 days of age, before the appearance of mucosal damage, which was previously shown by *Graham, S., et al., 2004,* to be present at day 30, but not at day 10 after birth. IFNγ level and IFNγ/IL-10 ratio were significantly higher in BBdp neonates than in BBc or WF rats. IFNγ may disrupt intestinal epithelial barrier function (Madara, J.L., and J. Stafford, 1989; Adams, R.B., et al., 1993; Bruewer, M., et al., 2003). Indeed, increased intestinal IFNγ levels preceded wheat gluten-induced gut impairment in CD and was in a strong

association with it, and the density of IFNγ mRNA positive cells correlated with the degree of CD in patients with T1DM (Nilsen, E.M., et al., 1995; Przemioslo, R.T., et al., 1995; Nilsen, E.M., et al., 1998; Westerholm-Ormio, M., et al., 2002; Westerholm-Ormio, M., et al., 2003). Exaggerated intestinal Th1 responses cause gut damage and correlate with increased gut permeability in CD, Crohn's disease and other chronic gut inflammatory conditions (MacDonald, T.T., et al., 1999). From this point of view an enhanced IFNγ production and IFNγ/IL-10 ratio in the duodenum of 10 days-old BBdp neonates as compared to BBc or WF rats, can be an early marker of the development of gut damage leading to increased gut permeability that precedes development of insulitis and T1DM in BBdp rats (Meddings, J.B., et al., 1999).

In accordance with our data, showing no influence of the diet on IFNγ production after weaning, no diet-induced differences in small intestinal permeability were observed in BBdp rats, although the rats fed with HC, had significantly lower diabetes incidence (Meddings, J.B., et al., 1999). IFNγ was initially thought to be produced exclusively by $CD4^+$ Th1 cells and NK cells, but now this list is extended to $CD8^+$ T cells, γδ T cells, NKT cells, B cells, macrophages and DC (Frucht, D.M., et al., 2001; Schroder, K., et al., 2004). CD-associated Th1 immune activation was seen in the LP and was suggested to be due to IFNγ-producing T cells, probably bearing α4β7 integrin, and TNFα-producing macrophages (Kontakou, M., et al., 1994; Savilahti, E., et al., 1999; Nilsen, E.M., et al., 1998; Westerholm-Ormio, M., et al., 2002). Although the surface epithelium and γδ-TCR^+ IEL showed no or little IFNγ expression in CD specimens, their increased density was in a good correlation with the density of INFγ-producing cells in the LP (Westerholm-Ormio, M., et al., 2002). However, the cells secreting INFγ predominate also in LP of healthy humans, suggesting their necessity to the normal homeostasis of intestinal mucosa (Hauer, A.C., et al., 1998; Carol, M., et al., 1998). It was demonstrated that IFNγ may be involved in establishment of oral tolerance, although contradictory data exist in this field (Kweon, M.-N., et al., 1998; Mowat, A.M., 1999). IFNγ or IFNα administration can prevent diabetes in BBdp rats and NOD mice (Sobel, D.O., and J. Newsome, 1997; Nicoletti, F., et al., 1998; Sobel, D.O., and B. Ahvazi, 1998; Sobel, D.O., et al., 1998; Sobel, D.O., et al., 2002). BBdp rats are not only deficient in

