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T Lymphocytes as Tools in Diagnostics and Immunotoxicology

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T Lymphocytes as Tools in Diagnostics and Immunotoxicology

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Chapter 1
The Use of T Cells in Hazard Characterization of Chemical and Drug Allergens and Integration in Testing Strategies

Foreword

Ian Kimber and Marc Pallardy

Abbreviations

APC	Antigen-presenting cell
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cells
IFN-γ	Interferon-gamma
LLNA	Local lymph node assay
MHC	Major histocompatibility complex
p-i concept	Pharmacological interaction concept
pMHC	Peptide major histocompatibility complex
TCR	T-cell receptor
TH1	T helper 1 lymphocyte
TH2	T helper 2 lymphocyte
Treg	T regulatory lymphocyte

1.1 General Introduction on the Role of T Lymphocytes

The activation of T lymphocytes expressing the αβ T-cell receptor (TCR) is a crucial event in the development of adaptive immune responses. T lymphocytes circulate throughout the organism and enter sequentially into contact with

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antigen-presenting cells (APC) displaying on their surface antigenic peptides bound to molecules of the major histocompatibility complex (MHC). The binding of TCR to specific peptide-MHC complexes (pMHC) triggers a cascade of intracellular signaling effectors leading to T-cell cytokine production, proliferation, and/or killing of target cells (Valitutti et al. 2010). However, for chemicals and drugs, the concept of antigenic structures presented to T cells by APC can vary compared to what is described for "classical" antigens. A chemical or a drug is not antigenic on its own because of its small size and it must first bind to a high molecular weight protein to be immunogenic. A stable covalent binding of a chemically reactive drug or chemical to a larger protein or peptide is then immunogenic and can be recognized directly by T cells after undergoing antigen processing of the hapten-carrier complex (hapten hypothesis). The chemical properties of hapten-like drugs are then crucial for the generation of antigenic epitopes and activation of the innate immune system. However, this is only one possibility for T-cell activation by chemicals, and other situations have been also described such as direct binding of chemicals to peptide loaded with HLA molecules, the p-i concept proposing that a drug is able to stimulate T cells directly without forming a hapten in a HLA-dependent manner, and the specific case of transition metals such as nickel forming coordination liaisons with HLA-loaded peptides and the TCR (Vocanson et al. 2009; Yun et al. 2012). These aspects will be addressed in this book by Christine Louis-Dit-Sully and Wolfgang W. A. Schamel in the chapter *Activation of the TCR Complex by Small Chemical Compounds* and also by H.-U. Weltzien, J.-F. Nicolas, and S. F. Martin in the chapter *T Cell Responses to Contact Allergens*. Identification of the haptenized peptides presented to T cells from circulating drug-bound proteins such as albumin found in allergic patients using specific methodologies such as mass spectrometry is also a very valuable approach to understand the mechanism underlying drug allergy, and this aspect is covered by C. J. Earnshaw, T. Pecaric-Petkovic, B. K. Park, and D. J. Naisbitt in the chapter *T-Cell Responses to Drugs and Drug Metabolites* in this book.

T cells are highly motile and scan most of the body spaces in search for rare APC presenting the antigens that will trigger their activation. Once the T-cell/APC cognate interaction is established, the spatial frame is restricted to the cellular interface, named the immunological synapse (Valitutti et al. 2010). At this specialized contact area, the TCR and multiple accessory molecules are simultaneously engaged, leading to the activation of varied and interconnected signaling pathways. These profound changes in spatial organization are instrumental for signaling in T cells and, most importantly, create a privileged site for cell–cell cooperation, allowing selectivity of T-cell responses. The time frame of T-cell activation encompasses molecular events happening within seconds and sustained for hours or days up to the acquisition of functional properties. Time plays a key role in the process of T-cell activation. The duration of TCR-mediated signal transduction in individual T cells (occurring on the scale of hours) and the duration of TCR/pMHC binding half-lives (on the scale of seconds) are crucial parameters in T-cell activation. Moreover, depending on the activation state of responding T cells, the quality of the presented antigen, the context of the antigenic stimulation, as well as duration

and "rhythm" of productive TCR signaling can profoundly influence activation and effector functions in T cells. Indeed, modification of the cellular microenvironment by chemicals can also influence and dictate the phenotype of DC and the type of immune response. When testing for T-cell activation by chemicals and drugs in vitro and according to what is known on T-cell biology and DC-T-cell interaction, the likely basis of an experimental model should be a DC-T-cell coculture model measuring T-cell activation parameters, T-cell proliferation, and cytokines/chemokines production. This paradigm is well exemplified in the chapter *Human T-Cell Priming Assay* described in this book but also in the chapter *Regulatory Role of Programmed Death-Ligand 1 in Dendritic Cell-T Helper Coculture Assays for Detection of Contact Allergy* by M. Peiser, M. Hitzler, and A. Luch. However, stimulating naïve T cells in vitro with chemicals is still a scientific and technical challenge.

The naive T-cell repertoire is extraordinarily diverse because it contains an enormous number of distinct T-cell clones, each represented by only a few cells (Arstila et al. 1999). This repertoire is mainly formed by positive and negative selections in the thymus leading to the generation of functional CD4+ T cells able to recognize foreign peptides presented by HLA class II molecules. Upon antigenic stimulation in secondary lymphoid organs, rare antigen-specific naive T cells undergo clonal expansion and differentiate to effector and memory cells. Thus, the memory T-cell repertoire contains a collection of expanded T-cell clones that reflect the antigenic experience of the individual. Molecular studies indeed established that the TCR diversity is at least 100-fold lower in the memory compared with the naive repertoire. The identification and characterization of antigen-specific T cells in the naive repertoire is of fundamental relevance to understanding the process of clonal selection and of practical relevance to predicting the immunogenicity of vaccines and therapeutic proteins. In chemical and drug allergy, it is tempting to speculate that the frequency of chemical- or drug-specific naive T cells present in a predisposed individual could be correlated to the strength of the T-cell response during the sensitization process (Nhim et al. 2013). However, due to the artificial nature of chemical T-cell epitopes, it is not clear whether selection of chemical-specific T cells is a common phenomenon or remains limited to few donors. Selection of chemical-specific T cells could be a relatively rare event accounting for the low occurrence of chemical allergy. On the other hand, a large T-cell repertoire found in multiple donors would underline the potential of chemicals to be recognized by many donors. These points are well described by S. F. Martin, S. S. Schmucker, and A. Richter in the *Tools and Methods for Identification and Analysis of Rare Antigen-Specific T Lymphocytes* chapter and by P. R. Esser, I. Kimber, and S. F. Martin in the *Human T-Cell Priming Assay. Correlation of Contact Sensitizer Potency with T-Cell Frequency and TCR Repertoire Diversity* chapter.

Regulatory T (Treg) cells function to maintain immune tolerance preventing autoimmune diseases and unwanted immune reaction to innocuous antigens and also prevent inflammatory diseases. Regulatory CD4+ T cells can be divided into two main subsets, natural Tregs (nTregs) and induced Tregs (iTregs), which develop in the thymus and the periphery, respectively (Campbell and Koch 2011).

Via the TCR, activated Tregs can exert non-antigen-specific bystander suppression of other T cells. Tregs have been shown to require cell-to-cell contact to suppress target cells in vitro. Binding of CTLA-4 on Tregs to CD80 and CD86 on effector T cells, infusion of cyclic AMP through gap junctions, or direct cytolytic mechanisms have been reported to be involved in Treg-mediated suppression. In addition, Treg expression of Neuropillin-1 facilitates cell contact-mediated suppression of DC. iTregs are generated from naïve CD4+ T cells in the periphery, where they acquire suppressive capacity. Data from several studies support the notion that iTregs are as important as nTregs in peripheral tolerance. iTregs are also important as they have been identified in situ and positively correlate with a clinical improvement of allergy symptoms. When developing in vitro assays to assess T-cell function in response to chemical allergens, it has been shown that deleting Tregs when initiating the assay may improve its sensitivity. M. Vocanson, A. Achachi, V. Mutez, M. Cluzel-Tailhardat, B. Levarlet, A. Rozières, P. Fournier, and J.-F. Nicolas have developed the human T-cell priming assay using these observations in the chapter *Human T-Cell Priming Assay. Depletion of Peripheral Blood Lymphocytes in CD25+ Cells Improves the In Vitro Detection of Weak Allergen-Specific T Cells*.

