

## Chapter 9

# On the Role of Co-inhibitory Molecules in Dendritic Cell: T Helper Cell Coculture Assays Aimed to Detect Chemical-Induced Contact Allergy

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**Abstract** T cells play a pivotal role in sensitization and elicitation of type IV allergic reactions. While T helper cells sustain and maintain the differentiation of further effector cells, regulatory T cells are involved in control of cytokine release and proliferation, and T killer cells execute cellular lysis, thereby leading to certain levels of tissue damage. According to their central role, the widely applied and OECD-supported test method for the assessment of the sensitization potential of a chemical, i.e., the local lymph node assay (LLNA), relies on the detection of the immune-responsive proliferation of lymphocytes. However, most sensitization assays recently developed take advantage of the initiators of sensitization, dendritic cells (DCs) or DC-like cell lines. Here, we focus on inhibitory molecules expressed on the surface of DCs and their corresponding receptors on T cells. We summarize insight into the function of CTLA-4, the ligands of inducible co-stimulators (ICOSs), and on the inhibitory receptor programmed death (PD). The targeting of immune cell surface receptors by inhibitory molecules holds some promise with regard to the development of T cell-based sensitization assays. Firstly, a broader and more sensitive dynamic range of detection could be achieved by blocking inhibitors or by removing inhibiting regulatory T cells from the assays. Secondly, the actual expression levels of inhibitory molecules could be also a valuable indicator for the process of sensitization. Finally, inhibitory molecules in coculture test systems are supposed to have a major influence on DCs by reverse signaling, thereby affecting their differentiation and maturation status in a feedback loop. In conclusion, inhibitory ligands of DC surface receptors and/or their cognate receptors on T cells could serve as useful tools in cell-based assays, directly influencing toxicological endpoints such as sensitization.

**Keywords** Dendritic cell • T helper cell • Co-stimulatory molecules • Test assay • Contact allergy

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## List of Abbreviations

|                  |                                           |
|------------------|-------------------------------------------|
| ACD              | Allergic contact dermatitis               |
| CTLA-4           | Cytotoxic lymphocyte-associated antigen-4 |
| DC               | Dendritic cell                            |
| DNCB             | 2,4-Dinitrochlorobenzene                  |
| DNFB             | 2,4-Dinitrofluorobenzene                  |
| ICOS             | Inducible co-stimulator                   |
| LC               | Langerhans cell                           |
| LLNA             | Local lymph node assay                    |
| MoDC             | Monocyte-derived DC                       |
| PBMC             | Peripheral blood mononuclear cell         |
| PD               | Programmed death                          |
| Th               | T helper                                  |
| T <sub>reg</sub> | Regulatory T cell                         |

## 9.1 Introduction

A population of T cells was originally named T helper (Th) cells to describe its main function in delivering essential support for different players of the innate and adaptive immune system. Among others, an important interaction occurs between Th1 cells and macrophages. Through the release of Th1 cell-derived IFN- $\gamma$ , macrophages will be primed for complete digestion of intracellular pathogens. In addition, the helper function is also mandatory to sustain immune cell-mediated hypersensitivity reactions. While type-I reactions have been found to rely on Th2 cell-mediated IL-4, IL-5, and IL-13 release, all of which are being necessary to promote differentiation and proliferation of IgE-producing plasma cells, the molecular and cellular support of delayed type IV reactions is still far from being clear. Various subsets of Th cells, i.e., Th1, Th17, and Th22, that express different co-stimulatory molecules such as CD28, CD40L, CD70, and 4-1BB are discussed to exert effector cell function in allergic diseases (Lenschow et al. 1996; Greenwald et al. 2005). Conversely, there is increasing evidence that regulatory mechanisms mediated by co-stimulatory molecules with a pronounced inhibitory function, also referred to as co-inhibitory molecules, are crucially involved in allergic asthma, allergic contact dermatitis (ACD), as well as in other kinds of hypersensitivity reactions (Chen and Shi 2006; Jutel and Akdis 2011). In the case of ACD, there is a fundamental lack of knowledge on the role of inhibitory molecules at the surface of regulatory effector cells.

In regulatory toxicology the mouse local lymph node assay (LLNA), along with two guinea pig assays are most commonly applied to measure the sensitization potential of chemicals (Kimber and Weisenberger 1989; Magnusson and Kligman 1969; Buehler 1965). Using the LLNA, the potency of a substance can be quantified

by measuring lymph node cell proliferation via incorporation of  $^3\text{H}$  thymidine in dividing cells. The great majority of cells populating the lymph nodes comprise different kinds of lymphocytes. It is thus not without reasoning that non-animal immune cell-based assays should also take advantage of lymphocytes to imitate *in vivo* conditions as closely as possible. In recent years, great efforts and achievements have been made to develop *in vitro* assays for the toxicological endpoint sensitization (Adler et al. 2011). However, so far all immune cell-based assays that reached prevalidation status are dedicated to the analysis of biomarker expression on the surface of or biomarker release from dendritic cells (DCs) or DC-like cells. A major issue raised against these “DC-only assays” is that only the phenotype, not a functional response based on the generation of effector cells, is being detected. Considering a crucial physiological role of T cells in ACD and that the LLNA actually could be regarded as T cell test, obviously new sensitization assays would greatly benefit from integrating T cells. This review summarizes advances in research on the role of T cells and on the potential function of inhibitory B7 molecules in ACD and their perspectives for the development of future antigen-presenting cell (APC)/T cell-based *in vitro* assays.

