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Immunomodulation induced by oral uptake of nickel (Ni)

Dissertation

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To my family

Contents

Abbreviations

ACD	Allergic contact dermatitis
APC	Antigen-presenting cell
DTH	Delayed-type hypersensitivity
NAH	Nickel allergic hypersensitivity
MHC	Major histocompatibility complex
LTT	Lymphocyte transformation test
TCR	T cell receptor
Treg cell	T regulatory cell
DC	dendritic cell
DLN	draining lymph nodes
ALN	axillary lymph nodes
MLN	mesenteric lymph nodes
7-AAD	7-amino-actinomycin D
IDO	indoleamine 2,3-dioxygenase
1-MT	1-Methyl-DL-tryptophan
DNFB	2,4-dinitrofluorobenzene
MFI	mean fluorescence intensity
MEST	mouse ear-swelling test
Ni^{high} mice	Conventionally bred and reared mice (in stainless steel cages with stainless steel water bottle tips) that received additional 10 mM $NiCl_2$ for 4 weeks.
Ni^{low} mice	Conventionally bred and reared animals without additional nickel supplementation.
$Ni^{very\ low}$ mice	Mice bred and reared for two generations in a nickel-poor environment by using cage covers and water bottle tips from plastic and glass.

1. Introduction

1.1 Allergy and delayed-type hypersensitivity (DTH)

The basic property of immune system is that it can recognize nonself from self, a power that promotes survival and exists in a delicate balance between tolerance to self and rejection of nonself (Middleton et al., 1998; Janeway et al., 2001). Allergy is an overreaction in certain individuals by a specific defense mechanism responding inappropriately to environmental encounters, resulting in annoying and sometimes debilitating secondary effects. Hypersensitivity reactions are strong immune responses to normally harmless self-or exogenous- antigens. These antigens predispose the immune system to cause cell damage and tissue injury upon re-exposure with the antigen (Middleton et al., 1998). The original Gell and Coombs classification lists four types of hypersensitivity (immunopathologic) reactions: I, immediate (IgE mediated); II, cytotoxic (IgG, IgM-mediated); III, immune (IgG, IgM complex-mediated); and IV, delayed (T-cell-mediated). Type I: The immediate hypersensity reaction uses the release of mast cell or basophil mediators to create immediate and delayed (4 or 8 hours) responses to sensitizing allergens. Anaphylactic responses require allergen-specific IgE antibody to attach to IgE receptors on mast cell or basophil surfaces providing the means for triggering the cascade of cellular events after allergen binding, as with anaphylaxis to penicillins or with allergic rhinitis to ragweed pollen. Type II: Antibody-mediated cytolytic reactions involve IgG and IgM to cell surface antigens on erythrocytes, neutrophils, platelets. The sensitizing antigens in these cases can be natural cell surface antigens, modified cell surface antigens, or haptens attached to cell surfaces. There are three categories of immunopathologic reactions. The first occurs by opsonization, which is facilitated by complement activation; the second induces complement lysis; the third is mediated by ADCC. Clinical examples of this type of reactions are thrombocytopenia, penicillin-induced autoimmune hemolytic anemia. Type III: IgG and IgM antibodies, activated complement, and neutrophils are participants in immune complex-mediated reactions. The immune complexes of antibody and antigen, activated complement components, and chemotaxis of neutrophils are important participants in this hypersensity reaction. Type IV: The delayed hypersensitivity reaction is mediated by sensitized T cells, in particular the CD4+ (helper) cell population. A representative clinical example of reaction is contact dermatitis resulting from poison ivy Rhus antigen. This form of hypersensitivity reaction is commonly considered an allergic reaction but is CD4+ T cell-mediated depending on a Th1 type of response rather than Th2 cells. There is also an increasing awareness of the participation of other immune and inflammatory cells and cytokines in delayed hypersensitivity reactions. In the skin, for example, the DR+

Langerhans′ cells presents antigen to T cells, and IFN-γ, IL-1, and TNF all modulate the intensity of the immune response (Turk et al., 1980; Middleton et al., 1998; Janeway et al., 2001).

1.2 Nickel and allergic hypersensitivity

In the overall category of contact allergens (natural or man-made), metals and their compounds represent a small proportion. Among these, however, is nickel which has been confirmed in recent epidemiological studies as the most prevalent contact allergen in the general population of the industrialized world (Hostynek et al., 2002). Nickel has been classified as a medium-level hazard. The risk of developing nickel allergic hypersensitivity (NAH), however, is high in industry as well as in the general population due to nickel's ubiquitous occurrence in tools and articles of everyday use, leading to frequent and sometimes intimate, potentially long-term exposure (Hostynek et al., 2002). The results of studies of unselected populations show overall percentages of NAH in 12 % (age group 15-34 years) (Nielsen et al., 1992). Among first-year female university students, 39% were patch-test positive to nickel (De Groot Ac, 2000; Mattila et al., 2001). Longitudinal surveys also indicate that there is an increase in NAH due to fashion and habits such as intimate skin contact with metal objects and practices such as skin piercing (Hostynek et al., 2002). With regard to the high prevalence of nickel allergy in the population and numerous cases of occupational disability due to nickel allergy (Menne,et al., 1979), it is of economical importance to understand the underlying immunological mechanisms, in order to enable us to make adequate risk assessments, improve diagnostic tools and, perhaps, develop new prevention strategies and therapeutical approaches.

Allergic contact dermatitis (ACD) is triggered by an encounter between an epidermal Langerhans cells (LC) and a hapten-carrier complex, i.e., between a xenobiotic agent (most often an eletrophilic or electron-seeking, organic compound) and a native, electron-rich group or nucleophile (e.g., a protein), which have formed a stable covalent bond by sharing an electron (Hostynek et al., 2002). Nickel is an example of an electrophilic agent avidly seeking to combine with electrons available in nucleophilic groups such as amino acid residues in native proteins. Nickel-protein complex can be recognized as non-self by the immune system. Nickel ACD is defined as a T lymphocyte-driven, delayed-type hypersensitivity (DTH) response, which is elicited by dermal exposure to Ni^{2+} ions. The latter may be released from metal alloys when they come into contact with water or bodily fluids, such as sweat and saliva (Jensen et al., 2003).

Classical T cells recognize nickel in the form of neoantigens that are generated by binding of Ni^{2+} ions to molecules of the major histocompatibility complex (MHC) and/ or MHC-embedded self-peptides (Lu et al., 2003; Loh et al., 2003; Gamerdinger et al. 2003). Ni^{2+} ions fail to undergo covalent protein binding, but form coordination complexes with protein. Within a given metal-protein coordination complex, one Ni^{2+} ion may bind to up to six amino acid side-chains as ligands. As the preferred ligands show a considerable variability, in principle each of the ligands within a given nickel-protein complex could have a different chemical group, the basic chain of histidine just being one out of a variety of candidate ligands. Therefore, the amino acid side-chains engaged in complex formation by Ni^{2+} may not only vary within a given coordination complex, but also from complex to complex (Lu et al., 2003; Loh et al., 2003; Gamerdinger et al. 2003). Nonetheless, there is evidence for a preferential engagement of certain nickel-binding elements of the human T cell receptor (TCR) in the formation of the bimolecular complexes and, hence, the T cell recognition of nickel-induced neoantigens (Fig. 1). The $CD4^+$ T cells obtained from the peripheral blood or skin of some patients with severe nickel-induced ACD were found to exhibit a significant over-expression of certain TCR Vβ17 sequences capable of binding nickel (Vollmer et al., 1997; Büdinger et al., 2001; Werfel et al.,1997). This conforms with the concept that Ni^{2+} ions may, indeed, form and stabilize inter-molecular bridges, such as those between a nickel-binding TCR on the one hand side and a certain MHC-embedded self-peptide and/or a conserved portion of the MHC molecule on the other hand side (Vollmer et al., 1997; Werfel et al.,1997; Büdinger et al., 2001).

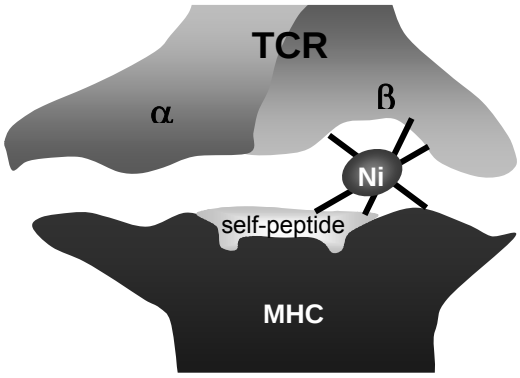


Fig. 1. Concept of Ni^{2+} ions acting as a 'bio-inorganic glue' in the MHC-TCR interaction, based on the work of Weltzien and colleagues (Vollmer et al., 1997; Büdinger et al., 2001). Ni^{2+} ions are loosely bound (Artik et al., 1999; Gamerdinger et al., 2003) to the surface of an antigen-presenting cell (APC) or target cell by engaging a sub-maximal number of ligands provided by MHC-embedded self-peptides and/or a conserved portion of the MHC molecule itself (Gamerdinger et al., 2003). These Ni^{2+}-induced neoantigens are recognized by the TCR so that the TCR engagement results in signalling and T cell activation. This would not

be the case in the absence of Ni^{2+} ions, as the TCR affinity for MHC-embedded self-peptides is too low (i.e., T cells are tolerant to unaltered self).

The clinical manifestations of ACD show a broad variety of symptoms ranging from redness and strong itching in the acute state up to eczema and skin thickening in the chronic state. These skin reactions result from a T cell-mediated damage of keratinocytes (Traidl et al. 2002). However, the mechanism underlying the sensitization to this common contact allergen is obscure. In the murine nickel allergy model, special measures had to be taken for successful sensitization of mice to nickel, such as breeding and rearing the animals in a nickel-free environment or administering exceedingly high concentrations of nickel salts, as well as nickel at higher oxidation states (Van Hoogstraten et al., 1993; Artik et al., 1999).

1.3 Oral tolerance in mouse model

Oral tolerance refers to systemic antigen hyporesponsiveness that occurs after oral antigen administration (Mowat et al., 1999). it is considered an important physiological mechanism that protects against hypersensitivity to food antigens and antigens from the microflora of the gastrointestinal tract (Weiner et al., 1997). Gut associated lymphoid tissue (GALT) is a well-developed immune network consists of vital immune components such as Peyer's patches (PP), mesenteric lymph nodes (MLN), the intraepithelial lymphocytes (IELs) and the lamina propria (LP) (Spahn et al., 2004). Antigens may act directly at the level of the GALT or may exert their effects after absorption (Weiner, 2000). Oral tolerance can be induced to a wide range of antigens, and a number of host factors, such as age, genetic background, and nutritional status, influence the induction and maintenance of oral tolerance (Samoilova et al. , 1998; Faria et al. , 2003). Like tolerance itself, oral tolerance is an active immunological process that is mediated by multipe mechanisms and is dose-dependent (Weiner, 2000). Low doses of antigen generate cytokine-secreting regulatory cells, such as Th2 cells (IL-4), Th3 cells (TGF-β), Tr1 cells (IL-10) and CD4+CD25+ cells (Weiner, 2000), whereas high doses induce anergy and/or deletion, as well as receptor downregulation (Chen et al., 1996; Samoilova et al., 1998; Zhang et al., 2001; Faria et al., 2003; Bertrand et al., 2003; Iriani et al., 2004). In addition to antigen dose, the nature of the antigen, the innate immune system, the genetic background and the immunologic status of the host, mucosal adjuvants and adjuvant costimulation during secondary antigen challenge influence the immunologic outcome following oral antigen administration (Weiner, 2000; Iriani et al., 2004).
The mucosal route is extremely attractive from a clinical standpoint, as it is easily administered to patients and accesses a major part of the immune system. Indeed, mucosal

administration of antigens has been shown in animal models to ameliorate not only classic autoimmune processes, but also stroke, Alzheimer's disease, and more recently, atherosclerosis (Weiner 2000). Therefore, elucidation of the basic immunological mechanisms associated with orally administered antigen has significance for the treatment of human diseases.

1.3.1 Orally induced tolerance to nickel

A prerequisite for the investigation of nickel tolerance in mice was the fact that Prof. Gleichmann's group had developed a mouse model for induction of allergic contact hypersensitivity to nickel (Artik et al., 1999), because tolerance, i.e., the selective absence of an immune response, can only be demonstrated if control animals show that particular immune response. It was showed that prolonged oral administration of a high dose of nickel (10 mM $NiCl_2$ in the drinking water) induced a complete, long-lasting immunological tolerance to nickel ions, which could not be broken by an injection of $NiCl_2$/ H_2O_2. Thus, the nickel tolerance induced in these so-called Ni^{high} mice prevented the subsequent induction of nickel hypersensitivity.

1.3.2 Adoptive transfer of nickel tolerance

The tolerance of the Ni^{high} mice can be adoptively transferred to non-sensitized Ni^{low} recipients by means of T cells obtained from the Ni^{high} donors; thereafter, the recipients can no longer be sensitized to nickel by injection of $NiCl_2$/ H_2O_2 (Artik et al., 2001; Ishii et al., 1993). On the day of cell transfer, the donor cells that are responsible for suppressing the allergic reactivity to nickel can be sorted, identified, and analyzed before being injected into the recipients. This is one of the advantages of working with mice. In humans, the analysis of nickel-specific Treg cells cannot be easily performed in vivo, but is confined to the lymphocyte transformation test (LTT) in vitro, whose diagnostic value is limited. Notwithstanding this difficulty, considerable progress in identifying human nickel-specific Treg cells has been achieved by Cavani and coworkers (Cavani et al., 2003).

1.3.3 Dominant, infectious nickel tolerance

A hallmark of peripheral T cell tolerance is its dominance, and this is based on the T cell capacity for specific suppression of an immune response and cellular spread of the tolerance. Following the adoptive transfer of T cells from specifically tolerized donors, the recipients' own T cells not only become unresponsive to immunization with the specific antigen studied, but they also acquire the capacity for suppression of new cohorts of specific T cells. The term infectious tolerance has been coined for this phenomenon observed in vivo (Gershon et al., 1971; Qin et al., 1993).

It was found that as few as 10^2 bulk T cells are needed to transfer the nickel tolerance of Ni^{high} mice to non-sensitized Ni^{low} recipients (Roelofs-Haarhuis et al., 2003). This strongly suggested that a powerful amplification mechanism takes place in the recipients. Indeed, an infectious tolerance spread was formally shown to underlie the tolerization of the numerous nickel-reactive T cells of the recipients (Artik et al., 2001). Moreover, in order to successfully transfer the nickel tolerance of Ni^{high} mice to Ni^{low} recipients, the Ni^{high} T cell inoculum must contain both $CD4^+$ and $CD8^+$ T cells (Roelofs-Haarhuis et al., 2003).