The differentiation of the CD4+ T-cell lineage into effector cells underlies successful adaptive immune responses aimed at distinct categories of pathogens. Their functional specialization is coordinated by genetic programs that use different transcription factors to direct expression of distinct soluble mediators and surface molecules that support interactions with other immune cells (Murphy and Stockinger 2010). The first paradigm for this functional diversification was the description of TH1 and TH2 CD4+ effector subsets by Mosmann and Coffman in 1986. TH1 cells were thought to be responsible for delayed-type hypersensitivity, activating macrophages through release of interferon (IFN)-γ and enabling them to kill intracellular pathogens. TH2 cells were considered the classical helper T cells providing help to B cells to generate class-switched antibodies. In the case of beta-lactam allergy, M. J. Torres Jaén, C. Mayorga, N. Blanca-López, and M. Blanca Gómez are showing that T cells are key factor in all types of hypersensitivity reactions to beta-lactams, regulating both IgE production or acting as effector cells, with a different profile of cytokine production. Indeed, in the chapter *Hypersensitivity Reactions to Beta-Lactams*, they described that a TH1 pattern is observed in non-immediate reactions to beta-lactams, whereas a TH2 pattern is expressed in CD4+ T cells in immediate reactions that are mostly IgE mediated.

1.2 How to Integrate In Vitro Tests Using T Cells in a Testing Strategy?

An excellent working definition of allergy is the adverse health effects that may result from the stimulation of an immune response.

For the practicing toxicologist, the main issues are chemical, protein and drug allergy, and the need to identify and characterize hazards and to develop effective risk assessment and risk management strategies.

Allergy develops in two phases. In the first phase, exposure to an allergen (at sufficient levels and via a relevant route of exposure) causes immunological priming and the acquisition of sensitization. If subsequently the now sensitized (primed) subject is exposed to the same material (again by a relevant route of exposure), then an allergic reaction can be elicited.

A key objective is, therefore, to have available methods that support the identification and characterization of materials (in the context of this book small molecular weight chemicals and drugs) that have the potential to induce the development of sensitization.

Historically animal models have been used. These have proved effective for the safety assessment of skin-sensitizing chemicals but less so for chemicals that cause sensitization of the respiratory tract and drug allergy. More recently, however, the scientific landscape has changed, and for various reasons there is an appetite (and in some instances a regulatory need) to develop alternative methods that will support effective safety assessments without a requirement for animal models.

The development of effective nonanimal methods for hazard identification has proven to be a significant challenge, and despite a very substantial and sustained investment, there are currently no fully validated alternative methods available (although the situation may change in the next 12 months). A greater challenge still is designing alternative test methods that, in addition to providing an accurate identification of hazard, also give an estimate of relative potency (an important first step in the risk assessment process).

Many strategies for alternative test method development have been described, and although attention to date has focused largely, although not exclusively, on skin sensitization, it is clear that success in that area would pave the way for innovations in the assessment of the potential for sensitization of the respiratory tract and drug allergy.

With the overall objective of designing a method, or a panel of methods, that will provide a sound basis for safety assessment, the aim is to model in vitro or in silico pivotal events or processes that are required for the effective acquisition of sensitization. Using skin sensitization as an example, it is possible to identify a variety of critical steps along the biological pathway to sensitization, and these include (a) the ability of chemicals to gain access to the viable epidermis; (b) the native or acquired ability of chemicals to form stable associations with macromolecules to generate an immunogenic complex; (c) the triggering by chemicals of danger signals and the innate immune system as a prelude to the initiation of an adaptive immune response; (d) the activation, mobilization, and maturation of DC populations that are able to process, deliver, and present antigen to the adaptive immune system; and (e) the elicitation of T lymphocyte responses of the necessary vigor and quality to induce immunological priming and the acquisition of sensitization.

In each of the above areas, in vitro and/or in silico approaches have been proposed. It is beyond the scope of this short introduction to provide a survey of

the various experimental systems that have been developed. Rather, the focus here is on the last of these approaches and the activation by allergen of T lymphocyte responses.

T lymphocyte activation (and subsequent clonal expansion and functional differentiation) is the hallmark of sensitization. Thus, for instance, the animal assay that is most commonly used for the assessment of skin-sensitizing potential (the mouse local lymph node assay LLNA) has as an endpoint the proliferation of lymphocytes in lymph nodes draining the site of exposure to a chemical. This works well, in vivo, and it has been shown that the vigor of proliferative responses in regional lymph nodes correlates causally with the acquisition of sensitization.

Translating this to an in vitro approach is, however, a challenge and the subject of this book.

It is true that it is possible to measure antigen-/allergen-induced T lymphocyte responses in vitro when the responder cells are taken from the peripheral blood of a subject that is already primed immunologically. Such responses are facilitated by the fact that prior immunological priming has caused a selective clonal expansion of antigen-reactive lymphocytes. That is, there has already been in vivo a quantitative increase in the number of cells that are able to respond the priming antigen. For this reason there are sufficient numbers of responsive cells to allow discernment of antigen-induced proliferation in vitro.

Such recall proliferative responses by lymphocytes in vitro (frequently referred to as lymphocyte transformation tests) require that expanded cells are available from a primed donor. In the context of developing alternative approaches to skin sensitization testing based on T lymphocyte responses in vitro, it is, of course, not possible to use animals (or humans) exposed previously to the test chemical. The need, therefore, is to develop experimental strategies that will permit the elicitation by chemicals of primary responses by naïve T lymphocytes, that is, responses by T lymphocytes that have not previously been primed and expanded in response to the relevant chemical allergen. This is a significant technical challenge, and addressing that challenge is the major focus of this book.

Given the scale of the technical problems associated with measuring naïve T-cell responses to chemical allergens (and in particular ensuring an appropriate signal to noise ratio), it could be argued that other experimental approaches may be more attractive and certainly more tractable. In answer, however, there are two strong reasons why it is appropriate to persevere with approaches based on measurement of T lymphocyte responses in vitro.

The first is that, as indicated above, the T lymphocyte response to allergen is the hallmark of sensitization. The other reason is this. For effective hazard characterization, it is important to understand the relative sensitizing potency of an allergen, and indeed an appreciation of potency is a key requirement for effective risk assessment (Kimber et al. 2012). The LLNA has been shown to provide a basis not only for the identification of skin-sensitizing hazards but also for the measurement of relative potency. This is because the vigor of T lymphocyte proliferation in draining lymph nodes correlates causally and quantitatively with the development of sensitization, and because of this proven quantitative relationship, it is possible to

derive from the LLNA an accurate assessment of sensitizing potency. The expectation is, therefore, that if primary T lymphocyte responses can be measured accurately in vitro, then it might be possible not only to identify potential hazards, but also to support effective risk assessment through provision of information on sensitizing potency.

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Chapter 2
Activation of the TCR Complex by Peptide-MHC and Superantigens

Christine Louis-Dit-Sully, Britta Blumenthal, Marlena Duchniewicz, Katharina Beck-Garcia, Gina J. Fiala, Esmeralda Beck-García, Markus Mukenhirn, Susana Minguet, and Wolfgang W. A. Schamel

Abstract Drug hypersensitivity reactions are immune mediated, with T lymphocytes being stimulated by the drugs via their T-cell antigen receptor (TCR). In the nonpathogenic state, the TCR is activated by foreign peptides presented by major histocompatibility complex molecules (pMHC). Foreign pMHC binds with sufficient affinity to TCRαβ and thereby elicits phosphorylation of the cytoplasmic tails of the TCRαβ-associated CD3 subunits. The process is called TCR triggering. In this review, we discuss the current models of TCR triggering and which drug properties are crucial for TCR stimulation. The underlying molecular mechanisms mostly include pMHC-induced exposure of the CD3 cytoplasmic tails or alterations of the kinase-phosphatase equilibrium in the vicinity of CD3. In this review, we also discuss triggering of the TCR by small chemical compounds in context of these general mechanisms.

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2.1 Introduction

Drug hypersensitivity reactions are a health problem worldwide, but they are difficult to predict and to cure. These reactions are immune mediated, i.e., the drug stimulates the immune system in an undesired way. The major responsive cells are T lymphocytes, being either directly or indirectly stimulated by the drug via their T-cell antigen receptor (TCR).