### 9.1.1 *T Cells in Contact Allergy*

ACD is a bi-phaseal inflammatory skin disease mediated by cells contributing to the innate and adaptive immune system (Saint-Mezard et al. 2004; Vocanson et al. 2009; Fonacier et al. 2010). In the initial early phase, individuals are getting sensitized by first-time exposures against allergic substances such as haptenic chemicals. Initiated by inflammatory cytokines that are released by danger-recognizing keratinocytes, antigen-bearing DCs residing in the skin start to migrate into the regional lymph nodes (Nestle et al. 2009; Aiba and Katz 1990). Antigen-challenged dermal DCs immediately move into the outer paracortex of B cell follicles, whereas Langerhans cells (LCs) in general rather populate T cell areas in lymph nodes with some delay (Kissenpfennig et al. 2005; Kaplan et al. 2008). As a consequence of antigen presentation, co-stimulation, and the release of polarizing cytokines, cytotoxic T cells and hapten-specific precursors of specific subpopulations of T helper cells, i.e., Th1, Th17, and Th22, start to differentiate and to proliferate (Vocanson et al. 2009). These initial processes in chemical-induced contact allergy, that is, penetration of the allergenic compound through the skin, cutaneous metabolism where applicable, interaction with reactive proteins (i.e., haptenization), phagocytosis (by DCs), migration (of antigen-bearing DCs), antigen presentation (by DCs), and the development of armored effector T cells, occur within the first 10–15 days after exposure during the so-called “sensitization” phase. In the subsequent, so-called “elicitation” phase, the work done by certain T cell populations becomes clinically evident. Skin affection and lesions including erythema, edema, vesicles, and pain are caused by perforin, granzyme, and inflammatory cytokines released from cytotoxic T cells (Yawalkar et al. 2001). In addition

to these executors of allergen-triggered epidermal damage, different subsets of Th1, Th17, and Th22 are also known to play a major role in hypersensitivity reactions of human skin.

### ***9.1.2 T Helper Subsets in ACD***

It is well known for nearly 40 years that beyond APCs and blood-derived leukocytes, allergen-specific T cells are required for the induction of ACD. Ground-breaking studies in mice revealed that animals deprived of T cells by thymectomy or irradiation were unable to develop contact allergy upon chemical-mediated sensitization (Zembala and Asherson 1973; Asherson and Zembala 1970). In addition, lymphocytes transferred from sensitized donors to irradiated recipients were capable of inducing skin affections in the recipients. The role of cytotoxic T cells in ACD has been well established, and additional studies demonstrated that CD8<sup>+</sup> suppressor cell proliferation occurs in allergen-specific, IL-2-dependent T cell clones isolated from blood of ACD patients (Kalish and Morimoto 1988; Dennert and Hatlen 1975; Grabbe and Schwarz 1998; Blauvelt et al. 2003). By contrast, the exact role of CD4 (Leu3A, OKT4)-positive (CD4<sup>+</sup>) cells in ACD remained ambiguous. Studies using mouse models and depletion of CD8<sup>+</sup> cells suggested that distinct CD4<sup>+</sup> cells might contribute as effector cells as well (Gocinski and Tigelaar 1990). In the past, the contribution of hapten-reactive Th cells in the induction phase of allergic skin reactions was only speculative (Takahashi et al. 1977). A closer look revealed CD4<sup>+</sup> Th cells as prominent population of skin infiltrating cells in patients suffering from ACD or nickel-induced ACD (Sinigaglia et al. 1985; McMillan et al. 1985). In the aftermath of the proposal of a Th cell bias separating Th1 from Th2 cells (Mosmann et al. 1986; Murphy and Reiner 2002; Zhu et al. 2010), nickel-specific T lymphocytes were found to secrete IFN- $\gamma$ , IL-2, TNF- $\alpha$ , and GM-CSF, but little or no IL-4 and IL-5. This pointed to a Th1 phenotype being involved in ACD rather than a Th2 phenotype (Kapsenberg et al. 1992). Thus, ACD was originally regarded as a response of the adaptive immune system, preferentially under the participation of cytotoxic T cells and Th1 cells. However, T cell populations carrying a pronounced Th2 phenotype were also described to contribute to skin allergy reactions in mice and humans (Xu et al. 1996; Probst et al. 1995). Today, the existence of additional human Th cell subpopulations, i.e., Th17, Th22, and Th9, has been proposed. Each of these subpopulations expresses a specific pattern of transcription factors, chemokines, cytokines, and cognate receptors (Acosta-Rodriguez et al. 2007; Wilson et al. 2007; Duhon et al. 2009; Trifari et al. 2009; Bluestone et al. 2009; Putheti et al. 2010). With the exception of Th9 cells, which are supposed to play a role in allergic airway inflammation (Chang et al. 2010), all of these particular Th cell subpopulations were demonstrated to be involved in ACD. So, specific patterns of IL-17, IL-22, and IFN- $\gamma$  were detected in Th cells derived from peripheral blood mononuclear cells (PBMCs) and in skin T cell lines derived from patients suffering