1.4 Costimulatory molecules and sensitization versus tolerance to nickel

It is well accepted that activation of naive T cells requires two distinct signals. Signal one is derived from recognition of MHC-peptide complexes by the TCR and signal two is provided by the binding of the T cell-expressed costimulatory molecule CD28 to its liglands B7.1 (CD80) and B7.2 (CD86) on APC (Abbas et al., 1999; Alegre et al., 2001; Sharp et al., 2002). In the absence of costimulation, recognition of antigen can lead to the induction of tolerance. Tolerance is brought about by failure of the T cells to efficiently expand to antigen, and by the surviving cells entering into a state of hypo-responsiveness to subsequent antigen encounter (Lee et al., 1998). Cytotoxic T lymphocyte antigen-4 (CTLA-4), which belongs to the CD28 family, is induced on T cells upon activation. CD80 and CD86 ligands expressed on APCs are shared by the positive and negative receptors (CD28 and CTLA-4, respectively). (Salomon et al., 2001; Chang et al., 2002). CTLA-4 delivers a negative signal to the activated T cells to downmodulate CD28-mediated positive co-stimulatory signal. (Salomon, et al., 2001; Chang et al., 2002; Benhamou et al., 2002). An important advance in the area of T cell costimulation is the identification of several new functional members of the B7 family, including B7H1 (PD-L1), B7DC (PD-L2), B7H(ICOSL)(Dong et al., 1999; Okazaki et al., 2002; Liu et al., 2003; Loke et al., 2003). It was known that both B7H1 and B7DC were found to interact with PD-1, a putative negative regulator for immune function

and may involved in peripheral tolerance (Okazaki et al., 2002; Liu et al., 2003; Dong et al., 1999; Loke and Allison, 2003; Salama et al., 2003; Ansari et al., 2003; Shin et al., 2003; Yamazaki et al., 2002; Freeman et al., 2002; Nishimura et al., 2001), whereas B7H was found to bind to ICOS (Yoshinaga et al., 1999; Rottman et al., 2001). Both PD-1 and ICOS are expressed by activated T cells (Yoshinaga et al., 1999). Engagement of PD-1 with its ligands PD-L1 or PD-L2 delivers a negative signal to inhibit the activation of T cells . C57BL/6 mice that lack PD-1 develop lupus-like arthritis and glomerulonephritis (Nishimura et al., 2001). In murine experimental autoimmune encephalomyelitis (EAE), a prototypic Th1-mediated demyelinating disease that is used as a model for human multiple sclerosis. In this model, antigen-specific CD4+ Th1 cells mediate inflammatory damage in the central nervous system (CNS), with consequent demyelination, manifested clinically by progressive paralysis (Salama et al., 2003). PD-1 blockade resulted in accelerated and more severe disease with increased CNS lymphocyte infiltration. Interestingly, PD-L2 but not PD-L1 blockade also led to disease augmentation (Salama et al., 2003). Blockade of the ICOS-B7RP-1 pathway during the efferent immune response to proteolipid protein (PLP) abrogates clinical symptoms, CNS leukocyte infiltration and induction of pro-inflammatory cytokines and chemokines in the CNS. In contrast, ICOS-B7RP-1 blockade during antigen priming polarizes a Th1 response to PLP, enhances expression of pro-inflammatory cytokines and chemokines in the CNS and exacerbates brain leukocyte infiltration and clinical symptoms. In addition, some members of the TNFR superfamily, including OX40, CD27 have been shown to transmit a costimulatory signal for T cell proliferation and cytokine production like CD28 (Akiba et al., 2000; Bansal-Pakala et al., 2001; Yuan et al., 2003). It has been reported that signaling through OX40 (CD134) breaks peripheral T-cell tolerance (Bansal-Pakala et al., 2001). OX40L and CD70 are the ligands for OX40 and CD27, respectively (Akiba et al., 1999; Tesselaar et al., 2002). The major costimulatory molecules are summaried in Table 1.

Table 1 The major costimulatory molecules

APC	T cell	Effect
CD80/CD86 (B7)	CD28	stimulatory
	CTLA-4	inhibitory
ICOS L	ICOS	stimulatory
CD40	CD40 L	stimulatory
PD-L1/PD-L2	PD-1	inhibitory
OX40 L	OX40 (CD134)	stimulatory
CD70	CD27	stimulatory
4-1 BBL	4-1 BB	stimulatory

Prof. Gleichmann's group developed a mouse model for experimental induction of allergic contact hypersensitivity and immunological tolerance to nickel, respectively. It was found that conventionally-reared mice, now termed Ni^{low} mice, can readily be sensitized with $NiCl_2$ plus H_2O_2. The latter can be considered an internal adjuvant since it is locally produced in large amounts during inflammation. In contrast, when $NiCl_2$ is administered alone, it fails to sensitize the conventionally-reared Ni^{low} mice (Artik et al., 1999). It was proposed that the functional role played by H_2O_2 is to induce the expression of co-stimulatory molecules such as CD80 and CD86 on APC (DCs and B cells), in the process of successfully sensitization of mice together with $NiCl_2$, whereas $NiCl_2$ alone has no or small effects on the up-regulation of costimulatory molecules. However, there are no available data from literature to directly address this question. Therefore, it is deserved to investigate the expression patterns of co-stimulatory molecules by APC after stimulated with $NiCl_2$ alone, $NiCl_2$ together with H_2O_2 in more details. More recently, it was demonstrated that prolonged oral administration of a high dose of nickel (10 mM $NiCl_2$ in the drinking water) induced a complete, long-lasting immunological tolerance to nickel. Moreover, The tolerance can be adoptively transferred from the orally tolerized donors, now termed Ni^{high} mice, to naive syngeneic recipient mice, i.e. the Ni^{low} mice. (Artik et al., 2001). It is obvious that orally administration of $NiCl_2$ can induce the production of Nickel- specific T suppressor cells or regulatory T cells. It has been reported that anergic T cell and T regulatory cells can down-regulate the expression of co-stimulatory molecules on dendritic cells (Li et al., 1999; Cederbom et al., 2000; Chang et al., 2002; Najafian et al., 2003; Misra et al., 2004). In orally nickel tolerized mice, it is unknown wethere or not the resistance of sensitization with $NiCl_2$ plus H_2O_2 is due to, at least partially, the down-rgulatory effects of T suppressor cells on the expression of co-stimulatory molecules by APCs upon immunization with $NiCl_2$ together with H_2O_2. The purpose of this study is trying to describe the picture, and elucidate the mechanisms underlying co-stimulatory molecules involved in the interaction between APC and T suppressor cells relevant to nickel allergy versus tolerance.

1.5 Nickel-specific T regulatory (Treg) Cells

The immune system must distinguish between innocuous and pathological antigens to prevent unnecessary and self-destructive immune response. (Parijs et al., 1998; Vendetti et al., 2000). This discrimination is mediated by immune tolerance, the mechanism that prevents the immune system from pathogenic reactivity against self-Ags. Indeed, the immune system has evolved a number of mechanisms to control self-reactive T cells that

escape negative selection in the thymus. However, T cell nonresponsiveness to self-antigen does not appear to result exclusively from clone deletion, T cell anergy, or T cell ignorance (Sakaguchi, 2000). There is accumulating evidence that the generation of regulatory T cells is necessary to maintain self-tolerance and prevent the onset of autoimmune disease (Baecher-Allan and Hafler, 2004). The past decade has seen the discovery of several T cell populations that appear to have major regulatory effects on T cells responding to both self-antigens and those derived from infectious agents. They can broadly be divided into IL-10-producing Tr1 and TGF-β-producing Th3 cells that appear to require antigen-specific, MHC-restricted stimulation, to down regulate immune responses, and the CD4+CD25+ T regulatory cells that comprise 5-10% of total peripheral CD4+ T cells in normal adult mice and expresses surface CD25 (IL-2 Rα) before activation (Sakaguchi et al., 1995; 2001; Levings et al., 2001; Hu et al., 2004).

Treg (or T suppressor) cells play a pivotal role in the acquisition and maintenance of the T cell tolerance that is acquired through T cell contact with antigen in the absence of costimulatory signals. Consistent with this, the T cells of Ni^{high} mice were found to contain potent Treg cells which down-regulate the costimulatory molecule CD80 on the dendritic cells of Ni^{low} mice co-cultured in vitro (Fang, 2004). In another co-culture system in vitro, lymph-node cells of nickel-sensitized mice were restimulated with $NiCl_2$ and their proliferation determined. When purified T cells, as potential Treg cells, were added to these indicator lymph-node cells, a hierarchy of $Ni^{high} > Ni^{low} > Ni^{very\ low}$ with respect to suppressive capacity was observed. Whereas the T cells of the Ni^{high} mice almost completely suppressed the restimulation of the lymph-node cells and those of Ni^{low} mice showed a moderate suppressive effect, those of the $Ni^{very\ low}$ mice failed to suppress at all, but even enhanced the $NiCl_2$-induced proliferative response of the indicator cells (Wu et al., 2004).

Hence, given the lack of nickel-specific Treg cells in the $Ni^{very\ low}$ mice, it becomes understandable why Ni^{2+} ions alone can sensitize those animals. On the other hand, the limited resistance of the Ni^{low} mice to sensitization (i.e., resistant to $NiCl_2$, but susceptible to $NiCl_2$/ H_2O_2) appears to be due to the presence of a suboptimal number or type of nickel-specific Treg cells in these animals. Taken together, the results appear to indicate that oral nickel exposure dose-dependently induces different numbers or types of nickel-specific Treg cells, which possess different levels of suppressive capacity. In other words, the higher the oral uptake of nickel, the higher the suppressive capacity of Treg cells and, hence, the more difficult it is to sensitize the mice to nickel.

A particular $CD4^+$ subset of Treg cells in men and mice is characterized by the constitutive expression of the cell surface marker $CD25^+$. Whereas the $CD4^+CD25^+$ T cells obtained from persons, who were non-allergic to nickel, profoundly suppressed the proliferative response of nickel-specific indicator T cells in vitro, those obtained from allergics exhibited only weak suppressive capacity (Cavani et al., 2003; 2001). In contrast, no difference between nickel allergics and non-allergics was detectable, when their $CD4^+CD25^+$ T cells were co-cultured with alloreactive control T cells (Cavani et al., 2003). As is the case with the T effector cells causing the allergic response, one also has to distinguish an induction phase and an effector phase of Treg cells. Whereas the effector phase of Treg cells can be studied in both mice and humans, their induction phase can hardly be studied in humans in vivo. The mouse model of oral nickel tolerance provides the unique possibility to investigate both the induction and the effector phase of nickel-specific Treg cells (Roelofs-Haarhuis, et al. 2003; 2004).

1.6 Indoleamine 2,3-dioxygenase (IDO) and immune tolerance

Dendritic cells (DCs) are the most important antigen-presenting cell type (Steinman et al., 2003). Although DCs are known to be critical for inducing T cell immunity in immunized or infected individuals, there is increasing evidence that, in certain circumstances, DCs can suppress immunity, or promote tolerance by inhibiting T cell responses to auto-antigens and allo-antigens (Steinman et al.,2002; 2003). Moreover, antigen challenge at mucosal surfaces, such as the gastro-intestinal tract and the maternal-fetal interface is associated with tolerant, rather than immune responses to foreign antigens (Hayday et al., 2000; Mellor et al., 2000). However, the mechanisms by which DCs suppress T cell immune response and induce T cell tolerance are unclear. There are several determinants that lead to immune tolerance of antigens presented by DCs, one is the maturation status of DCs at the time of antigen presentation to T cells, namely, immature DCs induces the immune tolerance. Furthermore, It has been demonstrated that, under steady state conditions, DCs induce peripheral T cell unresponsiveness in vivo (Hawiger et al., 2001), and a subset of DCs expressing CD8a is responsible for inducing peripheral self-tolerance to tissue-associated antigens (Belz et al., 2002). More recently, it has been reported that indoleamine 2,3-dioxygenase (IDO)-producing dendritic cells can modulate the proliferation of human and murine T cell by affecting tryptophan catabolism (Munn et al., 1998; Mellor and Munn, 2004).

IDO is a cytoplasmic, the rate-limiting enzyme involved in tryptophan catabolism and utilization, respectively, and is induced by interferon-γ (IFN-γ) (Ozaki et al., 1988). IDO catalyzes the first step reaction of tryptophan catabolism, the conversion of tryptophan to N-formylkynurenine, and subsequently kynurenine (Mellor and Munn, 1999; Grohmann et al.,

2003) . Therefore, IFN-γ exerts effect on tryptophan metabolism by increasing the rate of degradation of trytophan. IDO is strongly expressed in macrophages and dendritic cells (DCs) (Munn et al., 1999; Hwu et al. 2000), and has been shown to be responsible for downmodulating T cell activation and proliferation, through depletion of tryptophan from the microenvironment (Hwu et al. 2000). Murine studies have also suggested that products of tryptophan catabolism (quinolinic acid and 3-hydroxyanthranilicacid) can induce apoptosis of T cells (Lee et al., 2002)

IDO expression and tryptophan catabolizing activity can be induced by exposing DC to IFN-γ, CTLA4-Ig, or by culturing them with CD4+CD25+ Treg cells expressing surface CTLA-4, which binds and triggers B7 and induces IDO expression by ' reverse-signaling' pathways in DCs (Finger et al., 2002; Munn et al., 2004). Cytotoxic T lymphocyte antigen-4 (CTLA-4) is transiently expressed on T cells after activation, and can bind both B7.1 (CD80) and B7.2 (CD86) on antigen presenting cells (APCs), with higher affinity than CD28 (Oosterwegel et al., 1999). CTLA-4 is a negative regulator of T cell activation, but the mechanisms underlying its inhibitory activity are still not fully understood(Oosterwegel et al., 1999) . Competition with CD28 for binding to B7 molecules and induction of a direct inhibitory signal by CTLA-4 are among the suggested mechanisms (Oosterwegel et al., 1999). Recombinant soluble forms of CTLA-4 (CTLA-4-Ig) have been shown to induce IDO expression in murine (Grohmann et al., 2002; Fallarino et al., 2003), and, more recently, human DCs (Munn et al., 2002). CTLA-4 bound B7 molecules on DCs and triggered IFN-γ production, resulting in the induction of IDO espression.Thus, CTLA-4 expressed on activated T cells can stimulate IDO in DCs, providing feedback control of T cell activation itself (Grohmann et al., 2002; Fallarino et al., 2003)

A hallmark of peripheral T cell tolerance is ist dominance. The mechanisms underlying this phenomenon can be particularly well investigated by adoptively transferring T cells from tolerant donor animals into naive recipients, which thereafter become unresponsive to immunization with the specific Ag studied. Infectious tolerance has been proposed to explain this phenomenon and denotes the ability of T cells from a tolerant donor to spread the unresponsiveness to Ag-specific T cells in naive recipients (Roelofs-Haarhuis et al., 2003). Recently, several lines of evidence support the idea that, the tolerant T cells ‘educated‘ APCs can act as messengers from Ts to naive T cells. In other words, the Ts cells endowed APCs with the capacity to tolerized new sets of T cells (Roelofs-Haarhuis et al, 2003). Using chimeric mice and a two-step culture system in vitro, One study performed more recently (Alpan et al., 2004), has clearly demonstrated that DCs can act as ‘ temporal bridges‘ to relay information from orally immunized memory $CD4^+$ T cells to

naive $CD4^+$ T cells. It was found that the conversion of a naive T cell occurs only if it can interact with the same APCs, although not necessarily the same antigen, as the effector cell.

With regard to the molecular mechanisms by which the spread of tolerance from T cell to APCs, it has been proposed that cytokines such as IL-4 and IL-10 may play a role. However, in some experimental models, they are apparently cell contact-dependent. As aforementioned, in addition to modulation of the expression of costimulatory molecules by Treg, IDO is another candidate molecule to mediated the spread of T cells tolerance to the DCs through the engagement of CTLA-4 of Treg with B7 on DCs. Moreover, IFN-γ is a critical inflammatory mediator for contact hypersensitivity to nickel, and a potent inducer of IDO expression. Therefore, it is conceivable that IDO may play a role in our nickel tolerance mouse model.

1.7 Aim of this study

Aim of this study was to test the hypothesis that costimulatory molecules (e.g., CD80 /CD86, PD-L1/PD-L2) and IDO may be involved in reciprocal interplay of T suppress and tolerogenic APCs induced by oral uptake nickel. To this end, the mouse model for oral induction of immune tolerance to nickel (established by Artik et al., 2001) was used to address the following questions:

(1) Are there differences in the expression of costimulatory molecules (CD86 /CD80, CTLA-4 and PD-L1/PD-L2) between nickel-orally tolerized mice and naive mice when immunization with $NiCl_2$/ H_2O_2?

(2) Do the regulatory T cells from nickel-tolerized mice down-regulate gene expression of the costimulatory molecules on antigen presenting cells?

(3) Does IDO play a role in mice that were orally tolerized to nickel and, if so, does IDO mediate the tolerance spread from T cells to APC?

2. Materials and Methods

2.1 Mice

Specific pathogen-free female C57BL/6J ($H2^b$) mice, which express $Ly5.2^+$ ($CD45.2^+$) were obtained from Janvier (Le Genest St. Isle, France). Congenic $Ly5.1^+$ ($CD45.1^+$) C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed at the institute for environmental medicine (IUF) during the experimental periode in accordance with institutional guidelines for animal welfare. They had free access to drinking water and standard rodent lab chow (No. 1324, Altromin, Lage/Lippe, Germany). No measures were taken to protect the animals from exposure to nickel: cages (made from plastic) were covered by stainless steel covers, and drinking water was provided by glass bottles covered with water outlets made from stainless steel. Mice were used between 9 and 20 weeks of age. All animal protocols were approved by the governmental committee on Use and Care of laboratory Animals.