The TCR is a multiprotein transmembrane complex comprising the TCRαβ (or TCRγδ), CD3εδ, CD3εγ, and CD3ζζ dimers (Alarcon et al. 2003; Kuhns and Davis 2012) (Fig. 2.1a) and facultatively the $TRIM_2$ dimer (Swamy et al. 2010). TCRαβ possesses variable immunoglobulin domains that bind the ligand, an antigenic peptide bound to major histocompatibility complex (pMHC) molecules. TCRαβ forms contacts to the peptide as well as to the MHC (Garboczi et al. 1996; Garcia et al. 1996) (Fig. 2.1b). The CD3 chains contain tyrosine residues in their cytoplasmic tails, that are phosphorylated upon successful ligand binding to TCRαβ and that transmit the signal inside the cell. These tyrosines are part of the immunoreceptor tyrosine-based activation motif (ITAM) (Reth 1989) and are phosphorylated by the kinase Lck belonging to the Src-family (Iwashima et al. 1994). Phosphorylation is the critical event in initiating downstream signaling cascades, since phosphotyrosines serve as binding sites for proteins with src homology 2 (SH2) domains. Consequently, these proteins (e.g., the kinase ZAP-70) are recruited to the receptor and activate signaling pathways, such as activation of phospholipase C γ (PLCγ, Fig. 2.1b) and consequent calcium influx into the cytosol, resulting in the activation of the T cell. In addition, the CD3ε chain has a proline-rich sequence in its cytoplasmic tail that can bind to the signaling molecule Nck (Gil et al. 2002). For the TCR (as well as for most receptors), it is still not well understood which biochemical changes are induced by ligand binding to the receptor, that are transmitted via the transmembrane regions to the cytoplasmic tails, thereby leading to the phosphorylation of the tails. However, a number of ideas and reasonable models have been put forward for the TCR, which we will discuss here.

For each model we discuss which properties a drug needs to possess, in order to stimulate the TCR. An overview on how drugs modify pMHC, in order to activate the TCR, is found in our second review entitled "Activation of the TCR complex by small chemical compounds."

2.2 Selection of the TCR Specificity in the Thymus

During the development of T cells in the thymus, the genes encoding for the TCRβ and TCRα chains are randomly rearranged (mutated), so that TCRs with any random specificity are generated. This includes TCRs that strongly bind to MHC loaded with self-peptides derived from endogenous proteins. Thus, T cells

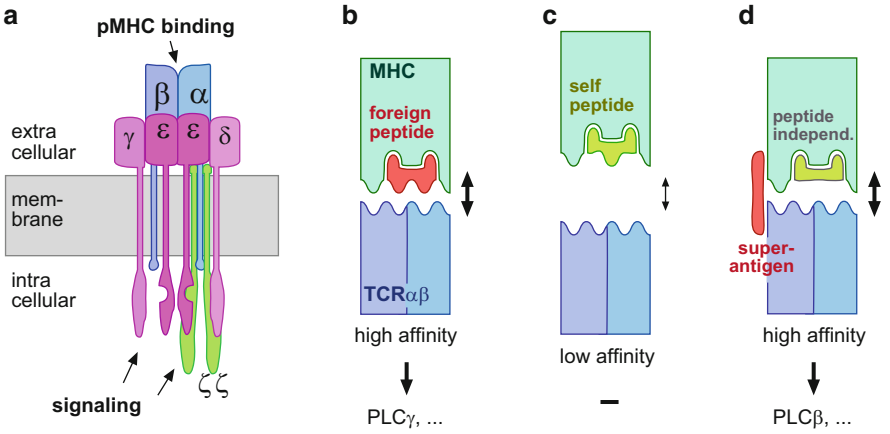

Fig. 2.1 The T-cell antigen receptor (TCR). (**a**) In its minimal form, the TCR is composed of the pMHC-binding TCRαβ and the signal-transducing CD3εδ, CD3εγ, and CD3ζζ dimers. (**b**) Foreign peptides bound to MHC have a high affinity for an appropriate TCR. Both the peptide and the MHC molecule have contacts with TCRαβ, triggering intracellular signaling events, such as the activation of PLCγ and other proteins, leading to T-cell activation. (**c**) Due to negative selection in the thymus, self-peptide-MHC only has a weak affinity for TCRs in mature T cells. Self-pMHC does not perfectly fit to TCRαβ, thus not triggering their TCR. (**d**) Superantigens simultaneously bind to MHC in a peptide-independent manner and to the constant regions of TCRαβ. Thus, pMHC is bridged to the TCR largely independent of pMHC-TCRαβ contacts. Superantigen stimulation leads to the activation of PLCβ and other proteins, resulting in T-cell activation

expressing these TCRs are stimulated in the thymus by self-pMHC, resulting in their death. This process is called negative selection. In sharp contrast, T cells possessing TCRs that weakly bind to self-pMHC survive this process and are positively selected (Starr et al. 2003). Further maturation and subsequent tissue allocation leads to the directed distribution of matured T cells in the body. This process ensures that TCRs do not bind strongly to any self-peptides loaded onto MHC molecules in the periphery, preventing autoimmune reactions (Fig. 2.1c). If a TCR binds strongly to pMHC in the periphery, it most likely is a foreign peptide, such as derived from viruses or bacteria, being loaded onto MHC (Fig. 2.1b). Hence, a T cell whose TCR is strongly triggered in the periphery will become activated and will initiate an immune response.

Thus, drugs that alter self-pMHC, so that a potent ligand for the TCR is generated, will stimulate T cells, and this leads to the hypersensitivity reactions observed for a number of different drugs or metal ions.

2.3 TCR Triggering Models

The TCR can be activated (i.e., the tyrosine residues of the CD3 chains can be phosphorylated) using a number of different ligands, such as membrane-bound pMHC, soluble pMHC multimers, anti-TCRαβ antibodies, and bacterial superantigens (Fig. 2.1d) (all binding to TCRαβ) or anti-CD3 antibodies. Consequently, a number of mechanistic models have been put forward to explain how the TCR is triggered, i.e., how ligand binding at TCRαβ causes CD3 phosphorylation.

2.3.1 The Homoclustering Model of TCR Triggering

Early experiments showed that bivalent anti-TCRαβ or anti-CD3 antibodies can activate T cells, whereas monovalent Fab fragments of these antibodies fail to do so (Chang et al. 1981; Kaye and Janeway 1984). Likewise, the TCR is only activated by bi- or multivalent soluble, recombinant pMHC (Boniface et al. 1998; Cochran et al. 2000). These experiments indicated that the soluble ligands for the TCR have to be multivalent, in order to be functional. This implies that two or more TCRs have to bind simultaneously to one ligand molecule in order to be activated. In conjunction with the hypothesis that individual receptor molecules are distributed equally on the cell surface, these findings led to the cross-linking (homoclustering) model of TCR activation (Ashwell and Klausner 1990) (Fig. 2.2a). However, it was shown that the TCR can be pre-clustered in the resting, non-ligand-bound state (Alarcon et al. 2006; Lillemeier et al. 2010; Molnar et al. 2010, 2012; Schamel et al. 2005), which is standing in contrast to this model. Nevertheless, these TCR nanoclusters can be co-expressed with TCR monomers (Fig. 2.2b).

According to the homoclustering model, small chemical compounds would need to bind to two adjacent self-peptide-MHC molecules, in order to allow both of them to simultaneously bind to two TCRs.