from the inflammatory skin disorders psoriasis, atopic eczema, or ACD (Eyerich et al. 2009). In the case of donors with known nickel allergy, proliferation of T memory cells was recently identified in both subpopulations, Th1 and Th17 (Larsen et al. 2009). Moreover, in biopsies obtained from such patients, CD4<sup>+</sup> T cells expressing IL-17 and IL-22 were detected. Although the definition of unique Th subsets seems to rely on the exclusive expression of transcription factors and cytokines like T-bet, GATA-3/IFN- $\gamma$ , and IL-4 for Th1/Th2, data from new studies point to a higher complexity. A certain degree of plasticity with flexible lineages capable of replacing irreversibly committed subsets is now assumed for Th cell differentiation (O'Shea and Paul 2010; Murphy and Stockinger 2010). Both analyses of the phenotype of T cells expressing co-stimulatory and co-inhibitory molecules and on the contributing role of Th1, Th22, and Th17 in ACD are currently under way.

### ***9.1.3 Co-inhibitory Molecules Expressed on the Surface of APCs and T Cells***

The diversity of the family of co-stimulatory molecules mirrors their fine-tuning role in the activation of distinct T cell populations toward differentiation and proliferation. In addition to the original B7 proteins, CD80 (B7.1) and CD86 (B7.2), further members of the B7 family, i.e., ICOS-L, PD-L1, PD-L2, B7-H3, and B7-H4, on APCs and their receptors/ligands on T and B cells were described (Lenschow et al. 1996; Greenwald et al. 2005). According to current models describing the interaction between APCs and T cells within the immune synapse, three or more signals necessary for the development of effector T cells have been proposed (Grakoui et al. 1999; Kapsenberg 2003). Accordingly, MHC/TCR interactions were assumed as “signal 1”; interactions between co-stimulatory molecules such as B7 and CD28 were regarded as “signal 2,” and the binding of certain cytokines to their cognate receptors was proposed as “signal 3,” enabling specific differentiation of T cells. Similar to the situation of a specific inflammatory immune response to pathogens or tumor cells, the complete signal pattern 1–3 has to be present in an allergic type IV reaction initiated by DCs. Conversely, certain agonist/receptor interactions that constitute “signal 2,” such as B7/CTLA-4 or PD-L1/PD-1, can also mediate tolerance against particular allergens through the limitation of Th cell-derived pro-inflammatory molecules and/or by inducing regulatory T (T<sub>reg</sub>) cell-derived anti-inflammatory cytokines such as IL-10 and TGF- $\beta$  (Cavani 2008). To propose new assay developments, the following sections are focused on “inhibitory signal 2 molecules” and their specific role in ACD and experimental coculture systems of APCs and T effector cells.

### 9.1.3.1 CTLA-4 in ACD

As the most prominent members of the B7 family, the co-stimulatory molecules CD80 and CD86 are well known to facilitate the interaction between APCs and T cells by contributing to “signal 2” in the immune synapse. This represents the principal way of how B7 molecules contribute to the initiation of pro-inflammatory responses to pathogens, allogeneic transplants, and tumor cells (Lenschow et al. 1996; Kapsenberg 2003). In addition to CD28, CD80 and CD86 also exhibit binding capacity to negative regulators such as CTLA-4 (cytotoxic lymphocyte-associated antigen-4) and programmed death (PD) ligands expressed on the surface of activated T cells, thereby mediating suppressive functions (Fife and Bluestone 2008). The transmembrane glycoprotein type 1 CTLA-4 (CD152) is a member of the Ig superfamily and exhibits an extracellular IgV domain with two binding sites for B7 (Teft et al. 2006). CTLA-4 shows a higher affinity for B7 when compared to CD28 and can bind to two molecules of B7. However, the homodimer of CTLA-4 is expressed at lower receptor density on the cell surface of T and B cells. Upon activation by CD28, T cells can be silenced via the interaction between CTLA-4 and CD80/CD86 and due to the downstream interference of cytoplasmic phosphorylated protein phosphatase 2 with PI3K-PKB/Akt signaling. The levels of expression were found higher in Th2 than in Th1 cells, and CTLA-4/B7 interactions were reported to attenuate Th2 cell differentiation (Pandiyani et al. 2004; Oosterwegel et al. 1999). In contrast to its role in type 1 allergic reactions, there are as yet only few reports available on the function of CTLA-4 in contact allergy. In a mouse model, the application of CTLA-4-Ig induced a long-lasting unresponsiveness to 2,4-dinitrofluorobenzene (DNFB) (Tang et al. 1996). This effect is thought to rely on a reduced number of Th1 cells that were found after administration of CTLA-4-Ig and DNFB. A different study addressed the question whether CTLA-4 is capable of mediating a UV-induced tolerance to contact allergens such as DNFB (Schwarz et al. 2000). Indeed, when transferring lymph node cells from sensitized mice into recipient animals, upon depletion of CTLA-4<sup>+</sup> cells, hypersensitivity reactions revealed uninhibited. In a further study, anti-CTLA-4 antibody enhanced Th1-mediated ear swelling elicited after sensitization, but not after reexposure to the same allergen (Nuriya et al. 2001). There were further hints indicating that CTLA-4 is involved in controlling the sensitization phase of ACD. For instance, the proliferation rate of antigen-specific T cells was found elevated in the presence of anti-CTLA-4. In patch test biopsies obtained from patients suffering from ACD to nickel or fragrances, CTLA-4 was detected after 3 days in a subpopulation of epidermal cells positive for CD4 and CD25 (Bangert et al. 2003). According to this phenotype it has been assumed that T<sub>regs</sub> are present in the eczematous lesions, albeit CD25 at intermediate expression may also be detectable on activated T cells different from T<sub>regs</sub>. Further evidence for the participation of T<sub>regs</sub> in ACD was delivered through the detection of FoxP3<sup>+</sup> cells in mice treated with picryl chloride and the anti-inflammatory drug ketoprofen (Atarashi et al. 2009). In experiments transferring lymph node cells from these mice to recipients, prior administration of