2.2 Reagents and buffer solutions

$NiCl_2$. $6H_2O$ (henceforth referred to as $NiCl_2$) and 2,4-dinitrofluorobenzene (DNFB) were purchased from sigma- Aldrich (Steinheim, Germany). 100 mM of $NiCl_2$ stock solution was prepared in 0.9% NaCl, sterilized by filtered through 0.2μM syringe filter (Pall Corporation), and stored at 4 °C until use. The working concentration of $NiCl_2$ for immunization was 10 mM. DNFB was prepared in a 4:1 (v/v) mixture of acetone and olive oil, working concentration for immunization is 0.5 % (w/v). 30 % hydrogen peroxide (H_2O_2) was obtained from E. Merk (Darmstadt, Germany). 1% H_2O_2 was used for immunization together with $NiCl_2$. 0.2 mg/ ml Streptavidin-APC was obtained from BD PharMingen (Heidelberg, Germany), and 1:200 dilution was used for second staining to detect biotin-labeled antibody in flow cytometry. 7-AAD (7-amino-actinomycin D) was purchased from eBioscience, it is a ready-to-use solution for the exclusion of nonviable cells in flow cytometric analysis. Fluorescence is detected in the far red range of the spectrum (650nm long-pass filter). Use at 5 μl per test (1x 10^6 cells) and incubate for 5 minutes before analysis. Albumin from bovine serum (BSA) lyophilized powder (further purified fraction V) was purchased from sigma. Saponin was obtained from E. Merk (Darmstadt, Germany). Formaldehyde, 37% solution (formalin) was purchased from sigma.
Indoleamine 2,3-dioxygenase (IDO)-inhibitor 1-methyl-DL-tryptophan pellet: slow-releasing polymer containing 1-methyl-DL-tryptophan pellets (200 mg, release time is 12 days, 16.7

mg / mouse/ day) and empty placebo pellets were purchased from Innovative Research of America (Sarasota, FL).

PBS buffer (10 L): NaCl 80g; Na_2HPO_4 11.6g; KH_2PO_4 2.0g; KCl 2.0g. dissolved in 10 L distilled water, and sterilized by filtered through 0.2 μM VacuCap 60 Filter Unit (Pall Corporation). Stored at 4 °C until use. All chemicals are from sigma unless mentioned otherwise

Hypotonic lysis buffer (Tris-NH_4Cl): 2.06g Tris; 8.55g NH_4Cl, dissolved in 1000 ml distilled water, and adjust pH to 7.2. Sterilized by filtered through 0.2 μM filter. Stored at 4 °C until use.

FACS staining buffer: PBS containing 1 % BSA, 0.02% NaN_3.

Saponin buffer: PBS, 2% BSA, and 0.5% saponin

MACS running buffer: PBS, 2 mM EDTA, and 0.5% BSA

2.3 Antibodies

The following anti-mouse mAbs were purchased from BD PharMingen: APC-labeled anti-CD3ε (clone 145-2C11), APC-labeled anti-CD4 (Clone RM4-5), FITC-labeled anti-CD8α (clone 53-6.7), FITC-labeled anti-TCRβ-chain (clone H57-597). PE-labeled anti-CD152 (CTLA-4, clone UC10-4F10-11), PE-labeled anti-PD-1 (clone J43), PE-labeled hamster IgG group 1, κ isotype control (anti-TNP), FITC- labeled anti-CD11c (clone HL3), FITC-labeled anti-CD19 (clone 1D3), PE-labeled anti-I-A/I-E (clone M5/114.15.2), PE-labeled anti-CD45.1 (clone A20), FITC-labeled anti-CD45.2 (clone 104), biotin-labeled anti-CD80 (clone 16-10 A1), biotin-labeled hamster IgG1, κ, isotype control (anti-TNP), biotin-labeled anti-CD86 (clone GL1), biotin-labeled rat IgG2c, κ, isotype standard (clone A23-1). Biotin-labeled anti-mouse PD-L1 (B7-H1), biotin-labeled anti-mouse PD-L2 (B7-DC) and biotin - labeled rat IgG2a isotype control (clone eBR2a) were purchased from eBioscience. Magnetically labeled anti-CD11c, anti-CD19, anti-MHC class II (anti-MHC-II), and anti-PE mAbs were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

2.4 Oral immune tolerance induction to nickel

Female C57BL/6J mice were treated with 10 mM $NiCl_2$ in drinking water for at least 4 weeks to induce oral immune tolerance to nickel as described previously (Artik et al., 2001), these mice were termed Ni^{high} mice. Age- and sex-matched control mice received tap water without any additional $NiCl_2$ termed Ni^{low} mice. The immune tolerance to nickel was verified

by mouse ear-swelling test (MEST). The animals were used to do experiments immediately after treatment.

2.5 Immunization of mice and preparation of draining lymph nodes cells

To determine the expression patterns of costimulatory molecules on APCs in draining lymph nodes induced by $NiCl_2/H_2O_2$, or H_2O_2 alone, Ni^{low} mice (at least 3 mice per group) were injected intradermally into both flanks (50 μl each) with saline (0.9% NaCl), 10 mM $NiCl_2$, 10 mM $NiCl_2$ in saline containing 1% H_2O_2 and 1% H_2O_2 alone, respectively. For DNFB treatment, mice were immunized by painting 0.5 % (w/v) DNFB on the shaved flanks (20 μl each). Thereafter, mice were sacrificed at different time (e.g., day 1, 2, 3, 4) and axillary lymph nodes were harvested for determining the costimulatory molecules expression.

To compare the expression levels of costimulatory molecules between Ni^{high} mice and Ni^{low} mice, groups of nickel orally tolerized Ni^{high} and untreated control Ni^{low} mice were immunized with $NiCl_2$, $NiCl_2$ /H_2O_2, and DNFB, respectively. Thereafter, axillary draining lymph nodes were harvested and single-cell suspensions were prepared. The expression of costimulatory molecules were determined by flow cytometric analysis.

The method used to prepare single-cell suspensions from draining lymph nodes (e.g. axillary lymph nodes) was as follows: mouse was sacrificed by asphyxiation with CO_2. Place it on its back on clean, dry, absorbent paper. Wet the fur with 70% ethanol to sterilize the area and reduce the possibility of contamination. Make a midline incision with iris scissors. Retract the skin above the head and below the thighs by pulling it with forceps. Identify the axillary lymph nodes and grasp the lymph nodes with curved forceps and pull them free of attached tissue. Place the lymph nodes in 1 ml PBS in 24-well cell culture plate. Press the lymph nodes with forceps to release the lymph node cells from the tissue, and pass the cell suspensions through a 70-μm nylon cell filter to remove cell clumps. Collect the cells and wash cells twice with PBS. Count the cell number and ready for flow cytometric analysis.

2.6 Immune flow cytometry

Cells were preincubated with anti-CD16/CD32 mAb (anti-FcγR mAb, 1:200 dilution) at 4 °C for 20 min to block nonspecific staining, then washed and incubated in PBS containing 1 % BSA, 0.02% NaN_3 (staining buffer) with the indicated mAb conjugates at 4 °C for 30 min. Cells were washed and analyzed on FACSCalibur (BD Biosciences) and analyzed with CellQuest software. To investgate the expression of costimulatory molecules on APCs in

the draining lymph nodes after immunization, mice were injected intradermally into both flanks (50 μl each) with pyrogen-free saline (0.9 % NaCl), 10 mM $NiCl_2$, 10 mM $NiCl_2$ containing 1% H_2O_2, or 1% H_2O_2. Single-cell suspensions of the draining axillary lymph nodes were prepared in PBS and preincubated with anti-CD16/CD32 mAb. To characterize DCs, the lymph node cells were stained with anti-CD11c (1: 200 dilution) and anti-MHCII (1: 4000 dilution), whereas for the characterization of B cells, anti-CD19 (1:100 dilution) and anti-MHCII were used. Subsequently, both subpopulations were stained with either anti-CD80 (1:100 dilution) or anti-CD86 (1:100 dilution) mAb. Flow cytometry was then performed, and the expression of CD80 or CD86 was calculated as the mean fluorescence intensity (MFI) on the DCs and B cells, respectively. Similarly, to characterize the expression kinetics of PD-L1 and PD-L2 on DCs, cells were stained with anti-CD11c, anti-MHCII and anti-PD-L1 (1:200 dilution) or anti-PD-L2 (1:200 dilution). DCs were defined by gated on $CD11c^+$ $MHCII^+$ cells, the expression of PD-L1 or PD-L2 was calculated as the mean fluorescence intensity (MFI). To phentype T cells, cells were stained with anti-CD3 (1:100 dilution), anti-CD4 (1:100 dilution) and anti-TCRβ(1:100 dilution). Intracellular staining for CTLA-4 expression by CD4+ T cells was performed as previously described (Levings M. K., et al., 2001) with some modification. In brief, lymphocytes prepared from draining lymph nodes were stained with anti-CD4-APC and anti-TCR-β-FITC in staining buffer for 20 min at 4 °C, pellet the cells by centrifuge with 1200 rpm for 8 min at 4 °C, and washed twice with staining buffer. Cells were fixed by incubation with 4% formaldehyde for 20 min at room temperature (in the dark), pellet the cells and wash twice with staining buffer. Membranes were permeabilized by incubation in saponin buffer for 10 min at room temperature. Pellet the cells and resuspend the cells with saponin buffer containing anti-CTLA-4-PE(1:100 dilution). Incubate cells at room temperature for 30 min in the dark. Wash the cells twice with saponin buffer. Cells were washed once in PBS, 1% BSA before analysis.

2.7 Mouse ear-swelling test (MEST)

To sensitize the mice for mouse ear-swelling test (MEST), mice (at least 5 mice per group) were injected intradermally into both flanks (50 μ l each) with 10 mM $NiCl_2$ (prepared in 0.9% NaCl saline) or 10 mM $NiCl_2$ in saline containing 1% H_2O_2. Ten days after immunization, mice were rechallenged by injecting 50 μl of 10 mM $NiCl_2$ in sterile, pyrogen-free saline into the pinna of each ear. Forty-eight hours after rechallenge with $NiCl_2$, delayed-type hypersensitivity reactions were determined by measuring the increment in ear thickness in comparison with the prechallenge values. To determine the prechallenge values, mice were anesthetized with di-ethyl-ether, whereas for the measurement after challenge, mice

were killed by asphyxiation with CO_2. Measurements were performed in a blind fashion using a micrometer gauge (Oditest D 1000 gauge; The Dyer Company, Lancaster, PA) as described previously (Artik et al., 2001). The following results represent the mean ear-swelling response from groups comprising five to six mice and are expressed in units of mm $\times 10^{-2}$ + SEM.

2.8 Sorting of T cells and APCs for adoptive transfer studies

To isolate T cells or APCs from the donor mice for performing adoptive transfer experiments, the donor mice (at least 2 nickel orally tolerized Ni^{high} mice or untreated Ni^{low} control mice) were sacrificed by asphyxiation with CO_2. Spleens were removed aseptically and kept on ice, and single-cell suspensions were prepared as follows: place the spleens in a 10 cm petri dish containing 10 ml PBS, single-cell suspensions were obtained by rubbing the tissue with a syringe plunger, and passed through a 70-μm nylon cell filter to remove cell clumps. Erythrocytes were removed by hypotonic lysis using hypotonic buffer (17 mM Tris-HCl (pH 7.2) and 160 mM NH_4Cl), and count the spleen cells for further T cells or APCs isolation. For the transfer of T cells, single-cell suspensions of erythrocyte-free spleen cells, which contained 30-35% T cells, 60-65% B cells, and 1-2 % DCs, were passed through nylon wool columns, followed by the depletion of $CD11c^+$, $CD19^+$, and $MHCII^+$ cells using a magnetic cell sorter (auto MACS; Miltenyi Biotec). For the transfer of APCs, single-cell suspensions of erythrocyte-free spleen cells were depleted of $CD4^+$, $CD8^+$, and $CD90^+$ T cells using the auto MACS. Hereafter, these cells, containing 90-95% $CD19^+MHCII^+$ B cells and 1-3% $CD11c^+$ $MHCII^{high}$ DCs, will be referred to as APCs. The purity of the resulting T cell and APC fractions were then clarified by FACS analysis and were found to be contaminated with $< 0.5\%$ $CD19^+$ $MHCII^+$ or $CD11c^+$ $MHC\text{-}II^{high}$ APCs and $<0.5\%$ CD4+CD3+ or CD8+CD3+ T cells, respectively. In the serial transfer assays using T cells and APCs (see 2.11), an additional depletion of $Ly5.1^+$ ($CD45.1^+$) cells was performed between the first set of recipients (i.e., the second donors) and the second set of recipients. After this depletion, the fraction of cells required for transfer contained < 0.1 % $Ly5.1^+$ cells.

2.9 Medium and in vitro cell coculture

The cell culture medium used for murine spleen lymph cells culture was RPMI-1640 complete medium: RPMI-1640 with L-Glutamine (GIBCO, Invitrogen life technologies), 10%

FCS (Sigma), 1 mM sodium pyruvate (sigma), nonessential amino acids (Sigma), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma), 50 μM 2ME (Sigma).

In vitro cell coculture: C57BL/6 mice were orally tolerized by 10mM $NiCl_2$ drinking water(Ni^{high} mice) or were left untreated (Ni^{low} mice). Spleen T cells, APCs (T cell-depleted spleen cells, containing dendritic cells) were obtained through MACS isolation as described above. Thereafter, coculture was performed in 24- well cell culture plate (Greiner bio-one, Cellstar) in 2 mL RPMI-1640 complete medium as follows: Ni^{low} APCs ($2x10^6$) with or without 75μM $NiCl_2$; Ni^{low} APCs($1x10^6$) plus Ni^{low} T cells ($2x10^6$) with or without 75μM $NiCl_2$, or Ni^{low} APCs($1x10^6$) plus Ni^{high} T cells ($2x10^6$) with or without 75μM $NiCl_2$. Each group has four wells. Cells were harvested after overnight coculture in 5% CO_2 at 37°C. CD80 and CD86 expressions by DCs were evaluated by FACScan (BD Biosciences) flow cytometer.

2.10 Implantation of slow-release pellets containing 1-MT into mouse skin

To observe the regulatory role of IDO in the process of infectious tolerance to nickel, serial adoptive transfer experiments were performed, and IDO-inhibitor 1-MT slow-release pellets were used to abolish the activities of IDO in the first recipients. Prospective first recipient mice (3 mice per group) were anesthesized by i.p. injection of mixture solution of rompune (2%) and ketamin (10%) in saline solution, 100μl per mouse. Slow-release pellets containing 1-MT or placebo pellets were implanted under the dorsal skin of the first recipient mice 1 day before T cell adoptive transfer. Closing of wound with sewing.

2.11 Serial adopive transfer studies

Prospective Ly5.1+ donor mice were orally tolerized to nickel (Ni^{high} mice) or were left untreated (Nil^{ow} mice). The first Ni^{low} recipient mice were implanted of IDO-inhibitor (1-MT) or placebo pellet into the skin as described above. Thereafter, T cells of the primary Ly5.1+ donors were transferred to a first set of Ly5.2+ recipients. Within 24 h after transfer, the first recipients were injected with $NiCl_2$ / H_2O_2. On day 11, APCs of the first recipients were isolated and depleted of donor Ly5.1+ cells, before they were transferred into a second set of Ly5.2+ recipients. All second recipients were then immunized with $NiCl_2$/ H_2O_2 and rechallenged, and the ear-swelling responses were determined.

2.12 Statistical analysis

Statistical significance of results was determined by ANOVA followed by Newman-Keuls test . The level of significance was set at $p < 0.05$.

3.Results

3.1 CD80 and CD86 expression pattern by APCs in draining lymph nodes after immunization of Ni^{low} mice with $NiCl_2/H_2O_2$

To determine the expression kinetics of CD80 and CD86 by APC (dendritic cells, B cells), groups of three mice were injected into both flanks with NaCl, $NiCl_2$ alone, $NiCl_2$ /H_2O_2, or H_2O_2 alone, as indicated. After 1, 2, 3, and 4 days, the draining axillary lymph nodes were prepared, and their cells were stained for surface MHC-II, CD11c, and CD80 or CD86. DCs were gated as $CD11c^+$ $MHC\text{-}II^{high}$ cells, as shown in Fig.1 (A, B).