2.3.2 Conformational Change Models of TCR Triggering

How can the requirement for a multivalent ligand and the presence of preformed oligomeric receptors be integrated into a unique model? One intriguing possibility is that binding of bivalent (or multivalent) ligands leads to a reorientation of one TCR in respect to the second TCR. The consequent alteration of the quaternary structure of the receptors might lead to conformational changes within the cytoplasmic tails of CD3, therefore exposing the tyrosines to allow phosphorylation (Minguet and Schamel 2008; Minguet et al. 2007). Otherwise, the CD3 cytoplasmic tails are in a closed conformation and not accessible to kinases. This model is called the permissive geometry model (Minguet and Schamel 2008) (Fig. 2.3). According

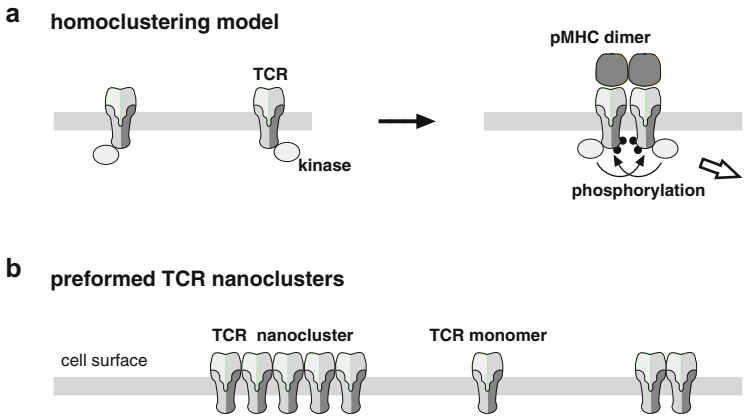

Fig. 2.2 The homoclustering model and TCR nanoclusters. (**a**) Monovalent TCRs are individually expressed on the cell surface. Stimulation by bivalent (or multivalent) pMHC leads to clustering of the TCRs. Consequently associated kinases can phosphorylate each other and the CD3 subunits. Monovalent pMHC do not cluster the TCRs and thus do not induce CD3 phosphorylation. *Black dots* represent phosphorylated tyrosine residues. The *open arrow* shows activation of downstream signaling cascades. (**b**) On the T-cell surface, the TCR is expressed as a mixture of monomers and nanoclusters of different sizes. Thus, the requirement for the homoclustering model is not fulfilled

to this model, monovalent ligands are not capable of reorienting one TCR in respect to another one and are thus inactive. The main inspiration for the conformational change model was the seminal finding that a proline-rich sequence in the cytoplasmic tail of CD3ε becomes exposed upon multivalent TCR stimulation (Gil et al. 2002). The permissive geometry model is supported by the finding that an artificially selected pMHC, binding in a different geometry to TCRαβ, cannot trigger the TCR (Adams et al. 2011).

A "lipid-based model" proposes that the tyrosines of the cytoplasmic tails of CD3ε and ζ are embedded in the inner leaflet of the plasma membrane and are thus not accessible for phosphorylation. Indeed, peptides corresponding to these tails bind to and partially integrate into artificial membranes (Aivazian and Stern 2000; DeFord-Watts et al. 2011; Xu et al. 2008). pMHC binding to the TCR would modulate the lipid environment of the TCR, so that the tyrosines become exposed and available for phosphorylation (Shi et al. 2013).

If drugs and other small compounds activate TCRs according to the permissive geometry model, they would have to approximate two pMHC molecules to two TCRs, in a way that structural changes in the TCR/CD3s are induced. Considering the "lipid-based model." the small chemical compounds have to mimic or modify pMHC in a way that binding to the TCR frees the cytoplasmic tails from being shielded by the membrane.

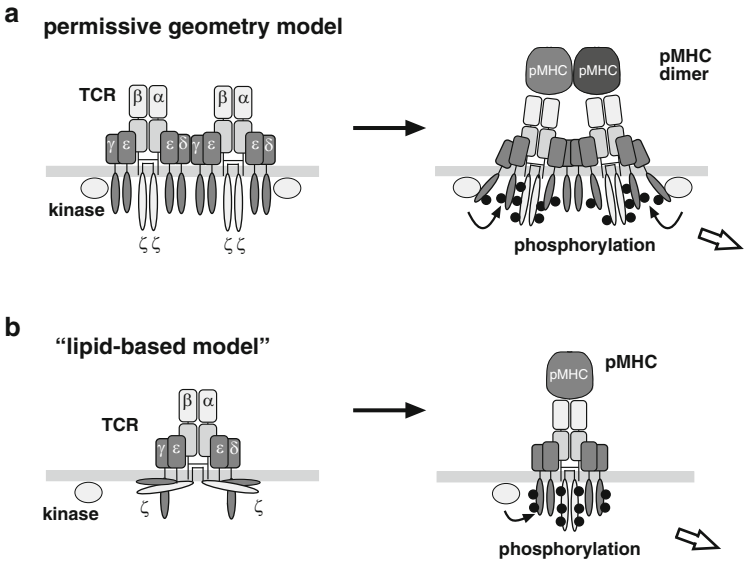

Fig. 2.3 Conformational change models. (**a**) The permissive geometry model. In the resting cell the cytoplasmic tails of CD3 are in a closed conformation, preventing phosphorylation. Bivalent (or multivalent) pMHC reorientates two TCRαβ towards each other without changing the structure of the individual TCRαβ. This reorientation induces conformational changes in the cytoplasmic tails of CD3, exposing the tyrosines, which now become available for phosphorylation. Since monomeric MHCp does not engage several TCRαβ simultaneously, it does not induce these conformational changes in CD3 (not shown). (**b**) In the "lipid-based model," the tyrosines of the cytoplasmic tails of CD3ε and ζ are embedded within the membrane, thus not being accessible for the kinases. pMHC binding to the TCR removes the cytoplasmic tails from the membrane, e.g., by modulating the lipids, thus making the tyrosines available for phosphorylation

2.3.3 The Pseudodimer Model of TCR Triggering

In addition to a possible foreign peptide-MHC, an antigen-presenting cell (APC) and other cells can also present self-peptide-MHC. In contrast to foreign pMHC, the affinity of a self-pMHC-TCR interaction is too low to stimulate a TCR. Mostly, foreign peptides are presented next to self-peptides, although there is a possibility for enriching foreign peptides in certain membrane areas (Lu et al. 2012). Importantly, self-peptides aid in the recognition of foreign peptides by the T cell (Irvine et al. 2002; Stefanova et al. 2002; Wülfing et al. 2002). Indeed, it was shown that soluble pMHC dimers with one agonistic (foreign, high affinity) and one self-peptide (low affinity) could stimulate TCRs (Krogsgaard et al. 2005). This underlying model is called pseudodimer model (Fig. 2.4a) (Irvine et al. 2002) and is a variant of the homoclustering model. Furthermore, the permissive geometry model could also allow simultaneous binding of one foreign and one self-peptide loaded onto MHC, in order to induce the structural changes at CD3 (Minguet and Schamel 2008).

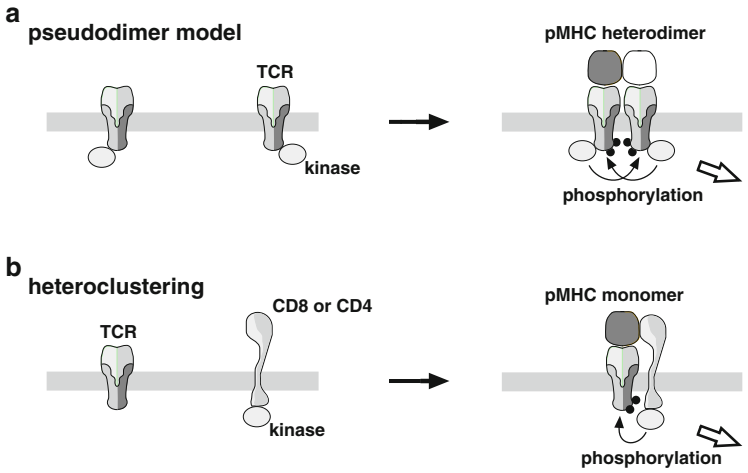

Fig. 2.4 The pseudodimer and heteroclustering models. (**a**) In the pseudodimer model TCRs are individually expressed. Upon bivalent (or multivalent) MHCp binding, TCRs become clustered and kinases can phosphorylate CD3 (see also the homoclustering model). On the APC foreign (high affinity), peptides are presented on MHC next to self (low affinity) peptides. Thus, heterodimeric pMHCs, with one foreign and one self-peptide, activate the TCR. In the original model, binding of CD4 to the heterodimeric pMHC aids in binding to the TCR (see also the heteroclustering model). (**b**) In the heteroclustering model, TCRs are not constitutively associated to kinases, but the co-receptors CD8 and CD4 are. Monomeric (and multimeric) pMHC simultaneously engages the TCR and CD8/CD4. Hence, the kinase is brought into the vicinity of the TCR allowing phosphorylation of CD3 with subsequent activation of the T cell

According to this model, it would be sufficient that small chemical compounds only bind to one pMHC of a preformed pMHC cluster, thus inducing high affinity binding of this pMHC with one TCR. The other TCR would be bound by a self-pMHC.