anti-CTLA-4 could inhibit the suppressive activity of  $T_{\text{regs}}$ , measured by an increased ear swelling in rechallenged mice. Elevated expression of CTLA-4 in a subpopulation of  $T_{\text{reg}}$  cells was suggested to prevent healthy non-allergics from hypersensitivity reactions after exposure to the strong contact allergen nickel (Cavani et al. 2003). Interestingly, nickel-stimulated  $CD4^+$  T cells from healthy donors restored their capacity to proliferate after depletion of  $T_{\text{regs}}$ , thereby counteracting the tolerance to a particular contact allergen. In conclusion, the negative regulator CTLA-4 was detected in human skin biopsies from rechallenged individuals. Transfer experiments in mice indicated that CTLA-4 expressed on the surface of activated T cells or  $T_{\text{regs}}$  is mandatory to control contact hypersensitivity reactions.

### 9.1.3.2 ICOS in ACD

The inducible co-stimulator ICOS (CD278) is a member of the CD28 family and expressed as a glycosylated disulfide-linked homodimer with IgV (Greenwald et al. 2005; Simpson et al. 2010). The protein shares 17 and 24 % sequence identity with CTLA-4 and CD28, respectively (Hutloff et al. 1999). ICOS is expressed on the surface of activated  $CD28^+$  and  $CD45RO^+$  T cells; its ligand ICOS-L (CD275, B7H2, B7RP-1) was detected on LCs from human skin and in immature monocyte-derived dendritic cells (MoDCs) (Witsch et al. 2002). ICOS was found to increase antigen-dependent  $CD4^+$  T cell proliferation (Hutloff et al. 1999; Dong et al. 2001). Preferentially, ICOS was described to control the effector function of Th2 cells in asthma and type 1 allergic reactions that are both not subject of this review. In addition to Th2 functions such as enhanced secretion of IL-4, IL-5, and IL-10 by T cells and IgM and IgG by B cells, effects that indicate Th1 responses were observed as well. For instance, anti-ICOS triggered the release of IFN- $\gamma$ , TNF- $\alpha$ , and GM-CSF but not IL-2 and an upregulation of molecules indicating T cell activation, e.g., CD40L, CD69, CD25, and CD70. In MoDC/T cell coculture systems, ICOS-L/ICOS interactions increased the secretion of IL-10, whereas CD28/B7 interactions decreased the release of this anti-inflammatory cytokine (Witsch et al. 2002). In contrast to lymph nodes of normal mice, ICOS expression was reported in the T cell area of the paracortex in mice that were sensitized against the contact allergen oxazolone (Yoshinaga et al. 1999). In addition, in oxazolone and ICOS-L-Fc-challenged mice, enhanced ear swelling indicating exacerbated contact hypersensitivity was found. However, another report demonstrated that ICOS-L $^+$  LCs from induced human skin more efficiently than dermal DCs IFN- $\gamma$ , TNF- $\alpha$ , and IL-4/IL-5 in naïve  $CD4^+$  T cells, thus indicating the control of ICOS-L/ICOS interactions on both Th1 and Th2 cells (Furio et al. 2010). Interestingly, only CD28/ICOS-L double-deficient mice were reported to show strongly impaired  $CD4^+$  T cell proliferation and IFN- $\gamma$  release in a model of delayed-type hypersensitivity (Wong et al. 2009). Taking this altogether, a hierarchical cooperation of B7/CD28 and ICOS-L/ICOS pathways in type IV allergic reactions should be assumed and therefore also considered in the development of novel DC-T cell coculture assays.

Recently, in hapten-stimulated mice, enhanced expression of ICOS and secretion of IL-10, IL-17, and IFN- $\gamma$  were found in a distinct subpopulation of T<sub>reg</sub> cells (Vocanson et al. 2010). These ICOS<sup>+</sup> T<sub>reg</sub> cells displayed a strong suppressive potential in vitro and in vivo by inhibiting CD8<sup>+</sup> T cell proliferation and the release of IFN- $\gamma$ . The question whether these ICOS<sup>+</sup> T<sub>reg</sub> cells may also exhibit the capacity to control allergen-stimulated effector Th cells in type IV reactions should be addressed in further studies.