In comparison with the capacity of inducing the expression of CD80 and CD86 by injection with NaCl, $NiCl_2$ alone, $NiCl_2/H_2O_2$, or H_2O_2 alone, I found that $NiCl_2/H_2O_2$ and H_2O_2 alone are strong stimulator for induction of expression of CD80 and CD86 by DCs and B cells. In contrast, $NiCl_2$ alone has a small effect to up-regulate the expression of CD80 and CD86 by DCs and B cells, whereas, NaCl failed to induction of expression of both CD80 and CD86 by DCs and B cells, as shown in Fig.1 (C, D, E, F)

The results have shown that the expression kinetic of CD80 is different from that of CD86, namely, the expression level of CD80 by DC peaked on day 1 after immunization, whereas, CD86 expression reached a peak on day 3 after immunization. The expression of CD80 and CD86 by B cells, both CD80 and CD86 increased day 1 after immunization, thereafter, the expression levels maintain at a relative high levels through day2 and 3 after immunization.

It was previously reported that attempts to induce contact hypersensitivity to nickel in Ni^{low} (naive) mice using Ni (II) Cl_2 alone often failed (Artik et al., 1999), whereas, the sensitization was achieved by injecting of $NiCl_2$ in combination with H_2O_2. It has been proposed that $NiCl_2$ alone fails to upregulate costimulatory molecules, whereas, the administration of $NiCl_2$ together with H_2O_2 would induce both nickel-specific signal 1 and costimulatory signal 2. Focusing on CD80 and CD86 as the recognized primary markers of costimulation, here, we determined the expression of CD80 and CD86 on APCs, in the draining axillary lymph nodes after immunization of Ni^{low} mice at the flanks. Indeed, the expression of CD80 and CD86 on DCs (Fig.1, C and D) as well as B cells (Fig.1, E and F) was higher after the injection of $NiCl_2$ / H_2O_2 or H_2O_2 alone than after injection of $NiCl_2$ alone or saline. These results confirmed the concept that, in the Ni^{low} mice, $NiCl_2$ alone induces signal 1 (neoantigens) on APCs, whereas $NiCl_2$ in conjunction with H_2O_2 induces both signal 1 and signal 2 (costimulation) and H_2O_2 alone induces only signal 2.

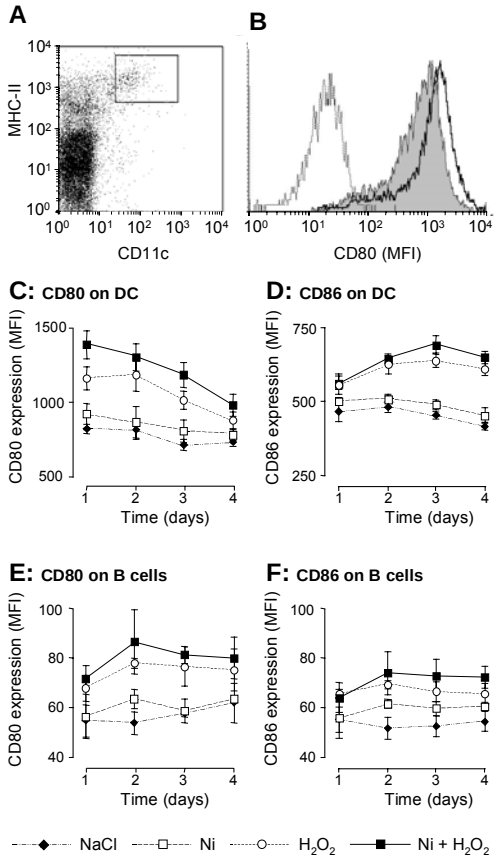


Fig. 1 Contrary to immunization with $NiCl_2/H_2O_2$, an injection of $NiCl_2$ alone fails to up-regulate CD80 and CD86 on the APCs in the draining lymph nodes. Groups of three Ni^{low} mice were injected into both flanks with NaCl, $NiCl_2$ alone, $NiCl_2/H_2O_2$, or H_2O_2 alone, as indicted. After 1, 2, 3, 4 days, the draining axillary lymph nodes were prepared, and their cells were stained for surface MHC-II, CD11c, and CD80 or CD86. DCs were gated as CD11c+MHCII ^{high} cells, as shown in A. The CD80 and CD86 expression of the gated DCs was plotted in histograms, as shown for CD80 in B. The bold curve represents the staining of DCs from mice immunized with $NiCl_2/H_2O_2$, the shaded curve shows the staining of DCs from mice immunized with $NiCl_2$ alone, and the dotted curve shows the the staining obtained with the isotype control mAb. The kinetics of CD80 and CD86 expression, shown as mean flurescent intensities, on DCs are displayed in C and D, respectively, whereas the kinetics of CD80 and CD86 expression on B cells (CD19+$MHCII^{high}$) are displayed in E and F, respectively. Results represent one of three experiments, which yields comparable results.

3.2 The expression profile of PD-L1 and PD-L2 on draining lymph nodes' DCs after immunization of Ni^{low} mice with $NiCl_2/H_2O_2$.

PD-L1 and PD-L2 were described as the ligands of PD-1 and have been shown to negatively modulate immune responses (Nishimura et al., 2001). I therefore asked whether the expression of PD-L1 and PD-L2 on DCs in the draining axillary lymph nodes can be induced after immunization with $NiCl_2/H_2O_2$. Fig. 2 showed that DCs exhibited an increase in the expression of both PD-L1 and PD-L2 after immunization with $NiCl_2$ / H_2O_2 or H_2O_2 alone in comparison with $NiCl_2$ or NaCl. Whereas they displayed differential kinetics between PD-L1 and PD-L2: administration of $NiCl_2$ together with H_2O_2 stimulates the expression of PD-L2 with much faster kinetics than PD-L1, inducing significant up-regulation after 1 day. In contrast, $NiCl_2$ /H_2O_2 stimulation of PD-L1 expression peaked until day 3. Of note, it was observed that $NiCl_2/H_2O_2$ is the most potent inducer of PD-L1 and PD-L2 expressions on DCs. Mixture of Ni (II) Cl_2 and H_2O_2 was known to result in the formation of Ni (III) (Artik et al., 1999) and hydroxyl radical are chemically more reactive than precursor, Ni (II) Cl_2 and H_2O_2, respectively and , hence, should be more powerful elicitors of the danger signal. As with costimulatory molecules such as CD80 and CD86, here, danger signal is also the strong inducer of coinhibitory molecules PD-L1 and PD-L2 expressions by DCs in the draining lymph nodes.

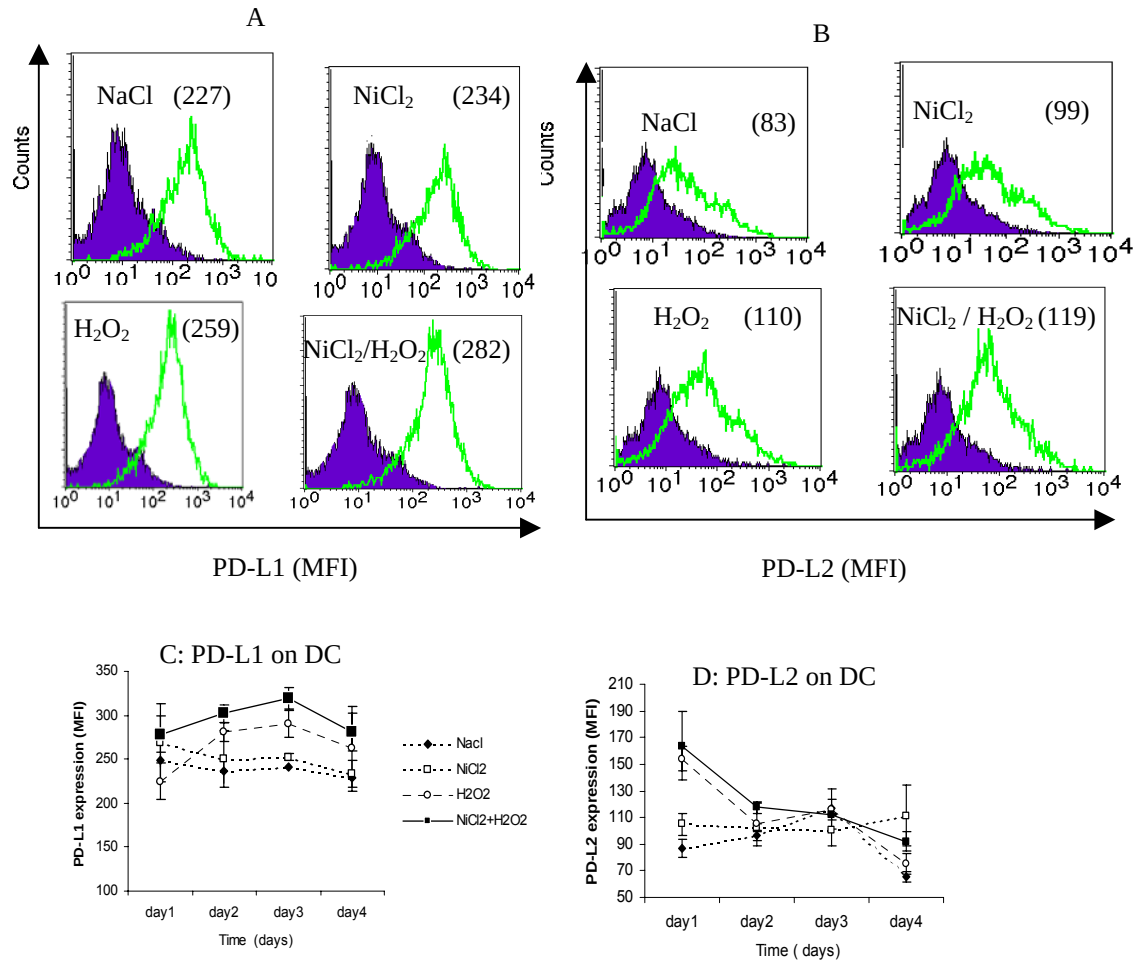


Fig. 2 PD-L1 and PD-L2 has different expression kinetics on DC after immunization with $NiCl_2$ alone, $NiCl_2/H_2O_2$, NaCl, H_2O_2, respectively.

Groups of three Ni^{low} mice were injected into both flanks with NaCl, $NiCl_2$ alone, $NiCl_2/H_2O_2$, or H_2O_2 alone, as indicted. After 1, 2, 3, 4 days, the draining axillary lymph nodes were harvested, and their cells were stained for surface MHC-II, CD11c, and PD-L1 or PD-L2. DCs were gated as $CD11c^{+}MHCII^{high}$ cells. The PD-L1 (as shown in A, day 3) and PD-L2 (as shown in B, day 2) expression of the gated DCs was plotted in histograms, respectively. The numbers shown in the the figues A and B represent MFI. The expression kinetics of PD-L1 and PD-L2 are shown in Figure C and Figure D, respectively. Results represent one of three experiments, which yields comparable results.

3.3 Draining lymph nodes' DCs from Ni^{high} mice display a tolerogenic phenotype after immunization with $NiCl_2/H_2O_2$.

The above described the kinetics of CD80/CD86 are displayed on the APC from Ni^{low} mice after immunization with $NiCl_2/H_2O_2$. The another question raised is whether there are different phenotype of APC between Ni^{high} and Ni^{low} mice. Indeed, phenotypic differences were detected among the APCs. Splenic DCs from Ni^{high} mice exhibited not only an increase in the expression of DEC-205, but they also displayed a profound decrease in CD40 (Roelofs-Haarhuis et al., 2003). These alterations point to an immature, potentially tolerogenic phenotype of the APC, albeit these cells did not show any alterations in the expression of CD28, CD80, CD86, and MHC-II (data not shown). However, it is not clear if DCs from draining lymph nodes display differential expression phenotype in terms of CD80, CD86, and MHC-II after immunization with $NiCl_2/H_2O_2$ because the expression of these molecules are inducible. To test this hypothesis, groups of nickel orally tolerized Ni^{high} and untreated control Ni^{low} mice were immunized with $NiCl_2$, $NiCl_2$ /H_2O_2, and DNFB, respectively. Thereafter, axillary draining lymph nodes were harvested and the expression of CD80 (on day 1), CD86 (on day 3) were determined after immunization. As shown in Fig. 3, the expression of CD80 and CD86 induced by $NiCl_2$ /H_2O_2 on draining lymph nodes' DCs from nickel orally tolerized Ni^{high} mice are significantly lower than that of control Ni^{low} mice, whereas immunization with $NiCl_2$ or DNFB did not evoke different expression levels of CD80, CD86 and MHC-II molecules between Ni^{high} and Ni^{low} mice. These results further confirmed that orally uptake of nickel can induce tolerogenic DCs, which may play a role at the nickel sensitization phase to prevent the indcution of nickel hypersensitivity.

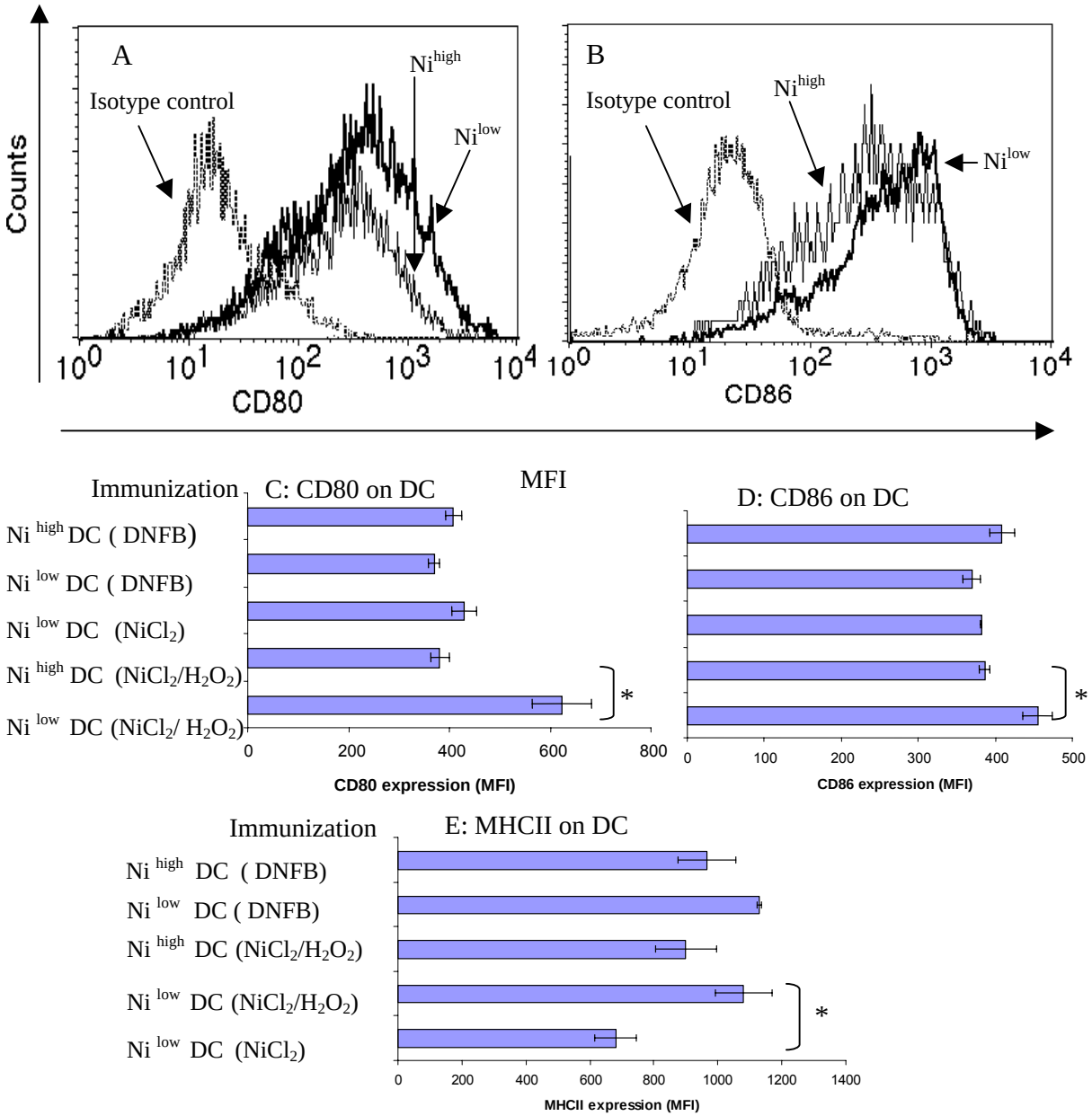


Fig. 3 Oral uptake of nickel induced tolerant phenotypic dendritic cells

C57BL/6 mice were orally tolerized by 10mM $NiCl_2$ drinking water (Ni^{high} mice) or were left untreated (Ni^{low} mice). Groups of three mice were injected into both flanks with $NiCl_2$ alone, $NiCl_2$ / H_2O_2, or were immunized by painting 0.5 % (w/v) DNFB on the shaved flanks as described in the Materials and Methods. After 1 day (for CD80 expression measurement), 3 days (for CD86 expression measurement), the draining axillary lymph nodes were prepared, and their cells were stained for surface MHCII, CD11c, and CD80 or CD86. DCs were gated as $CD11c^{+}$ $MHCII^{high}$ cells. The CD80, CD86 and MHCII (gated as $CD11c^{+}$ cells) expression was plotted in histograms, as shown in A and B. CD80, CD86 and MHCII expression levels (MFI, mean ± SD) were shown in Fig. C, D, E, respectively. The asterisk indicates a significant difference ($* \ p < 0.05$). Results represent one of three experiments, which yields comparable results.