2.3.4 The Heteroclustering Model of TCR Triggering

As well as binding to the TCR, pMHC also binds with its constant regions to the CD8 and CD4 co-receptors. The simultaneous binding of pMHC to the TCR and CD8/CD4 could lead to a heteroclustering of the TCR with CD8/CD4. The CD8 and CD4 cytoplasmic tails interact constitutively with Lck (Kim et al. 2003) that, when recruited to the TCR, could phosphorylate CD3 (heteroclustering model, Fig. 2.4b). The findings that monomeric pMHC does not activate TCRs (Boniface et al. 1998; Cochran et al. 2000) and that anti-TCRαβ or anti-CD3 antibodies stimulate TCRs clearly argue against the heteroclustering model.

In contrast to mature T cells, all Lck is CD4- or CD8-bound in thymocytes (Van Laethem et al. 2007). Thus, in thymocytes the co-receptors have to be recruited, in

order to bring Lck in the vicinity of the TCR. Most likely, this occurs simultaneously to another triggering event, such as exposure of the CD3 tyrosines.

If one aims to explain the activity of small chemical compounds using the heteroclustering model, then the compound would need to induce high affinity binding of one self-pMHC to one TCR and at the same time allow binding of the pMHC to either CD8 or CD4.

2.3.5 The Kinetic Segregation Model of TCR Triggering

The physiological ligand for the TCR is a membrane-bound pMHC molecule. However, due to technical reasons, most experiments to elucidate the mechanism of TCR triggering are done with soluble pMHC (see above). We think that the obtained results still hold true for membrane-bound pMHC, since key findings also hold true for stimulations with membrane-bound pMHC. For example, stimulation of T cells by APCs led to the exposure of the proline-rich sequence in CD3ε (Risueno et al. 2005), thus inducing a similar conformational change to stimulation with soluble pMHC multimers (Gil et al. 2002); and MHC class I and class II molecules form clusters on the surface of the APCs (Krishna et al. 1992; Schafer et al. 1995); therefore, the requirement for multivalent pMHC binding to TCRs seems to hold true. Anyhow, considering membrane-bound pMHC allows formulating additional models of TCR triggering (Choudhuri and van der Merwe 2007).

CD3 phosphorylation by kinases is counteracted by phosphatases (Mustelin et al. 2004). The main phosphatase in the plasma membrane of lymphocytes is CD45 (Trowbridge and Thomas 1994). Therefore, antigen binding could result in removal of the phosphatase from the vicinity of the TCR (Davis and van der Merwe 2006), thus initiating signal transduction. The TCR and pMHC are small molecules (Garboczi et al. 1996; Garcia et al. 1996), whereas CD45 has a bulky ectodomain (Davis and van der Merwe 2006). If the TCR and pMHC interact, then CD45 has to be "squeezed out" from these so-called close-contact zones (Davis and van der Merwe 2006) or microclusters (Yokosuka et al. 2005) (Fig. 2.5a). Indeed, confocal microscopy has shown that CD45 is excluded from TCR microclusters, where signaling is initiated at the immune synapse (Varma et al. 2006) (please note that formation of the synapse is a consequence of TCR triggering and not the cause). This model is called the kinetic segregation model since TCRs and kinases are temporally segregated from phosphatases, allowing CD3 phosphorylation. The model is supported by the finding that elongation of MHCp or shortening of CD45 results in inhibition of TCR triggering (Choudhuri et al. 2005; James and Vale 2012).

According to the kinetic segregation model, small chemical compounds presented by APCs need to be small enough so that the T cell and APC membranes come into close contact where the large phosphatases are excluded.

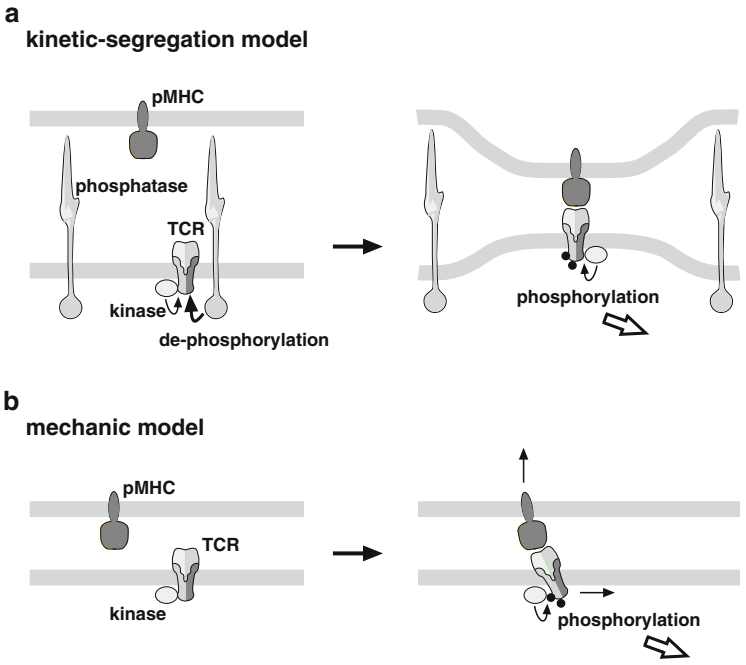

Fig. 2.5 The kinetic segregation and mechanic models. (**a**) TCRs, kinases, and transmembrane phosphatases intermingle on the membrane, resulting in constitutive phosphorylation and dephosphorylation of CD3. When the TCR interacts with membrane-bound pMHC, the two opposing membranes come into close contact. The small intermembrane distance at the TCR does not provide enough space for the large phosphatases. Therefore, phosphatases are segregated from the TCRs, resulting in accumulation of phosphorylated CD3. TCRs that do not bind to pMHC rapidly move out of these close-contact zones. In contrast, pMHC-bound TCRs stay for a sufficient amount of time inside the phosphatase-free areas. (**b**) Mechanical forces are applied to the TCR that change its structure allowing kinases to phosphorylate CD3. In the different mechanic models, it is either the APC that moves away from the T cell or it is the TCR being laterally moved by the actin cytoskeleton

2.3.6 Mechanical Models of TCR Triggering

Another model includes mechanical forces that are generated when pMHC binds to the TCR (Kim et al. 2009; Ma and Finkel 2010; Wang and Reinherz 2012). These forces distort the TCR, inducing conformational changes that are transmitted to the cytoplasmic domains (Fig. 2.5a). The forces are generated by a movement of either the TCR, e.g., mediated by the actin cytoskeleton, or the pMHC, e.g., by movements of the APC.

In contrast to the permissive geometry and other models, these membrane-bound pMHC-based models would be in line with monomeric pMHC stimulating the TCR. However, they fail to explain T-cell activation via soluble anti-TCRαβ or anti-CD3 antibodies (Chang et al. 1981; Kaye and Janeway 1984) or soluble bi- or

multivalent pMHC (Boniface et al. 1998; Cochran et al. 2000; Krogsgaard et al. 2005; Minguet et al. 2007).

According to the mechanical models, it would be sufficient if drugs modify self-pMHC in a manner that they would bind to TCRs with high affinity, without any further constraints.

2.3.7 TCR Triggering by Superantigens

Superantigens, also called enterotoxins, are secreted by *Staphylococcus aureus* and *Streptococcus pyrogenes* and can trigger a massive immune response that can lead to lethal toxic shock. This is caused by a peptide-independent activation of the TCR (Fraser and Proft 2008; Petersson et al. 2004). Enterotoxins bind both to the MHC class II and the outer face of TCR Vβ domains, triggering the TCR (Fig. 2.1d). The activated T cells divide and produce cytokines. As superantigens activate all T cells expressing certain Vβ domains (up to 40 % of all T cells), they can cause the toxic shock. Since superantigens bridge the TCR and MHC, all of the abovementioned models potentially hold true. However, superantigens approximate the TCR and MHC in a different geometry compared to the TCR-peptide-MHC interaction (Saline et al. 2010; Wang et al. 2007) and simultaneously to the TCR and MHC also bind to the co-receptor CD28 (Arad et al. 2011).

These differences might contribute to the fact that superantigens partially stimulate different signaling pathways than pMHC. Superantigens induce the canonical "pMHC-TCR" signaling pathways, which includes phosphorylation of CD3 by Lck, recruitment of ZAP70, and activation of PLCγ (Fig. 2.1b). In addition, a second Lck-independent pathway is triggered, in which the small heterotrimeric G protein Gαq11 is activated, leading to the stimulation of PLCβ (Bueno et al. 2007, 2006) (Fig. 2.1d). Both, PLCγ and PLCβ, cleave inositol lipids resulting in the increase in cytoplasmic calcium concentrations—one of the important second messengers in T-cell activation.