peripheral T cells, but also in intraepithelial γδ T cells and intraepithelial NK (IENK) cells that bear regulatory ART2$^+$CD25$^+$ phenotype and spontaneously secrete IFNγ and IL-4 (Angelillo, M., et al., 1988; Hornum, L., et al., 2002; MacMurray, A.J., et al., 2002; Ramanathan, S., et al., 2002; Todd, D.J., et al., 2001; Todd, D.J., et al., 2004). IENK deficiency therefore is accompanied by decreased IFNγ and IL-4 production by these cells (Todd, D.J., et al., 2004). It was hypothesized that diabetes development in BBdp rats starts from genetically-determined bone-marrow derived defect in regulatory gut IENK, followed by the appearance of abnormal MLN populations that proceed to induce insulitis and β cell destruction (Todd, D.J., et al., 2004). Interestingly, splenic bitypic NK/DC regulatory cells can transfer CD40L blockade-mediated protection against virus-induced T1DM in mice (Homann, D., et al., 2002). Murine natural killer DC (NKDC) populations with the molecular expression profile of both DC and NK cells that capable to secrete IFNγ after stimulation were recently described (Pillarisetty, V.G., et al., 2005; Taieb, J., et al., 2006; Chan, C.W., et al., 2006). Moreover, IFNγ-producing CD11c$^+$NK1.1$^+$ NKDC were found in MLN from C57BL/6 mice, and IFNα- or IFNγ-secreting killer DC (CD11c^{int}B220$^+$CD49b$^+$MHCII$^+$CD86$^+$CD40$^+$) were identified in intestinal LP, MLN, PPs and IEL fraction from BALB/c mice (Pillarisetty, V.G., et al., 2005; Chan, C.W., et al., 2006). It is therefore evident that many cell types in the intestine, including DC, can contribute to IFNγ production. It was also suggested that DC subsets CD11c$^+$CD4$^-$CD8α$^-$, which was previously shown to produce IFNγ in response to proper stimulation, can be, in fact, NKDC (Pillarisetty, V.G., et al., 2005; Fukao, T., et al., 2000; Hochrein, H., et al., 2001). IFNγ/IL-4 and IFNα/β/TNFα equilibrium was hypothesized to play a major role in maintaining immune homeostasis through generation of different DC subsets (Banchereau, J., et al., 2004). We could not see Th1-oriented response in BMDC towards wheat gluten digest in our *in vitro* experiments. But *in vivo*, especially in the presence of an increased amount of IFNγ, as it was seen in our study in the intestine of 10 days-old BBdp rats, a special DC subset can be generated that might favour activation and expansion of autoreactive T cells. It is also possible that co-stimulation by bacterial products is involved in DC response to wheat gluten *in vivo*, especially in the context of increased mucosal permeability in BBdp rats. This additional

stimulation may direct the development of specific mucosal DC subset(s). Besides, *in vivo* in steady-state intestinal epithelial cell-derived factors, including thymic stromal lymphopoeitin (TSLP) in a narrow window of concentrations, instruct mucosal DC to develop Th2 response (Rimoldi, M., et al., 2005). The entrance of *Salmonella typhimurium* across EC barrier lead to a large increase in local TSLP production that resulted in regaining the capacity of DC to secrete IL-12p70 and to promote Th1 response, whereas the majority of the patients with Crohn disease had undetectable TSLP mRNA level in endothelial cells, which correlated with enhanced IL-12p70 secretion from bacteria-activated DC, i.e. induced Th1 inflammatory response (Rimoldi, M., et al., 2005).

In our experiments diabetes-protecting HC diet tended to increase the level of IL-10 and CCL2 in small intestine of BBdp, BBc and WF rats and CXCL2 production in BBc and WF rats in comparison with the animals fed NTP-2000 or WG diets. BBdp rats fed HC diet had lower mean CXCL2 level than BBc or WF rats, which was significantly different to CXCL2 production in WF rats at 30 and 70 days of age. It is difficult to speculate whether this tendency is linked to the protective effect of HC diet on the development of T1DM. Again, if HC diet induces the development or expansion of a small cell population responsible for the protective effect in BBdp rats, it would be difficult to catch the difference in cytokine/chemokine production between diabetes-prone and control rats in our crude duodenal homogenate. Then our results can be a hint towards a future search of such a cell subset. Indeed, IL-10 mediates a protective effects of $CD4^+$ Tregs Th3 and Tr1 and $CD4^+CD25^+$ Tregs, participates in mechanisms of oral tolerance that was shown to be impaired both in IL-$10^{-/-}$ mice and in mice treated with neutralizing anti-IL-10R mAbs (Dubois, B., et al., 2005).

CXCL2 participated in tolerance induction by attracting NKT cells to the spleen, and this was absolutely required for the generation of the Ag-specific negative Tr cells (Faunce, D.E., et al., 2001). CCL2 is also involved in oral tolerance induction and maintenance (Karpus, M.J., and N.W. Lukacs, 1996; Karpus, W.J., et al., 1998; Abbas, A.K., et al., 1996). Out study demonstrated a significant increase of CCL2 production in the duodenum of 10 days-old BBdp neonates as compared to BBc or WF rats. CCL2 is chemotactic for monocytes, activated and memory T cells, NK cells, but not neutrophils,