3.4 Oral uptake of nickel upregulate PD-L1 expression on DCs from axillary lymph nodes after immunization with $NiCl_2/H_2O_2$

Because PD-1 ligands have been implicated in regulating effector T cells in the periphery (Nishimura et al., 2001), I decided to compare the expression of PD-L1 and PD-L2 on DCs in the draining lymph nodes between of Ni^{high} and Ni^{low} mice, upon immunization with $NiCl_2$, $NiCl_2/H_2O_2$, respectively. To do so, groups of Ni^{high} and Ni^{low} mice were injected with $NiCl_2$, $NiCl_2/H_2O_2$ on flanks, respectively. Thereafter, axillary lymph nodes were harvested at day1, day3 after injection, and the expression of PD-L1 and PD-L2 were determined by cytometric analysis. As shown in Fig. 4. In both Ni^{high} and Ni^{low} mice, $NiCl_2/H_2O_2$ induced higher level expressions of PD-L1 and PD-L2 than that of $NiCl_2$. In comparison of PD-L1 and PD-L2 expressions between Ni^{high} and Ni^{low} mcie, I found that PD-L1 expression is significantly increased in Ni^{high} mice compared with that of Ni^{low} mice after immunization with $NiCl_2$ / H_2O_2, whereas there is no difference of expression of PD-L2 after injection with $NiCl_2$ / H_2O_2. Upon immunization with $NiCl_2$ alone, there are no differences in terms of PD-L1 and PD-L2 expression levels by DCs, irrespective of Ni^{high} or Ni^{low} mice. These results suggest that, as with CD80 and CD86 expression, $NiCl_2$ combined with 'danger siganl' induced by H_2O_2 up-regulate the expression of PD-1 ligands, whereas $NiCl_2$ alone has small effects.

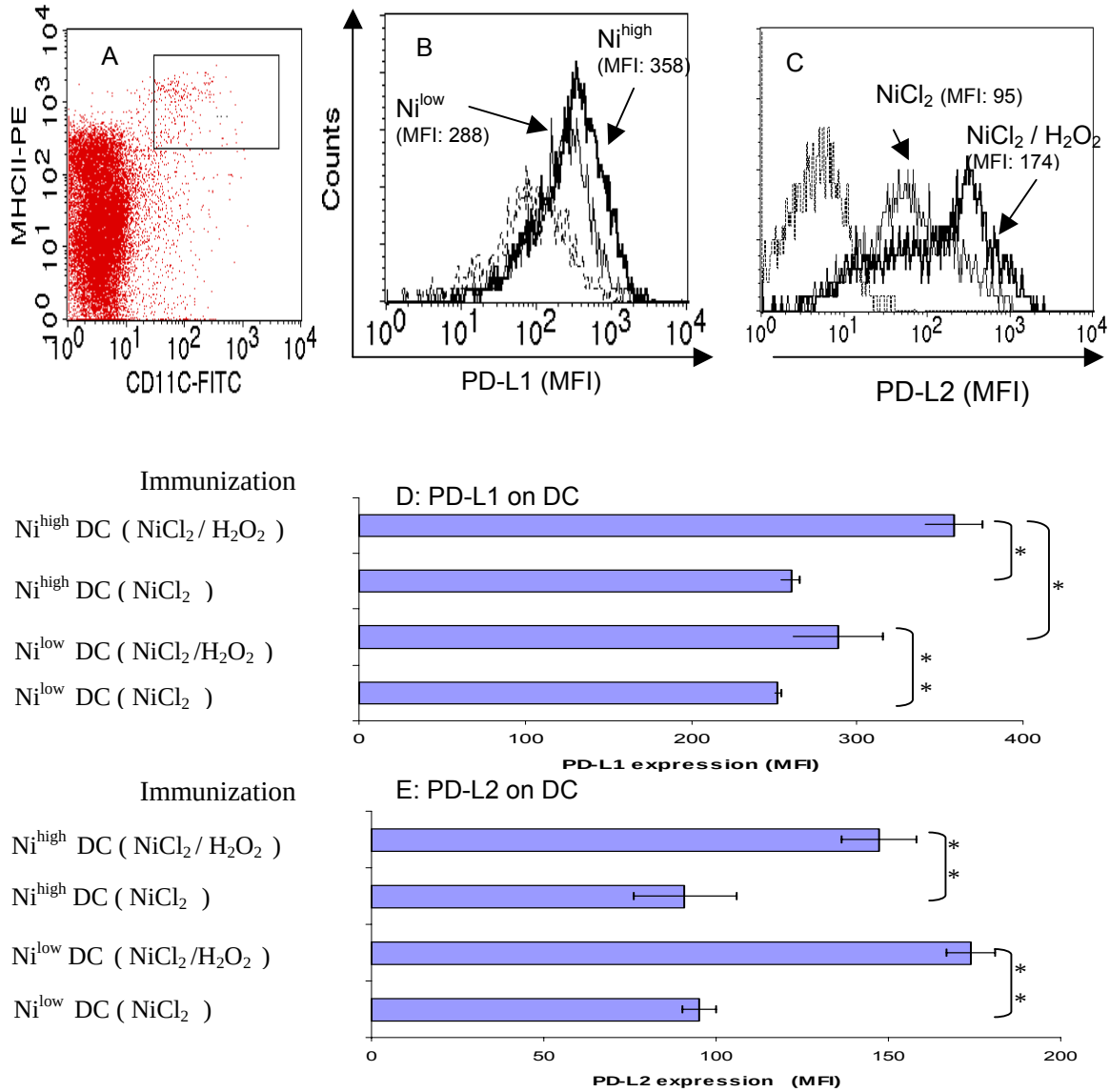


Fig. 4. Comparison of PD-L1 and PD-L2 expression by DCs between Ni^{low} mice and Ni^{high} mice after immunization with $NiCl_2$, $NiCl_2$ / H_2O_2.

Oral uptake of nickel can up-regulate the expression of PD-L1 by DCs in axillary lymph nodes after immunization with $NiCl_2/H_2O_2$. Group of three mice were injected into both flanks with $NiCl_2$, $NiCl_2/H_2O_2$, respectively. The draining axillary lymph nodes were prepared on day 1 (for PD-L1 expression measurement), day 3 (for PD-L2 expression measurement) after immunization, and their cells were stained for surface MHCII, CD11c, and PD-L1 or PD-L2. DCs were gated as $CD11c^+$ $MHCII^{high}$ cells, as shown in A. The PD-L1, PD-L2 expression was plotted in histograms, as shown in B, C. PD-L1 and PD-L2 expression levels were shown in bar graphs D, E, respectively. The asterik indicates a significant difference ($* p< 0.05$; $** < 0.01$). Results represent one of three experiments, which yields comparable results.

3.5 The modulatory effects of transferred T cells from Ni^{high} mice on the expression of CD80 / CD86 by DCs in Ni^{low} recipients.

Prof. Gleichmann's group has demonstrated that bulk T cells from the spleens of nickel-tolerant donors (Ni^{high} mice) were able to adoptively transfer the tolerance to Ni^{low} syngeneic recipients (Artik et al., 2001). Consequently, I hypothesized that one of mechanisms by which transferred Ni^{high} T cells to prevent Ni^{low} recipients to be sensitized by nickel is through modulation of costimulatory molecules expressions on APC. To test this hypothesis, 10^6 T cells from nickel orally tolerized Ni^{high} mice were transferred to the syngeneic Ni^{low} recipient mice. One day later, mice were immunized with either $NiCl_2$ or $NiCl_2$/ H_2O_2, respectively. Thereafter, axillary draining lymph nodes were harvested as described above, and costimulatory molecules expressions on DCs were determined by flow cytometric analysis. As represented in Fig. 5. induction of CD86 expression on DCs by immunization with $NiCl_2$ /H_2O_2 was significantly reduced after transfer of Ni^{high}T cells in comparison with Ni^{low} T cells transfer, whereas, the induction of CD80 expression between transfer of Ni^{high} T cells and Ni^{low} T cells showed some differences but not significantly. In contrast, the induction of expression of PD-L1 by immunization with $NiCl_2$/H_2O_2 did not show any alteration between Ni^{high} T cells transfer and Ni^{low} T cells transfer (data not shown). These results indicated that the Ni^{high} T cells transferred can partially block the up-regulation of costimulatory molecules after immunization of the Ni^{low} recipient mice with $NiCl_2$/ H_2O_2.

Fig. 5 A, B, C

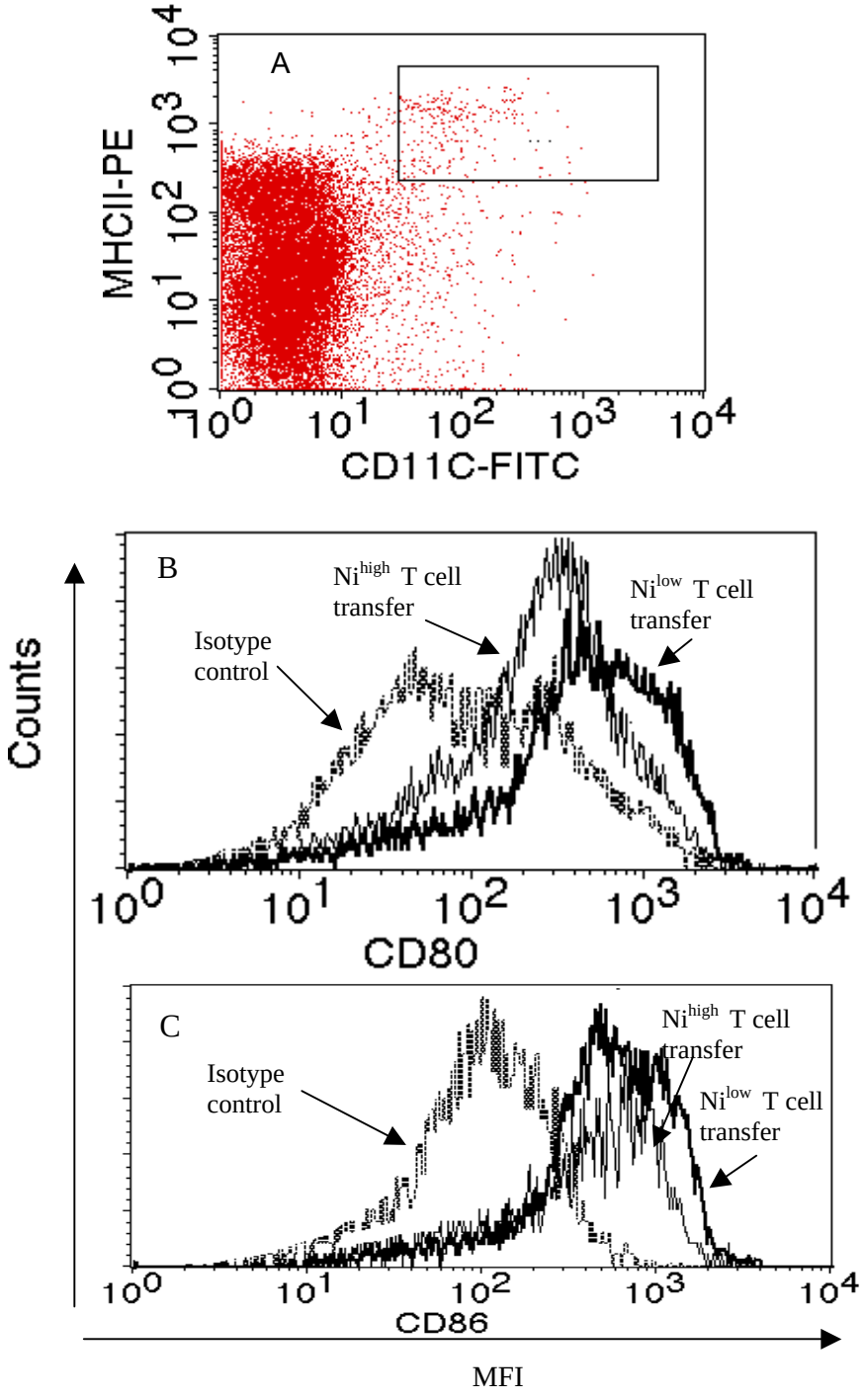

A
MHCII-PE
CD11C-FITC
B
Ni^high T cell transfer
Ni^low T cell transfer
Isotype control
Counts
CD80
C
Ni^high T cell transfer
Isotype control
Ni^low T cell transfer
CD86
MFI

Fig.5 bar graph

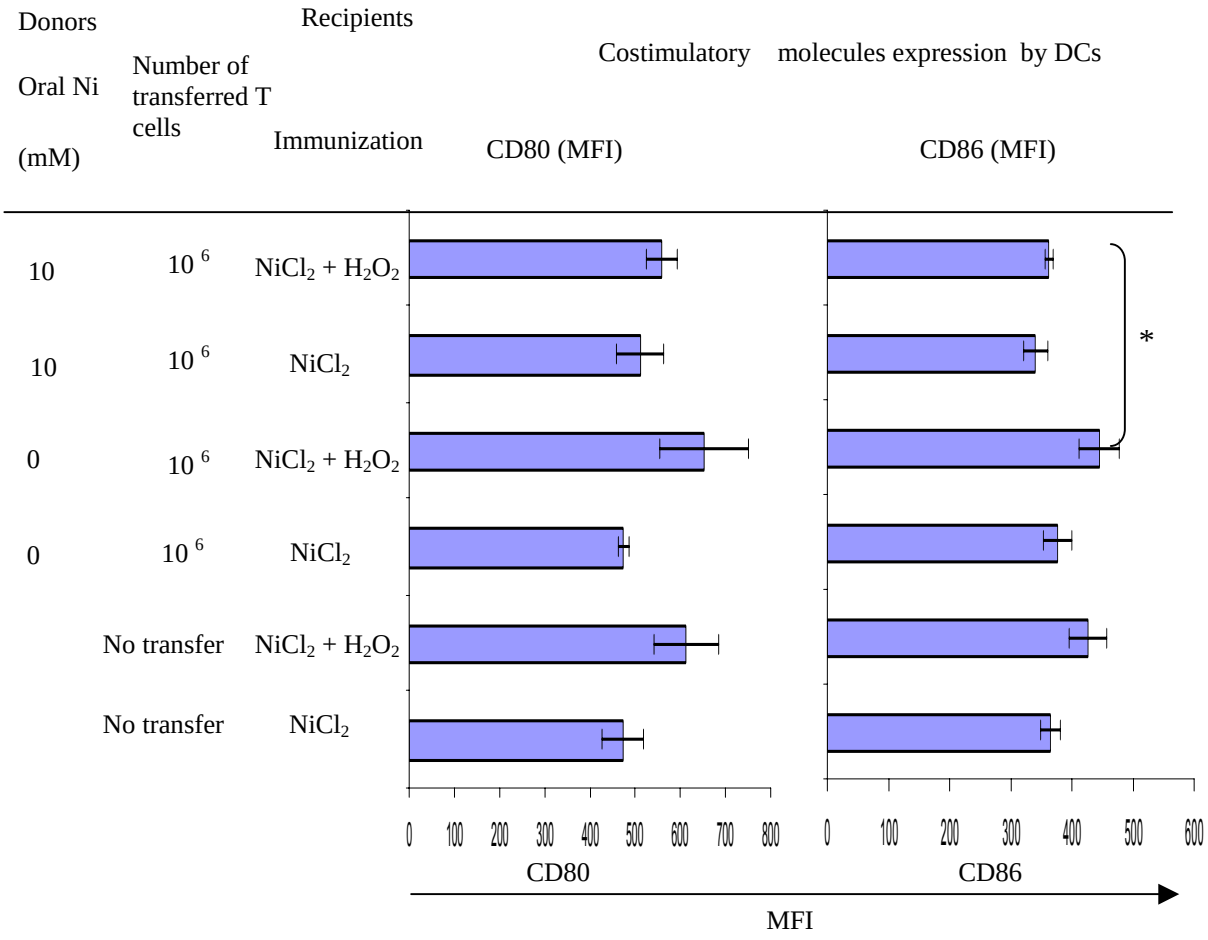


Fig. 5 Modulatory effect of transferred T cells from Ni^{high} mice on the expression of CD80, CD86 by DC in Ni^{low} recipients.