Considering superantigen-mediated TCR triggering, small chemical compounds could induce self-pMHC binding to TCRαβ in any orientation and may be even independent on any peptide bound to the MHC molecule.

2.4 TCRαβ Binds to pMHC in a Conserved Orientation

pMHC always binds in a diagonal orientation to TCRαβ, with variations in the binding angle of only 35° (Rudolph et al. 2006). The N-terminus of the peptide binds to TCRα and the C-terminus to TCRβ. Whether this orientation is germ line-encoded and dictated by semi-conserved TCRαβ-MHC interactions (Garcia 2012; Scott-Browne et al. 2009) or whether this orientation is necessary to activate the TCR (Minguet and Schamel 2008) is unknown.

Our data suggested that the diagonal orientation with which TCRαβ binds to pMHC is crucial for the structural change and the activation of the TCR (permissive geometry model) (Minguet et al. 2007). Since superantigens lead to an approximation of MHC with the TCR in different orientations, they might partially activate the TCR in a different manner. If true, then drug-induced TCR activation might be accomplished by pMHC binding to TCRαβ in different geometries.

2.5 The Kinetics of the pMHC-TCRαβ Interactions Determine the Stimulation Outcome

A high affinity pMHC-TCRαβ interaction leads to T-cell activation, as observed with foreign, agonistic pMHC. In contrast, a low affinity pMHC-TCRαβ interaction does not induce T-cell activation, as seen with self-peptide-MHC molecules, preventing autoimmune reactions (see above). How can the TCR interpret different affinities? One solution is the kinetic proofreading scheme (Dushek et al. 2011; McKeithan 1995). High affinity pMHC would be bound for sufficient time to a TCR, to allow forming an activatory signalosome at the TCR. Low affinity pMHC would only bind for short time, so that the signalosome cannot form and an activatory signal is not generated. Thus, the half-life of the pMHC-TCRαβ interaction is crucial in discriminating between high and low affinity ligands.

Recently, it has been argued that also the on-rate of the pMHC-TCRαβ interaction might play a role (Aleksic et al. 2010). Thus, small chemical compounds might need to alter self-pMHC, so that the modified pMHC binds for sufficient amount of time to the TCR. This would then translate into a high affinity interaction. Likewise, a fast on-rate might be beneficial.

2.6 TCR Triggering by Non-MHC Molecules

In mature T cells, a lot of the kinase Lck molecules are not bound to CD4 or CD8 and thus are free to interact and phosphorylate the TCR. In contrast, all Lck molecules are bound to CD4 and CD8 in developing T cells, called thymocytes (Van Laethem et al. 2007). Thus, stimulation of the TCR for positive selection in thymocytes requires the co-engagement of pMHC with the TCR and CD8/CD4, in order to allow Lck to approximate the TCR. Still, the TCR triggering models discussed above could hold true, such as that the cytoplasmic tyrosines of CD3 have to be exposed (Aivazian and Stern 2000; Minguet and Schamel 2008). In a recent experiment, MHC as well as CD4 and CD8 were deleted in the mouse, so that free Lck molecules are present in the thymocytes (Tikhonova et al. 2012). This then allowed T cells to develop that do not recognize pMHC, but for instance

CD155. This experiment clearly shows that TCRs can be triggered by other molecules than MHC.

Stimulation of TCRs by non-MHC molecules is also in line with the activating capacity of anti-TCRαβ and anti-CD3 antibodies (Chang et al. 1981; Kaye and Janeway 1984) or activation of a chimeric TCR by artificial ligands (Minguet et al. 2007).

These findings open the interesting possibility that drugs could also alter other proteins than pMHC in order to generate high affinity ligands for TCRαβ or even for CD3.

2.7 Conclusion

Although triggering of the TCR is one of the most important events in adaptive immunity, there is still no consensus on the underlying molecular mechanism. This is not due to lack of interest, but due to the fact that the TCR is one of the most complicated transmembrane receptors known to date. In fact, a large number of models have been put forward that mostly are not mutually exclusive. Thus, a combination of the proposed mechanisms might be close to what happens in reality.

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Chapter 3
Activation of the TCR Complex by Small Chemical Compounds

Christine Louis-Dit-Sully and Wolfgang W.A. Schamel

Abstract Small chemical compounds and certain metal ions can activate T cells, resulting in drug hypersensitivity reactions that are a main problem in pharmacology. Mostly, the drugs generate new antigenic epitopes on peptide-major histocompatibility complex (MHC) molecules that are recognized by the T-cell antigen receptor (TCR). In this review we discuss the molecular mechanisms of how the drugs alter self-peptide-MHC, so that neo-antigens are produced. This includes (1) haptens covalently bound to peptides presented by MHC, (2) metal ions and drugs that non-covalently bridge self-pMHC to the TCR, and (3) drugs that allow self-peptides to be presented by MHCs that otherwise are not presented. We also briefly discuss how a second signal—next to the TCR—that naïve T cells require to become activated is generated in the drug hypersensitivity reactions.

3.1 Introduction

Adverse drug reactions are a major health problem worldwide, but they are difficult to predict. The propensity for an individual to develop a reaction depends on the chemistry of the drugs or chemicals, on environmental factors, and on the biology of the patient. A good proportion of these reactions are immune mediated, which are also called allergic drug reactions or drug hypersensitivity reactions. These drug

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hypersensitivity reactions are due to several distinct immune mechanisms, but all types eventually involve stimulation of T cells by the drug.

For a T-cell-dependent immune response to occur, the drug must stimulate the T-cell antigen receptor (TCR). Drugs, which are usually small chemical compounds of low molecular weight (less than 1,000 Da) and metal ions, are thought to be too small to be antigenic per se. How they are able to stimulate an immune response often is an important open question left to answer.

The TCR is composed of the TCRαβ (or TCRγδ), CD3εδ, CD3εγ, and CD3ζζ dimers (Alarcon et al. 2003) (Fig. 3.1a). TCRαβ possesses variable immunoglobulin domains that bind the ligand, an antigenic peptide bound to major histocompatibility complex (pMHC) molecules (Garboczi et al. 1996; Garcia et al. 1996) (Fig. 3.1b). Foreign peptides, such as derived from viruses or bacteria, presented by MHC have a high affinity for their appropriate TCR and thus elicit an immune response. Peptides derived from endogenous proteins (self-peptides) are also presented. However, due to negative selection processes in the thymus, mature T cells only show weak affinity to self-peptide-MHC, so that T cells are not stimulated and autoimmune diseases are prevented. In addition, superantigens can bridge MHC and TCR peptide independently and stimulate T cells (Fig. 3.1c).

The CD3 chains contain tyrosine residues in their cytoplasmic tails, that are phosphorylated upon successful ligand binding to TCRαβ and that transmit the signal inside the cell. Phosphorylation of the tyrosines in these cytoplasmic tails is the critical event initiating signaling cascades. Phosphotyrosines serve as binding sites for signaling proteins with Src-homology 2 (SH2) domains.

The molecular mechanism as to how ligand binding induces CD3 phosphorylation is still a matter of debate. In the conformational change models (Aivazian and Stern 2000; DeFord-Watts et al. 2011; Gil et al. 2002; Minguet et al. 2007; Schamel et al. 2006; Xu et al. 2008), pMHC binding induces a structural change in CD3 that enables phosphorylation of the cytoplasmic domains. In other models the kinase-phosphatase equilibrium is disturbed in the vicinity of the TCR by excluding phosphatases (Choudhuri and van der Merwe 2007; Choudhuri et al. 2005; Davis and van der Merwe 2006; James and Vale 2012) or by enhancing the concentration of kinases (Boniface et al. 1998; Cochran et al. 2000; Irvine et al. 2002). These models are discussed in detail in the preceding review "Activation of the TCR complex by peptide-MHC and superantigens."

Here, we want to discuss models, which have emerged in the recent years to explain how small chemicals or metals can stimulate the TCR.

3.1.1 Chemicals Acting as Haptens

The term "hapten" was introduced by Landsteiner and Jacobs in 1935. Haptens are chemically reactive compounds that form a covalent bond with endogenous proteins. In 1992 it was demonstrated that hapten recognition by T cells required covalent hapten attachment to MHC-associated peptides (Ortmann et al. 1992).