regulates the expression of cell surface antigens CD11c, CD11b and IL-1 production (Daly, C., and B.J. Rollins, 2003; Stevceva, L., and M.G. Ferrari, 2005). Although its expression is reported in such autoimmune diseases, as atherosclerosis, multiple sclerosis, rheumatoid arthritis and diabetes, characterized by mononuclear cell infiltrates, CCL2 is constitutively present in normal human islet β cells in the absence of an inflammatory infiltrate, suggesting another function of this chemokine (Gu, L., et al., 1997; Chen, M.-C., et al., 2001; Piemonti, L., et al., 2002). CCL2, but not CCL3, CCL4 or CCL5, was expressed in the intestinal mucosa, PPs and MLN after antigen feeding and regulated oral tolerance by down-regulation of mucosal IL-12 expression, which could partially or completely block peripheral Th1 differentiation, with concomitant potentiation of mucosal IL-4 expression that would favour peripheral Th2 differentiation (Karpus, W.J., et al., 1998; Abbas, A.K., et al., 1996). It may therefore be suggested that the increase of CCL2 in small intestine of BBdp neonates as compared to BBc or WF rats reflects the attempt of gut immune systtem to compensate for genetically determined abnormal mucosal processes in BBdp neonates by maintaining oral tolerance. In support of this, down-regulation of serum CCL2 level was shown to be a risk factor for the development of T1DM in humans (Hanifi-Moghaddam, P., et al., 2005).

Our investigation showed the immune activation in upper small intestine of diabetes-prone BB neonates before the appearance of evident impairment in mucosal architecture and this can be a prerequisite for the development of autoimmune process in the pancreas later in life. The early period of life is very important for the future development of autoimmune disease, and oral administration of food antigens during this period may be beneficial (Scott, F.W., and H. Kolb, 1998). Thus, feeding of BBdp neonates with diabetes-promoting wheat-containing food or WG diet twice daily between 4 and 7 days of age could significantly delay the development of diabetes later in life in rats weaned onto the same diet, as compared to BBdp neonates treated with HC-based infant formula Pregestimil and subsequently received wheat-based or WG diet (Scott, F.W., et al., 2002). Neonatal oral administration of DiaPep277, a specific peptide of human Hsp60, once per day between 4 and 7 days of life combined with HC or cereal-based diet from weaning could reduce the incidence of diabetes in BBdp rats as compared to non-treated animals (Brugman, S., et al., 2004).

In vivo DCs are able to contact luminal antigens directly (Rescigno, M., et al., 2001; Niess, J.H., et al., 2005). DCs fulfil multiple functions: they are sensors of pathogens, commensal bacteria, tissue necrosis, apoptosis and local inflammation, they control activation of T cells, including NK T and Treg, B and NK cells, they actively participate in the maintenance of peripheral tolerance by presenting self-antigens to T cells in tolerogenic manner (Banchereau, J., et al., 2004). According to our data, wheat gluten digest and rhHsp60 caused maturation and immune activation of BMDC *in vitro*. BMDC interaction with rhHsp60 promoted strong Th1 response, supporting a point of view that autologous Hsp60 is a danger signal for innate immunity (Chen, W., et al., 1999; Matzinger, P., 2002). Autologous Hsp60 can be released from necrotic cells (Saito, K., et al., 2005). It is also expressed on the surface of B7-positive APC in the ileum and colon of patients with inflammatory bowel disease, on APCs during bacterial infection, and on the surface of stressed endothelial cells (Peetermans, W.E., et al., 1995; Belles, C., et al., 1999; Xu, Q., et al., 1994). Hsp60 located on the surface is a danger signal for the immune system, it is able to increase activation, i.e. IFNγ production, of T cells (Octerloh, A., et al., 2004). Hsp60-reactive CD8 T cells were found to cause inflammation of small intestine in mice (Steinhoff, U., et al., 1999). Wheat gluten in our study also induced maturation of BMDC that was not accompanied by production of potent Th1 or Th2 cytokines. Additional factors should be therefore involved *in vivo* to cause a pro-inflammatory reaction.

The microenvironment plays an important role in shaping DC responses. An enhanced IFNγ production by intestinal cells of BBdp neonates early in life observed in our study may facilitate the development of Th1 cells. Indeed, it was demonstrated by *P. Matzinger and colleagues* that IFNγ is required for priming DCs to secrete IL-12p70, stimulation through TLRs alone induced production of IL-12p40 but not intact IL-12p70 (Colonna, M., et al., 2006). Increased IFNγ level in the upper small intestine of 10 days-old BBdp rats seen in our study may be responsible for the impaired mucosal barrier function (Madara, J.L., and J. Stafford, 1989; Bruewer, M., et al., 2003). This can lead to the bacterial penetration. Interestingly, proximal small intestine that is normally sterile or contains only low numbers of bacteria, in treated and untreated CD had rod shaped bacteria on mucosal surface in 37% and 19% cases, respectively (Forsberg, G., et al.,

2004). IFNγ may reflect the early intestinal changes that can be genetically determined, which later cause an aberrant epithelial cell differentiation and binding of bacteria, however, there was no correlation between IFNγ mRNA and presence of bacteria (Forsberg, G., et al., 2004). Unfavourable immune reactions in response to wheat gluten may be easily seen in the presence of additional stimulation of DCs by bacterial products in the context of increased intestinal permeability observed in BBdp rats. In addition, gliadin and its peptides were shown to increase intestinal permeability (Thomas, K.E., et al., 2006).