Adoptive transfer of T cells from Ni^{high} donors can partially block the up-regulation of expression of CD80 and CD86 by DCs in Ni^{low} recipients after immunization with $NiCl_2$ /H_2O_2. Prospective C57BL/6 donor mice were Ni^{high} or Ni^{low} mice. 10^6 T cells from Ni^{high} or Ni^{low} donors were injected i.v. into syngeneic Ni^{low} recipients. Groups of three recipient mice were injected into both flanks with $NiCl_2$ alone, $NiCl_2$/H_2O_2. After 1 day (for CD80 expression measurement), 3 days (for CD86 expression measurement), the draining axillary lymph nodes were prepared, and their cells were stained for surface MHCII, CD11c, and CD80, CD86 and PD-L1. DCs were gated as CD11c+ $MHCII^{high}$ cells (A). The CD80, CD86 expression was plotted in histograms (B, C). The bar graphs represent the expression of CD80, CD86, respectively. The asterisk indicates a significant difference ($* \; p < 0{,}05$). Results represent one of three experiments, which yields comparable results.

3.6 The modulation of CTLA-4 expression by CD4+ T cells by oral uptake of nickel

Engagement of CTLA-4 with B7 play a crucrial role for down-regulation of T cell response. It has been shown that CTLA-4 is required for the induction of high dose oral tolerance (Samoilova et al., 1998). Therefore, I investigated whether oral uptake of nickel alter the expression of CTLA-4 on CD4+ T subsets . To this end, mice were treated with 10mM $NiCl_2$ in drinking water for 4 weeks, thereafter, mice were sacrificed, and spleen and draining lymph nodes such as mesenteric lymph nodes (MLN) and axillary lymph nodes (ALN) were harvested for determination of the expression of CTLA-4 expression on CD4+ T cells. As shown in Fig. 6, the intracellular expression of CTLA-4 on CD4+ T cells (as shown in A) in the mesenteric lymph nodes of Ni^{high} mice was marginally increased compared with Ni^{low} control mice (Fig.6 B), it remained unchanged on the spleen and ALN CD4+ T cells derived from the same animal (data not shown). Whereas the difference of expression of CTLA-4 on CD4+ T cells in axillary lymph nodes between Ni^{high} mice and Ni^{low} mice was increased after immunization with $NiCl_2$ /H_2O_2 on the flanks (Fig. 6 C). Furthermore, to test whether transfer of APCs from the Ni^{high} mice to the Ni^{low} mice can augment the CTLA-4 expression by CD4+ T cells in the recipients, 10^6 APC from Ni^{high} mice were transferred into the Ni^{low} recipients. One day after transfer, the recipients were immunization with $NiCl_2$ /H_2O_2 on the flanks. The expression of CTLA-4 by CD4+ T cells in ALN in the recipients that received APCs from Ni^{high} mice were marginally increased compared with recipients received the APCs from Ni^{low} donor mice (Fig.6 D). These results revealed that CTLA-4 may play a role in oral immune tolerance to nickel.

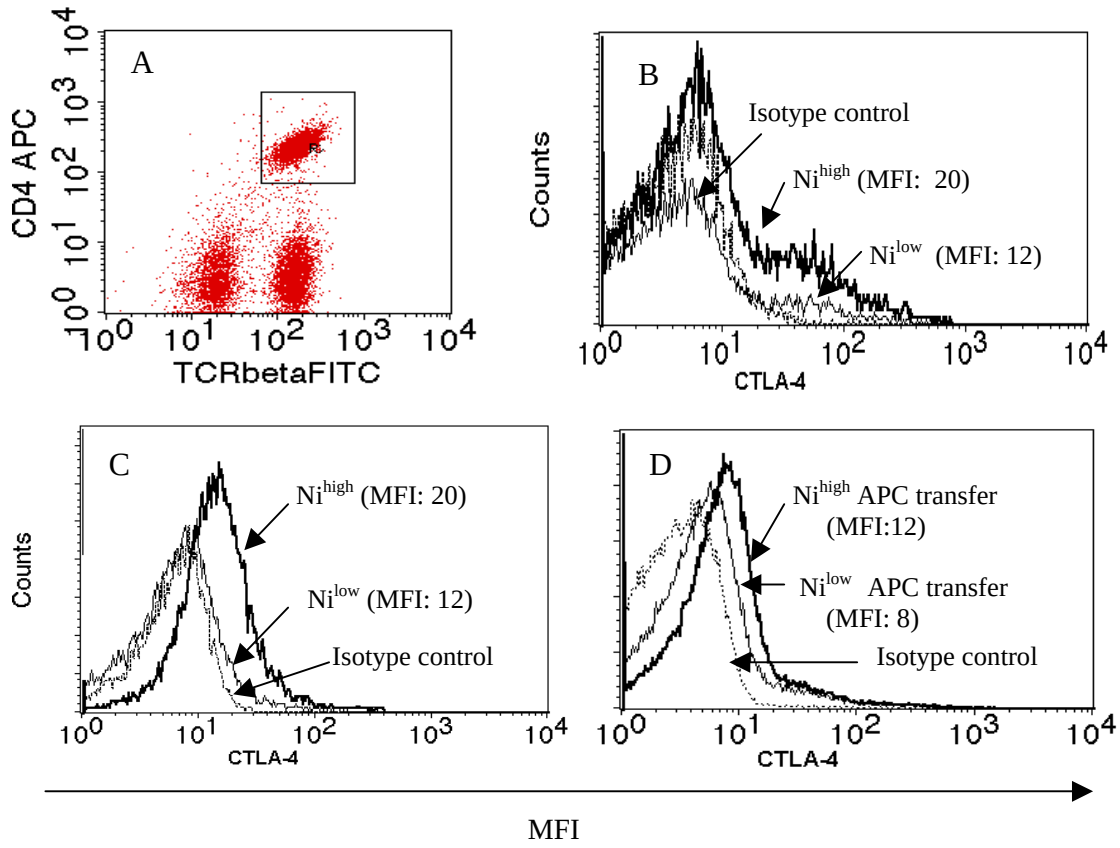
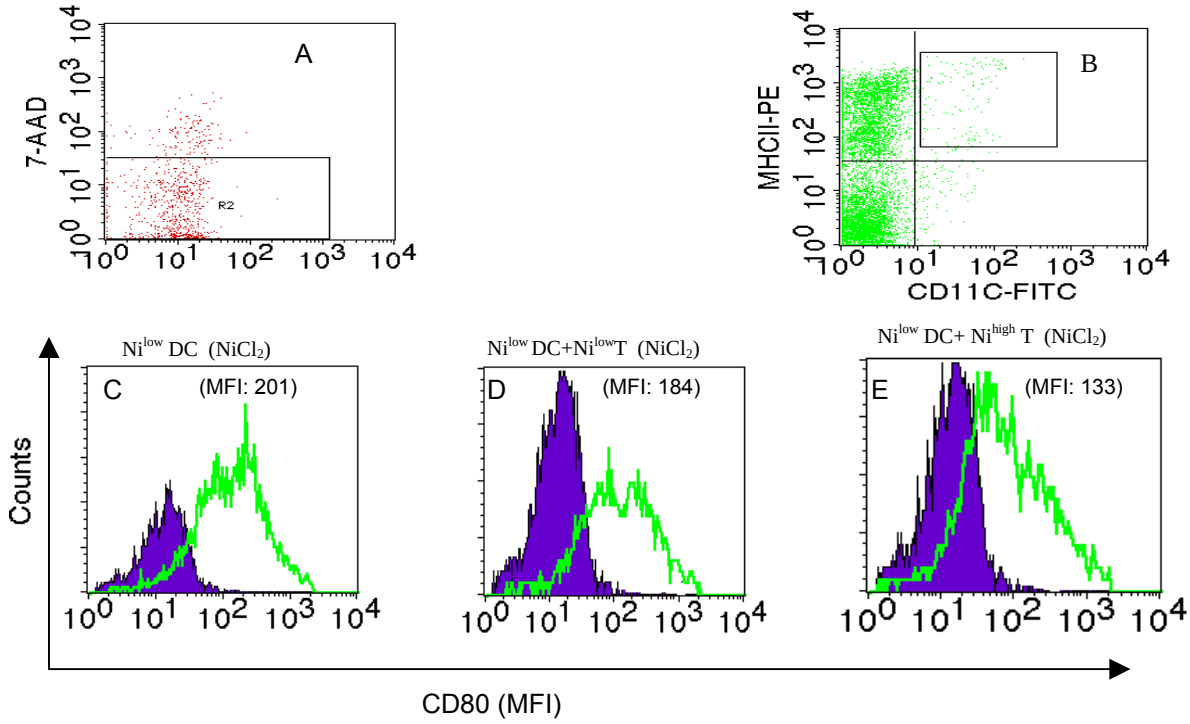
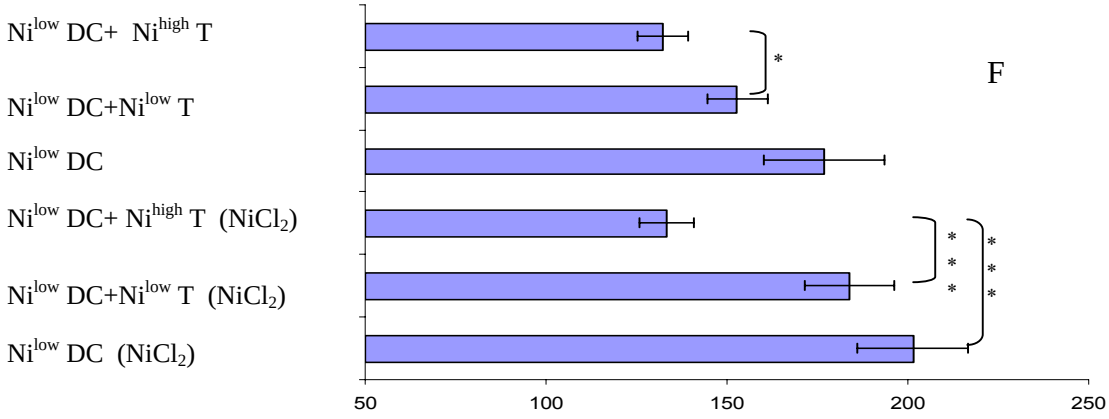


Fig. 6. Oral uptake nickel up-regulate the expression of CTLA-4 expression on T cells Lymph nodes (mesenteric lymph nodes or axillary lymph nodes) cells from Ni^{high} mice (bold line) and Ni^{low} mice (thin line) were analyzed for their expression of CTLA-4 on $CD4^+$ T cells. CD4+ T cells were gated by anti-CD4-APC and anti- TCRbeta-FITC staining, as shown in A. Expression by $CD4^+$ T cells of CTLA-4 was analyzed by intracellular staining. Dotted line represent stainings with isotype-matched control mAb. Histogram (B) shows the CTLA-4 expression on $CD4^+$ T cells of mesenteric lymph nodes between Ni^{high} mice and Nil^{ow} mice. Both Ni^{high} and Ni^{low} mice were injected with $NiCl_2$ / H_2O_2 on flanks, 3 days After immunization, axillary lymph nodes were prepared, cells were stained for CTLA-4 expression, as shown in C, oral uptake nickel can marginal up-regulate the expression of CTLA-4 expression. Further, $1x10^6$ APCs from Ni^{high} or Ni^{low} mice were injected i.v. into syngeneic Ni^{low} recipients, respectively. The latter were injected with $NiCl_2$ /H_2O_2 on flanks within 24 h after transfer. The axillary lymph nodes were harvested 3 days after injection with $NiCl_2$ /H_2O_2, cells were analysized for CTLA-4 expression on $CD4^+$ T cells. The results shown in D revealed that transfer of APC from Ni^{high} mice into Ni^{low} recipients can increase the CTLA-4 expression by $CD4^+$ in Ni^{low} recipients. Results represent one of three experiments, which yields comparable results.

3.7. T cells from Ni^{high} mice can block the up-regulation of costimulatory molecules expression by DCs from spleen of Ni^{low} mice in vitro cell coculture

Prof. Gleichmann's group previously has shown that the tolerance of the Ni^{high} mice can be adoptively transferred to non-sensitized Ni^{low} recipients by means of T cells obtained from the Ni^{high} donors; thereafter, the recipients can no longer be sensitized to nickel by injection of $NiCl_2$/ H_2O_2 (Artik et al., 2001). Next, I started to inquire into the possible mechanisms by which the transfer of Treg cells from the Ni^{high} mice prevents sensitization of the recipients. Therefore, I investigated the regulation of costimulatory molecules on DCs by Treg cells. As shown in Fig. 7, splenic APCs (which includes DCs) from naive Ni^{low} donors served as indicator cells throughout (they are termed 'Ni^{low} DC' in Fig.7). They were cocultured in vitro with T cells from Ni^{high} mice (termed ' Ni^{high} T') and Ni^{low} mice (termed ' Ni^{low} T'), respectively. While the most profound down-regulation in the expression of costimulatory molecules was observed when the Ni^{low} DCs were cocultured with T cells from the Ni^{high} mice, T cells from the Ni^{low} mice also exerted some suppression. The suppressive effects were somewhat stronger in the presence of $NiCl_2$ (indicated in brackets) than in its absence and were more distinct with CD80 than CD86.