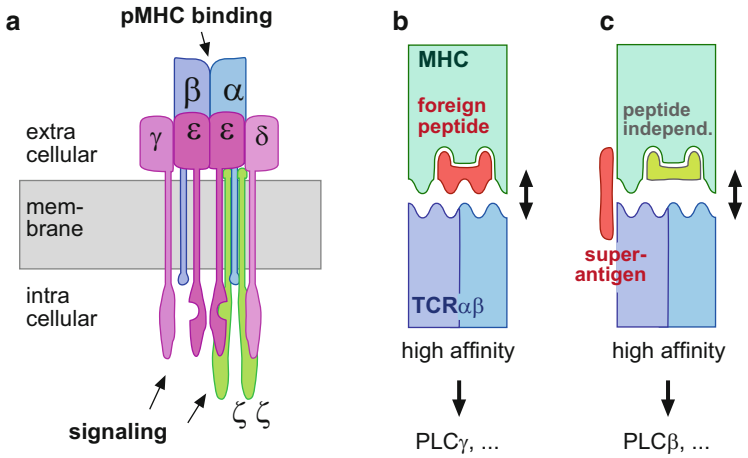

Fig. 3.1 The T-cell antigen receptor (TCR). (**a**) The TCR comprises the pMHC-binding TCRαβ and the signal-transducing CD3εδ, CD3εγ, and CD3ζζ dimers. (**b**) Foreign peptide-MHC has a high affinity for the responding TCR. The peptide and the MHC molecule have contacts with TCRαβ, triggering intracellular signaling events, such as the activation of PLCγ and other signaling proteins, leading to T-cell activation. (**c**) Superantigens simultaneously bind to MHC in a peptide-independent manner and to the constant regions of TCRαβ. Thus, pMHC is bridged to the TCR largely independent of pMHC-TCRαβ contacts. Superantigen stimulation leads to the activation of PLCβ and other signaling proteins, resulting in T-cell activation

In general hapten-modified proteins are processed by the antigen-presenting machineries and haptenated peptides displayed on MHC class I or II to T cells. In contrast to self-pMHC, which only weakly binds to the TCR and does not stimulate T cells (Fig. 3.2a), haptenated self-pMHC can possess strong binding to an appropriate TCR, and the T cell is stimulated (Fig. 3.2b). These haptenated self-pMHCs were absent during thymocyte development and negative selection, so that the specific TCRs reacting to these haptenated self-pMHCs were not removed from the T-cell population. One example for haptens are β-lactam antibiotics, such as penicillin, which covalently bind to lysine residues of many proteins, such as serum albumin (Batchelor et al. 1965; Jenkins et al. 2009; Levine and Ovary 1961; Schneider and De Weck 1965). One study, using the synthetic penicillin Flucloxacillin, showed that the modification of the lysine residues in human serum albumin occurs in a dose-, time-, and site-dependent manner (Jenkins et al. 2009). However, the exact mechanisms for the immune responses to penicillin are still not clear and create difficulties in the development of effective diagnostics methods (Blanca et al. 2009).

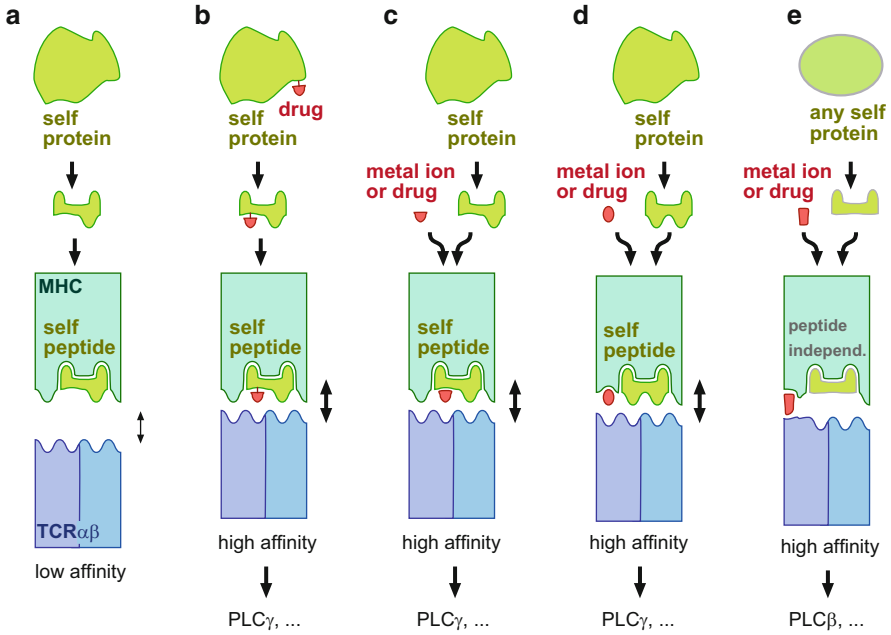

Fig. 3.2 pMHC-TCRαβ interactions mediated by small chemical compounds I. (**a**) Negative selection in the thymus ensures that self-peptide-MHC only has a weak affinity for TCRs in peripheral T cells. Self-pMHC does not perfectly fit to TCRαβ, thus not triggering their TCR. (**b**) Haptens bind covalently to endogenous proteins, thus generating haptenated self-peptides that together with MHC can form high affinity ligands for the TCR. (**c**) Transitional metal ions or drugs can non-covalently bind to the common self-peptide-MHC surface and thus potentially generate a high affinity ligand for the TCR. (**d**) Metal ions or drugs might also non-covalently bind to the MHC only. This could also form a complementary structure to some TCRαβ, forming a high affinity ligand. (**e**) Similar to superantigens, Fig. 3.1c, metal ions or drugs might bridge MHC with TCRαβ with high affinity without the involvement of the peptide. Thus, a large fraction of MHC might become competent in stimulation of TCRs. The stimulation of PLCγ (or PLCβ in case of superantigen-like ions/drugs) and other intracellular signaling molecules induced by high affinity TCR binding is indicated. This leads to activation of the T cell

3.1.2 Chemicals Acting as Prohaptens

Prohaptens are chemicals that only become active after a metabolic reaction. Often bioactivation of prohaptens involves oxidative processes, with the cytochrome P450-dependent metabolism playing a major role. In the liver, for example, sulfamethoxazole is converted by P450 to sulfamethoxazole hydroxylamine (Cribb and Spielberg 1992), and autooxidation of the latter compound generates the metabolite nitroso sulfamethoxazole (SMX-NO), which reacts with cysteine residues of proteins (Callan et al. 2009). Indeed, the peptides derived from the SMX-NO-modified proteins after antigen processing can possess high affinity for the appropriate TCR and thus are potent antigens to the specific T cells (Castrejon et al. 2010).

Prohaptens are a major problem for drug development as the different metabolic systems in a whole organism have to be investigated to make sure that an initially nonreactive drug does not become reactive upon metabolism.

3.1.3 Haptens as Tools in Basic Research

Traditionally haptens have been an important tool in studying the immune response. For example, trinitrophenol and dinitrophenol have been used to demonstrate the exquisite specificity of the immune system (Weltzien et al. 1996). More recently, haptenated peptides were synthesized to generate TCR antigens with defined properties. For example, photoreactive 4-azidobenzoic acid on the lysine of the peptide SYIPSAEKI was used and the haptenated peptide bound to MHC class I. Specific TCRs could bind, and using a short pulse of UV light, a covalent link between the peptide-MHC molecule and the bound TCR was made, thus "freezing" the otherwise transient peptide-MHC-TCR interaction (Gregoire et al. 1996; Luescher et al. 1995). Using this system we could show that bivalent peptide-MHC binding was required to induce an activatory conformational change in the CD3 subunits of the TCR (Minguet et al. 2007).

3.1.4 Transitional Metal Ions

Nickel (Ni) allergy is the most common and best studied of all metal hypersensitivities, again stimulating T cells. Reactions to other metals such as cobalt (Co), chromium (Cr), platinum (Pt), beryllium (Be), mercury (Hg), and gold (Au) are also reported. These transitional metals are only active as ions (e.g., Ni^{2+}) after their oxidation that can take place on the skin. Metal ions cannot form covalent bonds to peptides and thus are not classical haptens. Instead, metal ions form geometrically highly defined non-covalent coordination bonds with four or six electron donors. The electron donors are mainly nitrogen or oxygen in the amino acid side chains of proteins (Zhang and Wilcox 2002). Thus, metal ions can form very specific complexes with proteins. Ni is studied best (Thierse et al. 2005) and thus serves as an example here.