### 9.1.3.3 Programmed Death-1 and Its Ligands in ACD

Together with CTLA-4 and ICOS, transmembrane type 1 protein programmed death-1 (PD-1, CD279) is another member of the B7 family (Greenwald et al. 2005). However, its extracellular IgV domain demonstrates only 16 and 21 % amino acid identity with CTLA-4 and CD28, respectively, and PD-1 is active as monomer (Lazar-Molnar et al. 2008). Originally detected at the surface of apoptotic cells, PD-1 was subsequently also found on activated T cells and B cells but also on DCs and monocytes (Okazaki and Honjo 2007; Keir et al. 2008). In mice, PD-1 deficiency is accompanied by autoimmunity such as lupus-like arthritis, cardiomyopathy, and glomerulonephritis, providing evidence for a tolerogenic function of this protein. In humans, systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis were found to be linked to mutations in the corresponding gene (Sharpe et al. 2007). Binding of PD-1 to its ligands PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC) during T cell/DC interactions was shown inducing both inhibition and activation of T cells. In addition, the ligation of CD80 (B7.1) to PD-L1 caused reductions in T cell proliferation and the release of Th1-like cytokines (Butte et al. 2007). However, also directly opposed functions of PD-1 ligands expressed on T effector cells were recently observed in allergic diseases (Singh et al. 2011). In contrast to mice carrying a PD-L2-deficient phenotype and which suffer from severe lung inflammation, PD-L1-deficient animals revealed with restricted airway hyperreactivity (Akbari et al. 2010). Thus, in allergic asthma a negative effect of PD-L2 on Th2 cells via control of specific cytokines in the effector phase was proposed. In the context of type IV hypersensitivity reactions, expression of PD-1 was observed on immature human LCs, but not on migrating LCs (Pena-Cruz et al. 2010). Conversely, expression of PD-L1 and PD-L2 was found upregulated upon maturation signals delivered by cell migration, bacterial ligands, or contact allergens such as nickel and 2,4-dinitrochlorobenzene (DNCB) (Pena-Cruz et al. 2010). Previously, low expression of PD-L1 and lacking expression of PD-L2 were reported in the murine LC line XS106 (Kim et al. 2006). In the same study, both ligands were found increased on lymph node-resident DCs upon sensitization of mice by fluorescein isothiocyanate (FITC)-labeled hapten. Furthermore, the level of PD-L expression was demonstrated to affect functional properties such as proliferation of murine T cells. Using coculture assays and mixed leukocyte reactions with DNFB-primed CD3<sup>+</sup> T cells and APCs derived from draining lymph nodes of sensitized mice,

PD-L1 but not PD-L2 was crucial in the suppression of T cell proliferation (Tsushima et al. 2003). Further data supporting a role of PD-L1 in the control of T cell proliferation came from a study using transfected XS106 cells as APCs (Kim et al. 2006). These PD-L1<sup>+</sup> LC-like cells led to a decrease in the proliferation of total and CD8<sup>+</sup> T cells obtained from DNFB-sensitized mice. A PD-L1 blocking antibody, however, could restore the potential of both hapten-stimulated populations to divide in coculture with PD-L1<sup>+</sup> LC-like cells. In addition, a regulatory effect of PD-L1 on Th1-specific cytokines was reported. In the forecited report, experiments with blocking antibodies showed that secretion of IL-2 and IFN- $\gamma$  by CD8<sup>+</sup> and CD4<sup>+</sup> cells was inhibited by PD-L1, but not by PD-L2.

Congruently with the murine system, the inhibitory effect of PD-L1 was proven in allogeneic cocultures using human LCs and CD4<sup>+</sup> T cells (Pena-Cruz et al. 2010). In this system, however, LCs were activated by the process of migration out of the epidermal stripes from skin samples and not by stimulation with allergen. Interestingly, an effect of PD-1 engagement also on the APC was shown by experiments using bead-coupled PD-L Ig. In direct comparison to cells triggered with isotype-coupled beads, PD-L1 suppressed the release of IL-6 and MIP1 $\alpha$  from TLR2-activated LCs. Consequently, cytokines specific for differentiation of immune cell subsets other than Th1 and cytokines released by Th cell populations themselves were analyzed. In a recent study using cocultures of nickel-stimulated MoLCs and CD4<sup>+</sup> T cells, blockage of PD-L1 induced the release of high amounts of TNF- $\alpha$  and IL-22 (Hitzler et al. 2012). Upregulation of IL-17 secretion via blocking of PD-L1, however, was restricted to CCR6<sup>+</sup>/CCR4<sup>+</sup> T memory cells. Collectively, in mouse and human coculture systems, PD-L1 was found to function as a negative regulator in the proliferation and differentiation of specific Th cell subsets while responding to the challenge with particular contact allergens.

## 9.2 Inhibitory Molecules in Cell-Based Assays

### 9.2.1 *Dendritic Cells in In Vitro Test Systems*

DCs are usually generated from two different sources, CD14<sup>+</sup> monocytes isolated from peripheral blood or CD34<sup>+</sup> hematopoietic progenitor cells obtained from cord blood (Caux et al. 1992; Meierhoff et al. 1998). However, due to their ready availability, CD14<sup>+</sup> monocytes are usually the first choice for the development of in vitro test procedures. Apart from blood-derived DCs, various myeloid cell lines have been tested for their ability to detect sensitizers in vitro. Parameters such as upregulation of co-stimulatory molecules and increase of cytokine and chemokine secretion have been used to distinguish between sensitizers and nonsensitizers.