A
7-AAD
R2
B
MHCII-PE
CD11C-FITC
C
(MFI: 201)
D
(MFI: 184)
E
(MFI: 133)
Counts
CD80 (MFI)


F
CD80 expression (MFI)

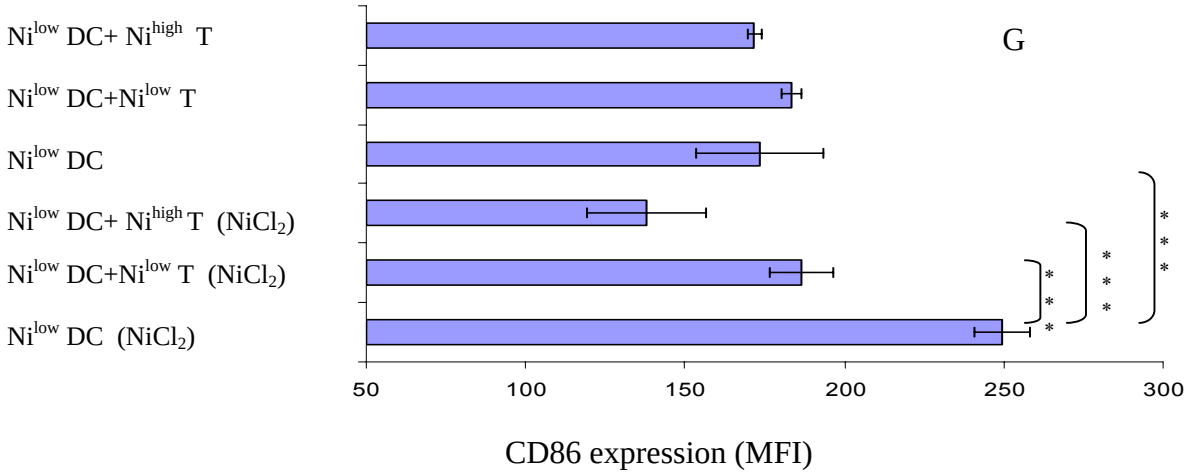

G
CD86 expression (MFI)

Fig. 7. T cells from Ni^{high} mice (Ni^{high} T cells here) prevent upregulation of the costimulatory molecules CD80 and CD86 by dendritic cells (DCs). C57BL/6 mice were orally tolerized by 10mM $NiCl_2$ drinking water (Ni^{high} mice) or were left untreated (Ni^{low} mice). Splenic T cells and APCs (T cell-depleted spleen cells) were obtained through MACS isolation. Thereafter, cocultures of both cell types were performed (in 24-well cell culture plates in 2 ml RPMI-1640 complete medium) as follows: Ni^{low} APCs ($2x10^6$) from Ni^{low} mice with or without 75 μM $NiCl_2$; Ni^{low} APCs ($1x10^6$) plus Ni^{low} T cells ($2x\ 10^6$) with or without 75 μM $NiCl_2$, or Ni^{low} APCs ($1x10^6$) plus Ni^{high} T ($2x10^6$) with or without 75 μM $NiCl_2$. Quadruplet determinations of each group were performed. Cells were harvested after overnight coculture in 5% CO_2 at 37 °C. Expression of CD80 and CD86 by DCs were evaluated by flow cytometer: Dead cells were excluded by staining with 7-amino actinomycin D(A), DCs were gated by anti-CD11c-FITC and anti-MHCII-PE staining(B). CD80 and CD86 expression levels are shown by mean flurescence intersity (MFI). C.D. E. represents the expression of CD80 by DCs alone ($NiCl_2$), DCs+ Ni^{low} T ($NiCl_2$) and DCs+Ni^{high}T ($NiCl_2$), respectively. The results shown in bar graph F (CD80 expression) and G (CD86 expression) represent one of three experiments, which yields comparable results. The asterisks indicate a significant difference ($* p < 0.05$, $** p< 0.01$, and $***$, $p < 0.001$).

3.8 Blockade of IDO can abolish the spread of immune tolerance of nickel from Ni^{high} T cells to Ni^{low} APCs

To investigate the role of IDO in the infectious tolerance of nickel in mouse model, serial adoptive cell transfer were performed as described previuosly (Roelofs-Haarhuis et al. 2003). These involved Ni^{high} mice as primary cell donors, a first set of recipient mice that in turn became the secondary cell donors, and, finally, a second set of recipient mice that were assayed for tolerance induction after immunization with $NiCl_2$ / H_2O_2 and rechallenge at the ears. To be able to distinguish the cells from the primary donors from those of the recipients, congenic C57BL/6 mice, which express Ly5.1 on the surface of all lympho-hematopoietic cells, were used as the primary donors, instead of the Ly5.2+ wild-type mice. To block the activity of IDO in the first recipient, slow-release pellets containing 1-MT, a inhibitor of IDO, or placebo pellets were implanted under the dorsal skin of the first recipient mice 1 day before T cell adoptive transfer. As shown in Fig.7. consistent with our previous study, Ly 5.1+ primary donor T cells can render the APC of the first recipient to be tolerogenic after immunization with $NiCl_2$/ H_2O_2, as evidenced that the tolerance can be transferred from the first recipients to the second recipients (Fig.7, bar 2). In contrast, when the IDO acitivity of the first recipient was abolished by 1-MT, the tolerance failed to spread from donor T cells to host APCs (Fig. 7, bar 6), whereas, the Ni^{high} donor T cells still can spread the tolerance to the APCs of the first recipient implanted with placebo (Fig. 7, bar 4). These results indicated that IDO activity is required for the spread of nickel tolerance from Ni^{high} T cells to Ni^{low} APCs.

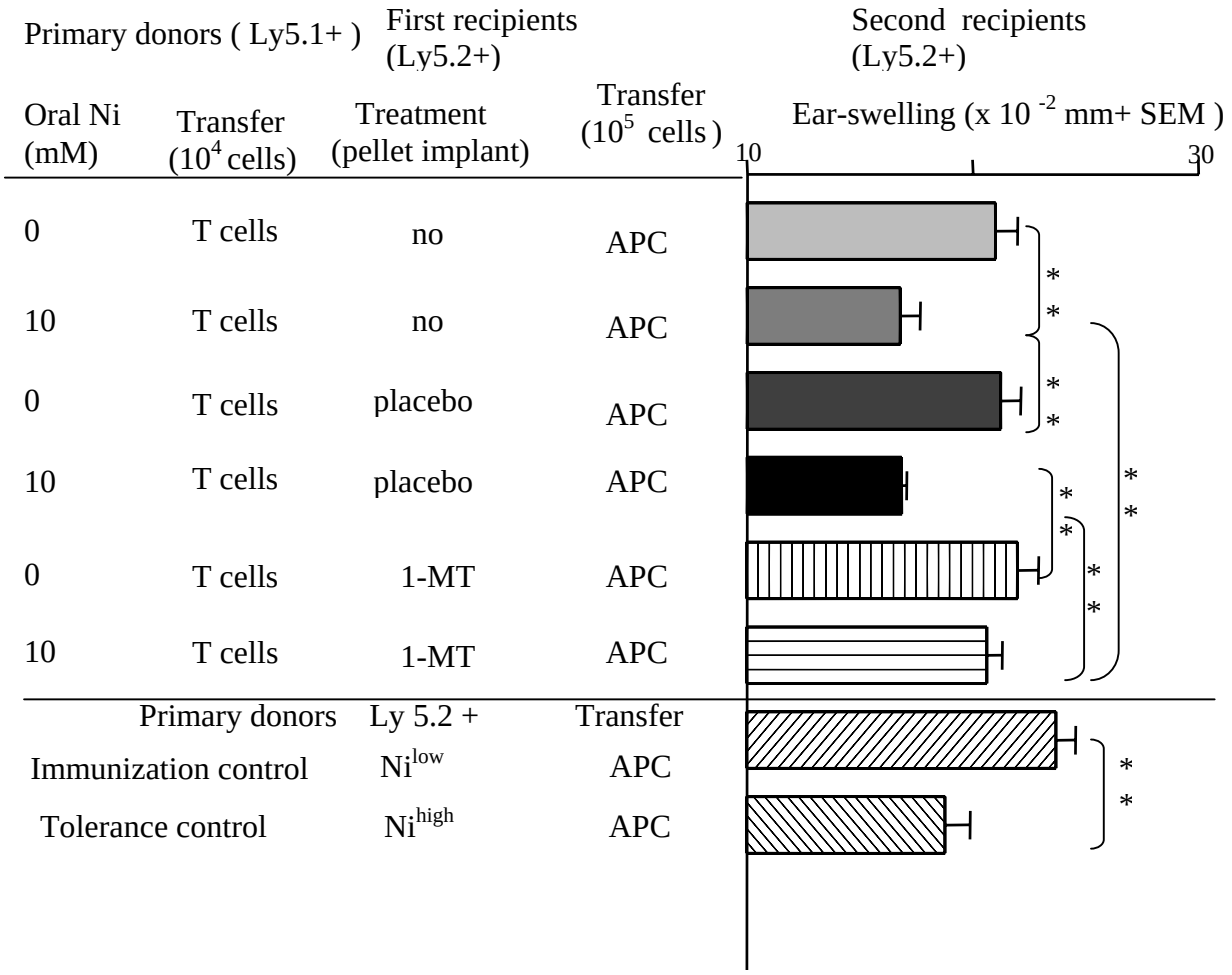


Fig. 8. IDO activity is required for the infectious spread of tolerance from donor T cells to host APCs. Prospective Ly5.1+ donor mice were orally tolerized to nickel or were left untreated, as indicated. The first recipient mice were implanted of IDO-inhibitor (1-MT) or placebo pellet into the skin, while some were left untreated as control. Thereafter, T cells of the primary Ly5.1+ donors were transferred (10^4 cells per recipient) to a first set of Ly5.2+ recipients. Within 24 h after transfer, the first recipients were injected with $NiCl_2$ / H_2O_2. On day 11, APCs of the first recipients were isolated and depleted of donor Ly5.1+ cells, before they were transferred (10^5 cells per recipient) into a second set of recipients. As control groups for tolerance and allergy (bottom), 10^4 isolated T cells from Ni^{high} or Ni^{low} Ly5.2+ donors were transferred into groups of Ly5.2+ recipients, as indicated. All second recipients were then immunized with $NiCl_2$/ H_2O_2 and rechallenged, and the ear-swelling responses were determined. Data shown represent the mean ear-swelling response+ SEM from groups of five mice each. Results represent one of two experiments, which yielded comparable results.

4. Discussion

4.1 Costimulatory molecules and nickel sensitization

In the present study, I first focused on the characterization of the expression kinetics of B7 family costimulatory molecules (CD80/CD86) and coinhibitory molecules (PD-L1/PD-L2) by APCs after immunization with NaCl, $NiCl_2$, H_2O_2 , $NiCl_2/H_2O_2$, respectively. The data presented here clearly show that the potency of induction of expression of B7 family molecules by these stimulators are quite different. Injection of $NiCl_2$ into the skin of conventionally-reared mice (Ni^{low} mice) induce low level of expression of CD80/CD86 by DCs and B cells of the draining axillary lymph nodes. In contrast, the administration of either H_2O_2 or $NiCl_2$ together with H_2O_2 can induce the high levels expression of CD80/CD86. In the sensitization phase, the presentation of nickel-induced neoantigen on antigen-presenting cells (APCs), together with up-regulation of costimulatory molecules on these cells, induces the differention of naive T cells into nickel-specific effector and memory T cells. Previously, It was reported that immunization with $NiCl_2$ failed to sensitize the Ni^{low} mice, whereas, the sensitization was achieved by injecting $NiCl_2$ in combination with either H_2O_2 or other adjuvant such as CFA, IL-12(Artik et al., 1999). Hence, it was proposed that $NiCl_2$ would be unable to up-regulation the expression of costimulatory molecules in Ni^{low} mice, whereas administration of $NiCl_2$ together with H_2O_2 would induce both signal 1 and signal 2. Here, the validity of this hypothesis has been confirmed. Clinical observations indicate that nickel allergy preferentially develops after nickel contact with an inflamed or irriated skin, whereas sensitization is unlikely to occur with the intact skin. Inflammation and skin irritation can be termed ' danger', which leads to up-regulation of costimulatory molecules and, hence, favors immune reactivity rather than immune tolerance (Artik et al., 1999). In our mouse model, conventionally-reared Ni^{low} mice are sensitized to nickel by injecting together with H_2O_2 (Artik et al., 1999). It is thought the combination of $NiCl_2$ and H_2O_2 by a Fenton-like reaction leads to the production of Ni^{3+}, the latter, however, is immediately reduced to Ni^{2+} again after contact with protein. Hence, it is the Ni^{2+} ions that ultimately form the nickel-induced neoantigens. H_2O_2 can be considered an endogenous adjuvant, since it is locally produced in large amounts during inflammation, to upregulate costimulatory molecules, such as CD80 and CD86, on APCs. In contrast, when $NiCl_2$ alone, i.e., without adjuvant, is administered to the Ni^{low} mice, it fails to sensitize them. These findings confirm the above-mentioned clinical experience in humans that nickel ions sensitize when exposed to the inflamed or irritated, but not the intact skin.

PD-L1 and PD-L2 are ligands for PD-1, a costimulatory molecule that plays an inhibitory role in regulating T cells activation in the periphery. Here, I found that administration of nickel in combination with H_2O_2 can dramatically augment the expression of PD-L1 and PD-L2 on DCs. It has been demonstrated that PD-L1 and PD-L2 expression is induced on monocytes and dendritic cells upon treatment with IFN-γ or IFN-γ/LPS, and transcripts for both ligands are detectable on lymphoid and nonlymphoid tissue (Loke et al., 2003). Whereas PD-L1 is easily inducible on a number of different cell types, PD-L2 is much more restricted DCs and can be highly up-regulated by IL-4 only on inflammatory macrophages. Furthermore, PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells (Loke et al., 2003). This suggests that PD-L1 might play a more general role in down-regulating activated T cells in the periphery as opposed to a more specific role for PD-L2. With regard to the possible regulatory role played by PD-L1 and PD-L2 in the sensitization phase to nickel, it was postulated that the augmentation of expression of PD-L1 and PD-L2 evoked by $NiCl_2$ together with H_2O_2 is one of negative feedback regulatory mechanisms by which preventing the host from sensitization to nickel. This is supported by a recent study (Tsushima et al., 2003) performed to examine the roles of PD-1 and its ligands, PD-L1 and PD-L2 in mouse hapten-induced contact hypersensitivity (CH). Administration of anti-PD-1 mAb at sensitization significantly enhanced and prolonged ear swelling. Treatment with anti-PD-L1 mAb, but not anti-PD-L2 mAb, also enhanced CH reactions.

4.2 Costimulatory molecules and nickel tolerance

It has been previously showed that oral up-take of nickel induced an increase in the expression of Dec-205 and a profound decrease in CD40 on splenic DCs from Ni^{high} mice (Roelofs-Haarhuis et al., 2003). However, upon immunization with $NiCl_2/H_2O_2$, i.e., at the nickel sensitization phase, the alterations of expressions of B7 family costimulatory molecules in Ni^{high} mice in comparison with Ni^{low} mice were not established. Here, I found that oral uptake 10 mM for 4 weeks also resulted in the impairment of up-regulation of CD80/CD86 induced by immunization with $NiCl_2$/ H_2O_2, whereas increased the expression of PD-L1 by the Ni^{high} DCs upon immunization. Hence, combined with previous work, several lines evidence now demonstrated oral uptake of nickel induce the production of tolerogenic APC, as evidenced by both phenotype analysis and funtional test studies: Firstly, compared with splenocytes of untreated control Ni^{low} mice, the APCs from Ni^{high} mice orally tolerized to nickel exhibited an increase in Dec 205 expression by DCs, an increase in CD38 expression on B cells, and a striking decrease in the expression of CD40 on DCs and in particular on B cells. Secondly, injection of $NiCl_2$/ H_2O_2 into orally tolerized Ni^{high} mice failed to up-

regulation of CD80/CD86 on the DCs of in the draining lymph nodes, whereas injection induce coinhibitory signal PD-L1/PD-1 on DCs. Thirdly, This tolerogenic phenotype of DCs and and B cells in the spleens of orally tolerized Ni^{high} mice conforms not only with the remarkable efficiency of their APCs to adoptively transfer the tolerance, but also with their reduced allostimulatory capacity. Furthermore, through serial adoptive transfers with Ly5.1+ donors and two successive sets of Ly5.2+ recipients, It has been demonstrated that nickel tolerance was infectiously spread from donor to host cells. After the transfer of either T cells or APCs from orally tolerized Ni^{high} donors, the spread of tolerance to the opposite cell type of the recipients (i.e., APCs and T cells, respectively) required recipient immunization with $NiCl_2/H_2O_2$. In other words, both signal 1 and signal 2 were required for the infectious spread of tolerance from donor T cells to host APCs and vice versa. Although signal 1 can be clearly defined as nickel-induced neoantigens, the relevant molecules induced by the danger signal remain unresolved. whether this is indeed CD80/CD86 or some other costimulatory or coinhibitory molecule (s) such as PD-L1/PD-L2 requires further investigation.