We therefore think that in the gut of individuals predisposed to the development of T1DM there is an early pro-inflammatory process that facilitates impairment of intestinal barrier functions, the appearance of intestinal inflammation, the establishment of inappropriate immune reactions to food antigens and break of oral tolerance mechanisms later in life. Under these circumstances wheat gluten and Hsp60 will exert an increased stimulatory capacity for DCs that might favour activation of Th1 and β cell-reactive T cells.

5 CONCLUSIONS

The main outcomes of the current study are:

1. Wheat gluten digest induced dose-dependent maturation of murine $CD11c^+CD11b^+$ DCs, which was independent of TLR2 or TLR4 receptors, and secretion of IL-1β, CXCL2, CXCL1 and CCL5 *in vitro*. Exposure to wheat gluten did not stimulate the production of pro-inflammatory mediators TNFα, CCL3 or the potent anti-inflammatory cytokine IL-10. Therefore, DC-wheat gluten interaction cannot be classified as pro-inflammatory, rather the secreted immune mediators appear to prepare the immune system for better reactivity. In the presence of appropriate genetic predisposition this may increase the risk of adverse immune reactions to wheat gluten itself or to other antigens present.
2. The protein rhHsp60 induced dose-dependent maturation of murine $CD11c^+CD11b^+$ DCs and the production of cytokines TNFα, IL-12p70, IL-1β, IL-10 and chemokines CCL2, CXCL1, CXCL2 and CCL5 *in vitro*. The receptor TLR4 was partly involved in Hsp60 signaling. DC-Hsp60 interaction can be classified as pro-inflammatory because of the presence of highly potent Th1 cytokines TNFα and IL-12 and minimal secretion of Th2 cytokine IL-10.
3. The level of the Th1 cytokine IFNγ and of the IFNγ/IL-10 ratio in the upper small intestine of diabetes-prone BBdp and control BBc and WF rats from 23 till 120 days of age was not dependent of the diet type, i.e. diabetogenic wheat gluten-containing WG and NTP-2000 or diabetes-retardant HC diets. Introduction to solid food, irrespectively of diet type, was accompanied by gradual increase of the IFNγ/IL-10 ratio in the duodenum of BBdp, BBc and WF rats up to 70 days of age.
4. BBdp pups at 10 days of age before weaning exhibited significantly higher levels of IFNγ, IFNγ/IL-10 ratio and CCL2 in the duodenum as compared to BBc or WF rats. The increased IFNγ production and higher IFNγ/IL-10 ratio in the duodenum of 10 days-old BBdp pups may favor an impairment of mucosal barrier function and the development of inflammation, and might be an important factor in the establishment of inappropriate immune response to selected antigens and in the development of diabetes later in life.

6 SUMMARY

Type 1 diabetes mellitus (T1DM) is characterized by an immune-mediated selective destruction of insulin-producing β cells in the pancreatic islets of Langerhans. Disease incidence in animal models can be broadly modulated by the diet, suggesting an important role of the gut immune system in the development of T1DM. Dendritic cells (DCs) are the main antigen-presenting cells that are considered as main regulators of mucosal immune responses and of oral tolerance.

The first part of the current study dealt with *in vitro* interaction of murine bone-marrow-derived DCs (BMDCs) with two antigens with relevance to gut immunity in general and T1DM in particular, wheat gluten (WG) and heat shock protein (Hsp) 60. An α-chymotryptic digest of WG was found to activate BMDCs and cause their maturation in a dose-dependent manner. These effects were not mediated by TLR2 or TLR4 receptors. Immune mediators secreted by WG-activated BMDC comprised IL-1β, CXCL2, CXCL1 and CCL5, but not TNFα or CCL2. This suggests that WG increases immune alertness, but does not deliver a pro-inflammatory signal. By contrast, the contact of rhHsp60 with BMDCs caused a pro-inflammatory response, which included the dose-dependent maturation of BMDCs that was partly TLR4-dependent, and the production of pro-inflammatory cytokines TNFα, IL-12p70, IL-1β, minimal amount of antagonistic IL-10, or of the chemokines CCL2, CXCL1, CXCL2 and CCL5.