Maturation of DCs is associated with upregulation of a distinct pattern of co-stimulatory and co-inhibitory molecules on the cell surface. Several

co-stimulatory and adhesion molecules have been found to be upregulated and have been thoroughly tested for their use in DC-based sensitization assays. The most prominent among them are CD86 and CD54 (Aiba et al. 1997). CD86 is upregulated not only in CD34<sup>+</sup> cells and MoDCs but also in the cell lines THP-1, U-937, and Mutz-3 that have been extensively characterized in the past (Ade et al. 2006; Azam et al. 2006; Python et al. 2007; Sakaguchi et al. 2006). In addition to co-stimulatory molecules, the co-inhibitory molecule PD-L1 was recently reported as a new suitable marker for the detection of sensitizers. This protein was shown to be significantly upregulated after challenge with DNCB, cinnamic aldehyde, or paraphenylenediamine in MoDCs (Williams et al. 2010). In the Mutz-3 cell line, PD-L1 was also increased after challenge with hydroquinone, hexyl cinnamaldehyde, and eugenol (dos Santos et al. 2009).

### 9.2.2 *T Cell-Based Assays*

The upregulation of maturation markers on the surface of DCs is necessary but not sufficient for the development of contact dermatitis. In vivo the activated DC migrates from the skin toward the local lymph nodes and then presents a chemically modified protein to T cells via MHC molecules. This results in the proliferation of Th cells and the induction of a specific cytokine pattern. Therefore an optimized T cell-based in vitro sensitization assay should be capable of recapitulating these key events occurring in the development of ACD, thereby providing even more relevant information about the sensitizing capacity of certain chemicals than any assay related to innate immunity. T cell assays are usually performed by coculturing of professional antigen-presenting cells with T cells and subsequent assessment of T cell proliferation and/or secretion of specific cytokines (i.e., IFN- $\gamma$ , IL-4, IL-17, or IL-22). A major issue in T cell-based test systems relates to the relatively low sensitivity of these assays. This might be partly due to the repression of T cell priming in vitro by naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. Congruently the depletion of such CD4<sup>+</sup>CD25<sup>+</sup> cells in test systems for contact sensitizers resulted in significantly pronounced effects regarding T cell proliferation and IFN- $\gamma$  secretion (Vocanson et al. 2008). Another promising way to enhance the sensitivity of such test systems could be the interference with co-inhibitory molecules expressed on the surface of DCs or T cells that are involved in fine-tuning or even complete suppression of a functional T cell response. On one hand, co-inhibitory molecules might be useful tools to regulate and terminate immunological reactions such as local inflammation in vivo; in vitro however they could even serve as valuable and indirect markers that help to characterize the sensitizing potential of chemicals. Inhibition of co-inhibitory molecules can enhance T cell responses in terms of proliferation and cytokine secretion, and thus this would help to detect sensitizers in vitro by means of test systems optimized in their sensitiveness.

### **9.2.3 *Co-inhibitory Molecules in In Vitro Test Systems:*** ***CTLA-4***

CD80 and CD86 are probably the best-characterized co-stimulatory molecules that activate T cells via interaction with CD28. However, when binding to CTLA-4 on T cells, the molecules can act as negative regulators of T cell activation, too (Walunas et al. 1994). In HIV models with PBMCs, blocking of CTLA-4 with specific antibodies resulted in higher numbers of virus-specific CD4<sup>+</sup> T cells and in an increased secretion of IFN- $\gamma$  and IL-2 (Kaufmann et al. 2007). In tumor immunology, antibodies against CTLA-4 are currently tested for cancer therapy. Cocultures of peripheral blood lymphocytes with DCs showed heightened proliferation when T<sub>regs</sub> were removed and CTLA-4 was blocked by specific antibodies. Interestingly the removal of T<sub>regs</sub> led to an elevation of CTLA-4 expression on the remaining T cells (Suarez et al. 2011).

### **9.2.4 *Co-inhibitory Molecules in In Vitro Test Systems:*** ***ICOS-L***

ICOS-L expressed on DCs is able to induce antigen-specific CD4<sup>+</sup> T cell proliferation and secretion of both Th1 and Th2 cytokines upon interaction with its receptor ICOS. However, through ICOS-L/ICOS interaction, IL-10-producing T<sub>reg</sub> cells become induced to subsequently execute their inhibitory function on T cell responses. In cocultures of naïve T cells with plasmacytoid DCs, the blocking of ICOS-L resulted in significantly reduced levels of IL-10. IL-10-producing T cells, induced by ICOS-L/ICOS interactions, were shown to be regulatory T cells by assessing their capacity to suppress primary T cell responses in cocultures (Ito et al. 2007).