The costimulatory molecules B7-1 (CD80) and B7-2 (CD86) can both up- and down-regulate the functions of T cells. Interaction of B7 with CD28 delivers positive signals that are required for T cell activation, whereas interaction of B7 with CTLA-4 may deliver negative signals that are crucial for immune down-regulation (Lee et al., 1998; Salomon et al., 2001). It has been reported that CTLA-4 is required for the induction of high dose oral tolerance (Samoilova et al., 1998). Consistently, in our nickel tolerant Ni^{high} mouse model, I found that the expression of CTLA-4 on CD4+ T cells of mesenteric lymph nodes (MLN) marginally increased after 4 weeks of oral uptake of nickel in comparison with Ni^{low} mice, and immunization with $NiCl_2/ H_2O_2$ further enhanced the difference of CTLA-4 expression on CD4+ T cells of axillary lymph nodes (ALN) between Ni^{high} mice and Ni^{low}. It was known that CD28 is constitutively expressed on the T cell surface, whereas CTLA-4 is up-regulated after T cell activation and reaches a maximum after 2-3 days (Egen et al., 2002). In several murine oral immune tolerance models, it has been shown that T cell pre-activation does occur during the inductive phase of oral tolerance (Samoilova et al., 1998; Iriani et al., 2004). It is plausible that this activation may be sufficient to up-regulate CTLA-4 expression but inadequate to provoke a complete immune response (Samoilova et al., 1998). Hence, CTLA-4 is likely involved in the induction of oral immune tolerance to nickel, and plays a role in limiting the nickel-specific T cells proliferation.

A large number of recent studies have focused on self-tolerance as maintained by CD25+CD4+ regulatory T cells (Treg). Treg cells express OX40, 4-1BB, CTLA-4 and GITR, as well as the Foxp3 transcription factor (Sakaguchi, 2000; McHugh et al., 2002; Hori

et al., 2003). It has been shown that CD4+CD25+ Treg also plays a role in oral immune tolerance (Zhang et al., 2001; Bertrand et al., 2003). Taking into account CD4+CD25+ Treg constitutively expressing CTLA-4, as well as the increased expression of CTLA-4 on recipient's CD4+ T cells of ALN after adoptive transfer of APCs from Ni^{high} mice to Ni^{low} recipient mice, I speculated that the donor tolerogenic APC can induce the production of CD4+CD25+ nickel-specific Treg in the recipients. In this respective, the investigation on the precise role played by CD4+CD25+ Treg in oral immune tolerance to nickel is ongoing.

4.3 Regulation of costimulatory molecules expression on APCs by nickel- specific T regulatory (Treg) cells

Once the specific Treg cells were induced through oral uptake nickel, activation of these Treg cells by injection of $NiCl_2$ and H_2O_2 might have promoted them to inhibit the up-regulation of costimulatory signals or induce coinhibitory signals (Roelofs-Haarhuis et al., 2003). Consistent with this, in the present study, it was found that injection of $NiCl_2/H_2O_2$ into both flanks of Ni^{high} mice failed to up-regulate CD80/CD86 expression on the DCs in the draining axillary lymph nodes. Hence, I proposed that tolerant T cells from Ni^{high} mice can impair the up-regulation of costimulatory molecules on DCs. This idea was supported by both the in vivo T cells adoptive transfer experiment and in vitro cell coculture experiment. Transfer to Ni^{low} recipients of 10^6 T cells from Ni^{high} mice, but not from the Ni^{low} mice, can prevent the up-regulation of costimulatory molecules, e.g. CD86, on the DCs that is seen normally when Ni^{low} mice are immunized with $NiCl_2$ and H_2O_2. Further, when T cells from Ni^{high} mice cocultured with spleen DCs from Ni^{low} mice in vitro, the up-regulation of costimulatory molecules of DCs can be blocked by the T cells. This capacity of the Treg cells from Ni^{high} mice to prevent costimulation could account for the failing nickel sensitization in both the Ni^{high} mice themselves and the recipients of their Treg cells (Roelofs-Haarhuis et al., 2003). Recently, there are several investigators have demonstrated that Treg such as CD4+CD25+ T cells, CD8+CD28- T cells, and anergic T cells can suppress the T cells immune response through down-regulation of costimulatory molecules on APC (Cederbom et al., 2000; Chang et al., 2002; Li et al., 1999; Najafian et al., 2003; Vendetti et al., 2000). In a murine experimental autoimmune encephalomyelitis (EAE) model, it was reported that CD8+CD28- T cells play a major role to prevent EAE development, the mechamisms of disease regulation appear to be related to inhibition of expansion of encephalitogenic CD4+ Th1 cells. The regulatory CD8+CD28- T cells prevent the upregulation of costimulatory molecules on APCs, thus prohibiting efficient costimulation of CD4+ T cells (Najafian et al., 2003). It has also been demonstrated that murine CD4+CD25+ T cells down-regulated the expression of the costimulatory molecules CD80 and CD86 on dendritic cells (Cederbom et

al., 2000). More recently, it was reported that human CD4+CD25+ T cells render the monocyte-derived DC inefficient as APCs despite prestimulation with CD40 ligand. After cocultured with CD4+CD25+ T cells, there was an increased IL-10 secretion and reduced expression of costimulatory molecules in DC. Hence, in addition to direct suppressor effect on CD4+ T cells, regulatory T cells may modulate the immune response through DC (Misra et al., 2004). With regard to the mechanisms by which Treg downregulate the costimulatory molecules expression, it has been proposed that activation of NF-κB family was shown to correlate with DC maturation in both mice and human (Neumann et al., 2000; Yoshimura et al., 2001; Kendra et al., 2003). NF-κB is a dimer composed of virtually any of the 5 mammalian Rel proteins (p65/RelA, c-Rel, RelB, p50/NF-κB1, and p52/NF-κB2), which was structurally characterized by the Rel homology domain (Baeuerle et al., 1994; Thanos et al., 1995). The nuclear translocation and activity of NF-κB factors is controlled by a family of cytoplasmic inhibitory proteins, the IκBs. The IκBs interact with NF-κB dimers, thereby blocking their nuclear translocation (Baldwin et al., 1996). The genes controlled by NF-κB factors encode molecules of principal importance for the immune systems, such as MHCI and II molecules, cytokines, cell adhesion molecules and costimulatory molecules(Baldwin et al., 1996). The Cytokine-induced maturation of dendritic cells resulted in an increased in nuclear RelB, p50, p52, and especially c-Rel (Neumann et al., 2000). Li J-F et al. have demonstrated that CD8+CD28- T suppressor cells downregulate CD86 expression by inhibiting NF-κB-mediated transcription of CD86 gene in APCs (Li et al., 1999). In my in vitro co-culture system reported in the present study, the mechanisms by which Treg from Ni^{high} mice block the up-regulation of CD80/CD86 on DCs is currently unclear, it can be speculated that it may also involved in DC and Treg interaction and signal transduction, such as NF-κB pathway, but I has yet to formally test this.

The in vitro co-culture system I established serves to study the mechanism underlying the spread of tolerance from Treg cells to APCs observed in vivo (Roelofs-Haarhuis et al., 2003). One possible mechanism by which Treg cells spread the tolerance to APCs is by the production of IL-10 and /or TGF-β. If so, the addition of neutralizing anti-IL-10 or anti-TGF-β mAb to this coculture of APCs and Treg cells should prevent the down-regulation of costimulatory molecules. Furthermore, the different cell types (APCs and T cells, respectively) can readily be separated by cell sorting. It can then be asked whether the APCs, indeed, were rendered tolerogenic by the treatment in vitro. If so, they should be able to induce tolerance in vivo. In the future, such systems might be applicable for the treatment of patients suffering from severe nickel allergy. Among the APCs, we can separate DCs and B cells and ask which population was rendered (more) tolerogenic, the DCs or the B cells.

4.4 Nickel infectious tolerance and IDO

In the orally nickel tolerized mouse model, Infectious tolerance was indeed found to account for the spread of tolerance upon cell transfer (Roelofs-Haarhuis et al., 2003). The infectious tolerance pathway was found to comprise a spread of tolerance from Ly5.1+ donor Ts cells to Ly5.2+ host APCs and vice versa, from tolerogenic Ly5.1+ donor APCs to Ly5.2+ T cells of the host. A prerequisite for the successful spread of tolerance was that the hosts were immunized with $NiCl_2$ /H_2O_2 before transferring their cells to the second set of recipients. The data presented here clearly show that IDO play a critical role for the spread of tolerance from donor Ts cells to host APCs. Taking into account that both immunization with $NiCl_2$ /H_2O_2 and IDO are required to spread the tolerance from Ts cells to APCs, I proposed that engagement of CTLA-4 on Ts with B7 (CD80/CD86) on DCs is the trigger for inducing the IDO expression by DCs. Obviously, immunization with $NiCl_2$ /H_2O_2 upregulated CTLA-4 expression and IFN-γ production by nickel specific T cells. Indeed, I found that CD4+ T cells from nickel orally tolerized mice increased the expression of CTLA-4 expression compared with the untreated mice after immunization with $NiCl_2$ /H_2O_2. Conceivably, upon adoptive transfer and immunization with $NiCl_2$ /H_2O_2, the engagement of CTLA-4 on the nickel-specific Treg cells from Ni^{high} donor mice with the B7 molecule on the DCs in the first recipient is responsible for the induction of expression of IDO on DCs, which can render the DCs to be tolerogenic. The latter mediated the transfer of the tolerance of nickel from the first recipient to the second recipient. This idea was supported by the evidence that increased production of IFN-γ in the spleen of first recipients received donor T cells from Ni^{high} mice (F. Kopp, unpublished result). Consequently, after second transfer of APCs (including IDO-expressing CD8α+ DCs) from first recipients to the second recipients, IDO-expressing CD8α+ DCs may exert a tolerogenic effects on T cells by two different mechanisms: first, they inhibit T cell proliferation due to the IDO-induced consumption of the essential amino acid tryptophan and, second, they may induce T cell apoptosis due to production of pro-apoptotic tryptophan metabolites. Taken together, our results demonstrate that infectious tolerance in vivo involes a reciprocal interplay of specific Treg cells and tolerogenic APCs that is mediated by IDO and driven by immunization. With regard to the functional role of IDO and the consquences of immunization in the effector phase of T cell suppression (defined here as the time period after adoptive cell transfer and the subsequent immunization of the recipients), I proposed that there is an reinforcing effects between IDO-expressioning regulatory DCs and CTLA-4-expressing Treg cells, which is enhanced by immunization with $NiCl_2$ /H_2O_2. Under these conditions, tolerogenic APCs and Treg effector cells engaged naive T cells and normal APCs, respectively, into the tolerization process so that unresponsiveness prevailed.

4.5 In summary

Taken together, results presented here demonstrate that oral uptake nickel water (10mM) for 4 weeks downregulate the expression of costimulatory molecules CD80 and CD80 expression by DCs, upregulate the expression of PD-L1 by DCs, and CTLA-4 on T cells, upon immunization with $NiCl_2/H_2O_2$, whereas, PD-L2 and PD-1 did not shown any alterations after immunization with $NiCl_2/ H_2O_2$. Adoptive transfer experiments have shown that, donor T cells from nickel-tolerized Ni^{high} mice have regulatory effects, they can partially block the upregulation of the expression of costimulatory CD80 and CD86 by DCs in Ni^{low} recipient mice induced by $NiCl_2/ H_2O_2$. This effects were further confirmed by the in vitro coculture experiments, in which, T cells from Ni^{high} mice can block upregulation of CD80 and CD86 expression on DCs from Ni^{low} mice in the presence of nickel. However, which subset of regulatory T cells (e.g., CD4+CD25+ Treg cells, or CD8+ T suppress cells) from nickel orally tolerized mice play a role, and how they function (e.g., cytokine dependent or cell contact dependent), are still to be determined. With regard to the role played by the IDO in the nickel infectious tolerance, using two steps adoptive transfer and IDO inhibitor 1-MT pellet, we demonstratred that IDO mediated the spread of nickel tolerance from T suppress cells to the DCs. In summary, from the views of costimulatory molecules and IDO, this study obtained some insight into the cellular and molecular mechanism underlying immunomodulation induced by oral uptake nickel.

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Zusammenfassung

Die Arbeitsgruppe von Prof. Gleichmann hat ein Mausmodell zur oralen Induktion der Nickeltoleranz etabliert. Sie haben gezeigt, daß die Erscheinung der Nickeltoleranz von der reziproken Interaktion von T-Suppressorzellen und tolerogenen APZ begleitet wird, die durch die Immunisierung ausgelöst wird. Studien aus der Literatur deuten an, daß kostimulatorische Moleküle (z. B. CD80/CD86, PD-L1/PD-L2) und Indolamin-2,3-dioxygenase (IDO) an diesem Prozess beteiligt sind.

In dieser Arbeit wurde die Expression von kostimulatorischen (CD80, CD86) und koinhibitorischen (PD-L1, PD-L2) Molekülen durch die APZ nicht-toleranter Mäuse (bezeichnet als Ni^{low} Mäuse) und oral tolerisierter Mäuse (bezeichnet als Ni^{high} Mäuse) mittels Immundurchflußzytometrie verglichen; die APZ stammten aus den drainierenden Lymphknoten (DLNs) nach der Immunisierung mit entweder $NiCl_2$ oder $NiCl_2/H_2O_2$. In Ni^{low} Mäusen induzierte die Immunisierung mit $NiCl_2/H_2O_2$ eine hohe Expression von CD80, CD86, PD-L1 und PD-L2, während die Injektion mit $NiCl_2$ nur einen geringen Effekt zur Folge hatte. Dies führte zu der Schlußfolgerung, daß die Behandlung mit Nickel und H_2O_2 sowohl Signal 1 als auch Signal 2 induziert, die für die Sensibilisierung der T-Zellen gegen Nickel erforderlich sind. Im Hinblick auf die Expressionskinetik von dendritischen Zellen (DZ) stimuliert die Immunisierung mit $NiCl_2/H_2O_2$ die Expression von CD80 und PD-L2 sehr viel schneller als die von CD86 und PD-L1. Verglichen mit den DZ unbehandelter Ni^{low} Mäuse zeigen die DZ von Ni^{high} Mäusen eine reduzierte Expression von CD80 und CD86 und eine erhöhte Expression von PD-L1, was auf einen tolerogenen Phänotyp hindeutet. Nach adoptivem Transfer von 10^6 T-Zellen von Ni^{high} Mäusen auf Ni^{low} Empfänger, gefolgt von deren Immunisierung mit $NiCl_2/H_2O_2$, wurde eine teilweise Blockierung der Aufregulation der Expression von CD80/CD86 durch die DZ der Empfänger beobachtet. Vergleichbare herunterregulierende Effekte wurden auch in vitro gefunden, wenn DZ unbehandelter Ni^{low} Mäuse zusammen mit T-Zellen von Ni^{high} Mäusen kokultiviert wurden. Diese beschriebenen Effekte waren stärker in Anwesenheit von $NiCl_2$ und waren für CD80 deutlicher ausgeprägt als für CD86. In Übereinstimmung hiermit konnte eine geringe Erhöhung der CTLA-4-Expression von $CD4^+$ T-Zellen in den DLN beobachtet werden, wenn Ni^{high} Mäuse mit $NiCl_2/H_2O_2$ immunisiert wurden. Es ist bekannt, daß DZ, die das Tryptophan-abbauende Enzym Indolamin-2,3-dioxygenase exprimieren, die Proliferation von T-Zellen supprimieren können. Durch Verwendung von Indolamin-2,3-dioxygenase-Inhibitorpellets, kombiniert mit seriellen adoptiven Transfers von $Ly5.1^+$-Spendern auf zwei aufeinanderfolgende Sätze von $Ly5.2^+$-Empfängern, konnte mittels des Mausohrschwellungstests gezeigt werden, daß IDO für die Übertragung der Nickeltoleranz von Suppressor-T-Zellen auf APZ erforderlich ist.

Aus diesen Ergebnissen ergibt sich die Schlußfolgerung, daß die orale Aufnahme von Nickel sowohl Treg-Zellen als auch tolerogene APZ induziert. Welche der bekannten T-Zellsubpopulationen, $CD4^+CD25^+$ Treg oder $CD8^+$ Treg, daran beteiligt sind, und wie sie in diesem Modell wirken, steht noch zur Untersuchung aus. Ähnliches gilt für CTLA-4 und PD-L1/PD-L2. Obwohl sie als "molekulare Brücken" dienen können, um die Toleranz von Treg auf DZ zu übertragen, ist nicht untersucht worden, ob DZ, die in vitro durch Treg modifiziert wurden, funktionell sind, und ob sie dazu in der Lage sind, die Nickeltoleranz in vivo zu induzieren. Ähnlich verhält es sich für IDO.

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