The second part of the current work concerned the intestinal immune response to diabetes-promoting wheat-gluten-containing food vs. diabetes-retardant hydrolysed casein (HC) diet in the upper small intestine (duodenum) of Bio-Breeding diabetes-prone (BBdp) rats of 23, 30, 45, 70, 95 and 120 days of age in comparison with non diabetes prone Bio-Breeding control (BBc) and Wistar-Furth (WF) rats, and of 10-days-old rats. The Th1 response in the duodenum estimated by IFNγ level and the IFNγ/IL-10 ratio was not modulated by the type of diet. Weaning and the introduction to solid food was accompanied by a gradual increase of the IFNγ/IL-10 ratio in the duodenum of BBdp, BBc and WF rats up to 70 days of age. BBdp pups at 10 days of age before weaning exhibited significantly higher levels of IFNγ, IFNγ/IL-10 ratio and CCL2 in the duodenum as compared to BBc or WF rats. This Th1 milieu in the BBdp gut might lead

to the impairment of mucosal barrier function and to the development of inflammation, and thus may contribute to the establishment of an inappropriate immune response to dietary antigens and promote the development of diabetes in BB rats later in life.

ZUSAMMENFASSUNG

Typ 1 Diabetes mellitus (T1DM) ist die Folge eines immun-mediierten Untergangs der Insulin-produzierenden ß-Zellen in Langerhans Inseln des Pankreas. In Tiermodellen kann die Entwicklung des Diabetes deutlich durch die Zusammensetzung de Nahrung moduliert werden, was auf eine wichtige Rolle des Darmimmunsystems bei der Pathogenese hindeutet. Dendritische Zellen (DCs) sind die wichtigsten Antigen-präsentierenden Zellen und damit bedeutende Regulatoren der Schleimhaut-assoziierten Immunität und der oralen Toleranz.

Der erste Teil der vorliegenden Arbeit befasst sich mit der Interaktion *in vitro* von aus Knochenmark der Maus kultivierten DCs (BMDCs) mit zwei Antigenen von Relevanz für die Darmimmunität und die Entwicklung des T1DM, Gluten aus Weizen (WG) und das Hitzeschockprotein (Hsp) 60. Ein mit α-Chymotrypsin behandeltes WG aktivierte BMDCs und induzierte deren Reifung dosisabhängig. Diese Wirkung wurde nicht über TLR2- oder TLR4-Rezeptoren vermittelt. An Immunmediatoren wurden IL-1β, CXCL2, CXCL1 und CCL5, aber nicht TNFα or CCL2 sezerniert. Dies spricht für eine Vorstimulation und gegen eine proinflammatorische Aktivierung des Immunsystems durch WG.

Im Gegensatz dazu induzierte die Inkubation von BMDCs mit rhHsp60 dosisabhängig eine proinflammatorische Reaktion und Reifung, mit partieller Beteilung von TLR4. Als Sekretionsprodukte fanden sich die proinflammatorischen Zytokine TNFα, IL-12p70, IL-1β, nur minimale Mengen des antagonistischen IL-10, sowie die Chemokine CCL2, CXCL1, CXCL2 und CCL5.

Der zweite Teil der Arbeit beschäftigte sich mit der Darmimmunreaktion bei Fütterung mit Diabetes-förderender WG-haltiger Nahrung vs. Diabetes-hemmender Nahrung auf der Basis von hydrolysiertem Kasein. Untersucht wurde das Duodenum von Diabetes-prädisponierten Bio-Breeding (BBdp) Ratten im Alter von 23, 30, 45, 70, 95 und 120

Tagen im Vergleich zu Diabetes-resistenten Bio-Breeding Control (BBc) und Wistar-Furth (WF) Ratten, und zusätzlich von 10 Tage alten Ratten der drei Stämme.

Die durch den IFNγ-Spiegel und den IFNγ/IL-10 Quotienten definierte Th1-Immunreaktivität wurde nicht durch die Art der Nahrung beeinflusst. Dagegen führt das Absetzen der Tiere und der Beginn der Aufnahme fester Nahrung zu einem graduellen Anstieg des IFNγ/IL-10 Quotienten im Duodenum von BBdp, BBc und WF Ratten bis zum Alter von 70 Tagen. Noch säugende BBdp Ratten im Alter von 10 Tagen wiesen im Duodenum signifikant erhöhte Spiegel von IFNγ, des Quotienten IFNγ/IL-10 und von CCL2 im Vergleich zu BBc oder WF Ratten auf. Dieses Th1-Milieu im Darm von BBdp Ratten könnte die Schleimhautbarriere beeinträchtigen und die Entwicklung einer Entzündungslage fördern. Als Konsequenz sind unerwünschte Immunreaktionen gegen Nahrungsantigene und dadurch ein Förderung der Entwicklung des T1DM denkbar.

7 LITERATURE

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

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