### **9.2.5 *Co-inhibitory Molecules in In Vitro Test Systems:*** ***PD-L1***

Another molecule with great impact on T cell activation, expressed on DCs, is PD-L1. The co-inhibitory properties of PD-L1 were first reported in 2000. In CD4<sup>+</sup> T cells stimulated with anti-CD3 antibodies, the addition of soluble PD-L1 resulted in a decrease of proliferation and impaired secretion of IFN- $\gamma$  and IL-10 (Freeman et al. 2000). On the other hand, after blockade of PD-L1/PD-1 interactions in anti-CD3-stimulated naïve CD4<sup>+</sup> T cells co-stimulated with macrophages, the in vitro production of IL-2 and IFN- $\gamma$  was found increased although the proliferation of T cells was reduced (Yamazaki et al. 2005); similarly in CD4<sup>+</sup> T cells cocultured with MoDCs, the blockade of PD-L1 with specific antibodies increased the amount of T

cell-derived IFN- $\gamma$  (Karakhanova et al. 2010). In models investigating the impact of rhinovirus infection on PD-L1 expression, the repressive properties of PD-L1 in T cell cocultures could be shown. Human rhinovirus induces an upregulation of PD-L1 on DCs leading to an impaired T cell proliferation in mixed leukocyte reactions. The T cell stimulatory function could be restored by blocking PD-L1 with specific antibodies (Kirchberger et al. 2005).

### ***9.2.6 DC-Like Cell Lines in T Cell Activation Assays***

Apart from DCs, also several other cell lines with DC-like phenotypes have been used in T cell activation assays. Advantages of cell lines are their unrestricted availability and the absence of donor variability compared to primary DCs. However the co-stimulatory capacities of all cell lines proposed so far reveal limited. Although as yet no reports exist on co-inhibitory molecule expression and blocking assays using DC-like cell lines, there is a great amount of data on the PD-L1 and CTLA-4 binding partners, that is, CD80 and CD86. As the implementation of inhibitory molecules to the established marker set analyzed in DC-like cell line assays is conceivable, relevant cell lines used in sensitization assays are proposed in the section below.

#### **9.2.6.1 Mutz-3**

The leukemia cell line Mutz-3 was derived from the peripheral blood of a patient with acute myeloid leukemia and has been known to be cytokine sensitive for quite a long time (Hu et al. 1996; Quentmeier et al. 1996). The Mutz-3 cell line is used in in vitro test systems usually in three different modes. The unmodified Mutz-3 cell line itself, often referred as “progenitors,” is comprised of three different subtypes. CD34<sup>+</sup>CD14<sup>-</sup>CD11b<sup>-</sup> cells constitute the proliferative compartment of the cell line with the ability to differentiate through a CD34<sup>-</sup>CD14<sup>-</sup>CD11b<sup>+</sup> stage to give rise to a morphologically large, nonproliferating CD14<sup>+</sup>CD11b<sup>hi</sup> progeny. The addition of a distinct cytokine cocktail to these CD14<sup>+</sup>CD11b<sup>hi</sup> precursors gives rise to a DC-SIGN expressing DC-like subtype and a langerin expressing LC-like subtype (Santegoets et al. 2006). The Mutz-3 cell line was not only shown to express several co-stimulatory and adhesion molecules such as CD80, CD86, CD40, CD54, and HLA-DR after cultivation with specific cytokines but also proven to be capable of antigen processing and presentation via MHC classes I, II, and CD1d. Stimulation of flu-specific cytotoxic T lymphocytes by Mutz-3 cells loaded with flu peptides demonstrated the ability of antigen presentation via MHC class I. The capability of antigen processing and presentation via MHC class II molecules was shown by stimulating tetanus toxoid-specific CD4<sup>+</sup> T cells. Alpha-GalCer-loaded Mutz-3 DCs resulted in an increase in the number of natural killer T (NKT) cells, thereby

showing the functionality of antigen presentation via the nonclassical antigen-presenting molecule CD1d (Masterson et al. 2002; Santegoets et al. 2008).

### 9.2.6.2 THP-1

The THP-1 cell line has been tested thoroughly for its potential to detect sensitizers by upregulation of distinct activation markers such as CD86 and CD54 (Ashikaga et al. 2006; Sakaguchi et al. 2006; Yoshida et al. 2003) and is known to express HLA-DR. Yet there are only few reports about the T cell stimulatory capacities of THP-1 cells. Janic and colleagues could show enhanced proliferation of CD3<sup>+</sup> T cells after cocultivation with THP-1 cells (Janic et al. 2008), whereas Cho and colleagues were able to detect increased IFN- $\gamma$  levels in CD8<sup>+</sup> T cell cultures incubated with *Mycobacterium tuberculosis*-infected THP-1 cells (Cho et al. 2000).

### 9.2.6.3 U937

The U937 cell line was derived from a patient with generalized histiocytic lymphoma in 1976 (Sundstrom and Nilsson 1976). U937 have been found to express HLA-DR (Virelizier et al. 1984) and several co-stimulatory and adhesion molecules such CD86 and CD54 among others that become upregulated after activation with contact allergens (Ashikaga et al. 2006). There are only few reports using U937 cells in coculture systems with T cells. Stonehouse and colleagues reported that U937 cells provide a co-stimulatory signal to resting tonsillar T cells; however, this effect was only detectable upon the addition of anti-CD3 antibodies (Stonehouse et al. 1999).

## 9.3 Perspectives

In the last decade great efforts were directed toward non-animal approaches aimed at identifying and characterizing a chemical's sensitization potential in the area of quantitative risk assessment for cosmetics. For hazard identification, a panel of alternative methods was proposed and classified in test methods addressing common mechanistic steps relevant in the development of contact dermatitis in vivo (Adler et al. 2010). In detail, for the cell-based methods, test systems detecting skin inflammation, activation of DCs, and migration of DCs were proposed as parts of a toolbox. Thereby the main focus of recently developed assays was on the initiators of sensitization, DCs and DC-like cells, and cell lines such as Mutz-3. Congruently, DC-specific surface molecules and mediators such as CD86, CD54, IL-8, and IL-1 $\beta$  were used in such assays. Up to now T cells were only considered in such a sensitization battery by measurement of hapten-specific proliferation, the common endpoint that has been exploited by the in vivo mouse LLNA. In addition to this

panel of methods, further T cell assays based on molecular mechanisms in T cell differentiation and polarization by detecting Th cell- and cytotoxic T cell-specific cytokines, chemokines, and transcription factors are deemed mandatory for a toolbox comprising late events in the process of sensitization. With respect to the strong influence of co-stimulatory molecule interactions both on DC maturation and T cell differentiation, a high relevance of assays considering such interactions could be assumed for co-inhibitory signals as well. Such signals are being delivered by the interaction of DCs with T cells via CTLA-4/CD28, ICOS-L/ICOS, and PD-L1 or 2/PD-1, respectively. Specialized cells with complete inhibitory function,  $T_{reg}$ s, modulate inflammatory responses to a large extent by the inhibitory mediators IL-10 and TGF- $\beta$ . Consequently the transfer of knowledge from basic research on inhibitory functions offers additional perspectives and improvements for sensitization assay development. The following applications with regard to inhibitory effects on T cells are conceivable. To receive a T cell response that could be validated against decays per minute or micrometers of ear swelling in the mouse LLNA and that could serve as constitutive basement for chemical classification comparable to the effective concentration (EC<sub>3</sub>) *in vivo*, a broad dynamic range of T cell parameters is likely to be required. Such a broader range for cytokine, chemokine, and proliferation measurement could be achieved by depletion of inhibitory  $T_{reg}$  cells from the assays by means of cell sorting. Another approach relies on the blocking of inhibitory interactions by specific antibodies or fusion molecules, thereby enlarging the range of mediator release and marker up- or downregulation. Further, the expression levels of inhibitory molecules such as PD-L1 and ICOS and their binding partners on DCs and T cells could be measured as direct indicators of sensitization. Moreover, co-inhibitory molecules, in parallel to co-stimulatory molecules, are supposed not only to signal in a one-way fashion from DCs to T cells. In contrast, inhibitory T cell-derived signals modulating a maturation status could also be assumed as a major influence on DCs by reverse signaling. Because of this feedback loop even in DC-only based sensitization assays, adding autologous T cells may have a great impact on the expression of DC-specific CD86 and other markers. Collectively, inhibitory molecules on DCs and T cells could be a useful part in a future sensitization test battery *in vitro*.

## 9.4 Conclusion

T cells are the pivotal cells in the process of sensitization and in elicitation of a type IV allergic reaction. Accordingly, the capacity of T cells to divide in response to allergens is exploited by the internationally accepted *in vivo* test method LLNA, an assay that measures proliferation of lymphocytes from draining lymph nodes in mice. By contrast, *in vitro* assays that are aimed at replacing the LLNA mainly apply DCs rather than T cells. This discrepancy is probably due to the complex handling of DC/T cell cocultures and the difficult readout of these assays. The development of *in vitro* assays using T cells is based on the complex mechanisms of

the adaptive immune system. There are several T cell subtypes involved, such as Th1, Th2, Th17, Th22, and T<sub>reg</sub>, and there is a complex interaction between the antigen-presenting cell and the T cell. Co-stimulatory molecules activate T cells and initiate the immune response, whereas co-inhibitory molecules regulate and terminate immune reactions. One of these co-inhibitory molecules, the negative regulator CTLA-4, was detected in human skin from individuals allergic against nickel or fragrances. In mice, CTLA-4 is mandatory to control contact hypersensitivity reactions. There exist further hints for the relevance of CTLA-4 in coculture systems afar from the area of allergy. In coculture systems of tumor cells and in HIV models, the interference of CTLA-4 signaling has been reported. Another co-inhibitor, ICOS-L, is expressed on DCs and induces IL-10-producing T<sub>reg</sub> cells, thereby executing an inhibitory role on T effector cell responses. The functional relevance of ICOS in hapten-induced ACD was demonstrated in ICOS<sup>+</sup> T<sub>reg</sub> cells that secreted IL-10, IL-17, and IFN- $\gamma$ . In addition, a strong suppressive potential was demonstrated in vitro and in vivo. Similar to ICOS and CTLA-4, PD-1 is a negative regulator of Th cell differentiation. Its ligand PD-L1 was found to inhibit proliferation of T cells and secretion of specific cytokines in both mouse and human coculture systems. Further, inhibitory capacities of PD-L1 were reported in model systems using viruses or particular contact allergens. Collectively, co-inhibitory molecules play an important role in the physiological shutdown of inflammation including ACD and have been already used in tumor and virus model cell assays. Therefore these molecules constitute promising tools for future sensitization assays when applying primary or cell line-derived DCs and T cells together in coculture systems.

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