T-cell activation by Ni-treated APCs did not require antigen processing in some cases (Moulon et al. 1995), being in contrast to the classical haptens. In some cases, Ni bound to the MHC molecule and the peptide, thus forming a new surface to be recognized by the TCR (Fig. 3.2c) (Lu et al. 2003). This might require a certain MHC haplotype, a limited set of peptides and some specific TCR. However, if the hypervariable regions of the TCR are not required to make the contact, then a large number of different TCRs (e.g., those containing a certain Vα and/or Vβ segment) might be stimulated (Vollmer et al. 1997). Unfortunately, a crystal structure of a pMHC-Ni-TCRαβ complex does not exist. However, mimotopic peptides have

been used to substitute for the Ni and the self-peptide. The structure shows that the same diagonal orientation of the pMHC-TCRαβ interaction as for the classical pMHC-TCRαβ exists (Yin et al. 2012). This might suggest that the canonical PLCγ pathway is used (Fig. 3.2c). Unfortunately, it is unknown whether metal ions also allow other orientations than the diagonal one. In this case the interaction might resemble more the one of superantigen-mediated TCR activation and thus also activate PLCβ (Bueno et al. 2006, 2007).

It could also be possible that the metal ion only contacts MHC and TCR (Fig. 3.2d).

In another case, Ni activated the TCR with the correct MHC haplotype, but independent of any peptide (Gamerdinger et al. 2003). Thus, it was proposed that Ni acted in a similar manner as superantigens (Fig. 3.2e). However, in this case the hypervariable regions of the TCR were used as a contact site; thus, only very few TCRs might be activated. This is in contrast to superantigens that can activate all TCRs of a given Vβ region (Fraser and Proft 2008; Petersson et al. 2004). It was suggested that self-pMHC first binds to the TCR and that Ni then stabilizes the complex, in order to generate a high affinity interaction (Thierse et al. 2005). Whether in these cases PLCβ is simulated is unknown.

3.1.5 The "Pharmacological Interaction of Drugs with Immune Receptors (p-i) Concept"

The mechanisms of generating high affinity pMHC ligands for the TCR that we have just discussed for metal ions (Fig. 3.2) might also hold true for small organic compounds that do not covalently bind to peptides but still activate T-cell responses. These mechanisms were proposed in the "pharmacological interaction of drugs with immune receptors (p-i) concept" (Adam et al. 2011; Pichler 2005) and have gained much experimental support in the last years.

This model accounts for the observation that T cells could be activated, in an MHC-dependent mechanism, even in the presence of glutaraldehyde-fixed APCs (unable to process antigens) and the nonreactive drug (Schnyder et al. 1997; Zanni et al. 1998). Furthermore, the speed in which T cells could be activated (visualized by calcium influx) after introduction of the drug was too fast for antigen processing to have occurred (Zanni et al. 1998). Thus, these chemicals form reversible, non-covalent bonds (electric, van der Waals, hydrophobic, and hydrogen bonding forces) with pMHC and TCRαβ.

3.1.6 Drugs Binding Non-covalently to pMHC

Strong associations between drug hypersensitivity reactions and specific HLA alleles (human leukocyte antigen, HLA, is the name for human MHC) have been described, although the exact mechanisms for the TCR-stimulating activity of the drugs are often unclear. For example, strong associations between HLA-B*5801 and allopurinol-induced or HLA-B*1502 and carbamazepine-induced Stevens-Johnson syndrome or between HLA-B*5701 and flucloxacillin-induced reactions have been reported (Chung et al. 2004; Daly et al. 2009; Tassaneeyakul et al. 2009). In fact, carbamazepine seems to bind directly but non-covalently to endogenous peptide-loaded HLA-B*1502 (Wei et al. 2012; Yang et al. 2007). Thus, the pMHC-binding mechanisms shown in Fig. 3.2c, d might hold true. The 5-carboxamide chemical moiety of carbamazepine was seen to be required for presentation with HLA-B*1502, and three key residues in the peptide-binding groove of HLA-B*1502 were identified (Wei et al. 2012).

3.1.7 Drugs Altering the Self-Peptide Repertoire Bound to MHC

The antiviral drug abacavir causes severe adverse reactions exclusively in HIV-infected individuals expressing HLA-B*5701. As one example for personalized medicine, it is now common practice to genotype a patient for HLA-B*5701 before prescribing abacavir. The adverse reactions are mediated by the activation of cytokine-producing cytotoxic CD8+T cells, and the specificity of the abacavir-HLA interaction was mapped to the F pocket of the peptide-binding cleft of the MHC molecule (Chessman et al. 2008). Several groups showed recently that abacavir binds non-covalently to amino acid residues within the F pocket and thus changes the shape and chemistry of the cleft (Illing et al. 2012; Ostrov et al. 2012), changing the repertoire of self-peptides bound to and presented by HLA-B*5701 (Fig. 3.3a). The X-ray crystal structure of HLA-B*5701 bound to pep-V in the presence of abacavir suggests that the peptide is bound in the MHC molecule in a normal antigen conformation allowing for conventional pMHC-TCR interaction (Ostrov et al. 2012). So, self-peptides, that can bind to HLA-B*5701 only in the presence of abacavir, will then form new pMHC complexes not present in the thymus during negative selection of T cells. Thus, the abacavir hypersensitivity syndrome is a model of drug-induced autoimmunity in which the drug alters the self-peptide repertoire presented by MHC and so drives responses of T cells recognizing these neo-self epitopes (Illing et al. 2012; Norcross et al. 2012; Ostrov et al. 2012).

A different mechanism for the presentation of new self-peptides was suggested for metal ions, in cases where antigen processing was required to stimulate Ni-specific T cells by APCs. It was suggested that the presence of Ni altered antigen processing, so that new self-peptides are presented (Fig. 3.3b). Thus, the T cells

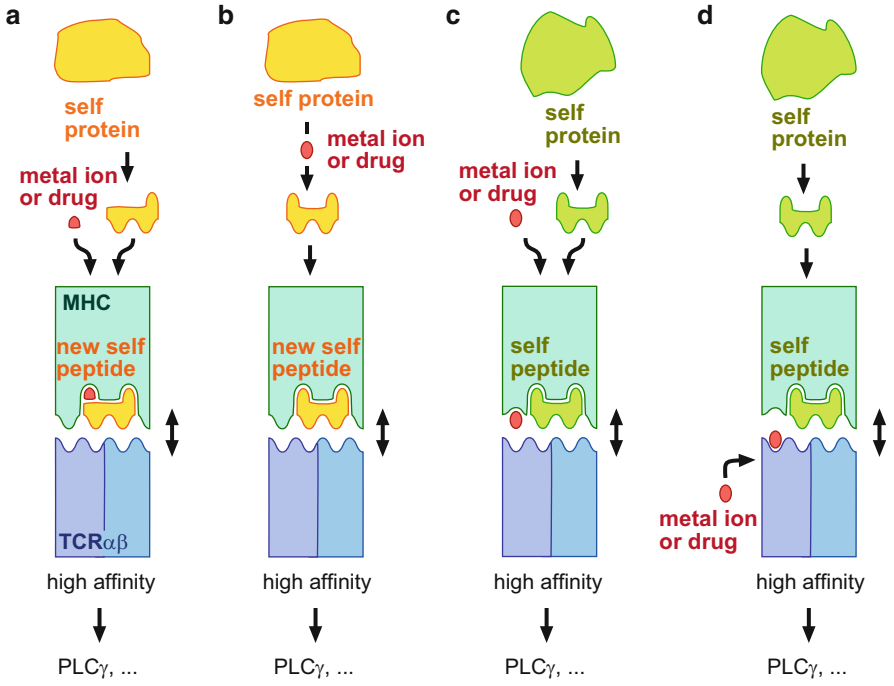

Fig. 3.3 pMHC-TCRαβ interactions mediated by small chemical compounds II. (**a**) In at least one documented case, the drug binds to the peptide-binding cleft of the MHC molecule. This allows peptides that otherwise cannot bind to the MHC, to bind and be presented. Since these new self-peptides are not presented in the thymus, reactive T cells are present in the periphery. (**b**) A drug or metal ion might influence proteins of the antigen processing pathways, such that new self-peptides are made and presented on MHC. (**c**) As seen in Fig. 3.2, metal ions or drugs can bind to pMHC and thus create a high affinity docking site for specific TCRs. (**d**) The same pMHC-drug-TCRαβ complex as in (**c**) can be accomplished, if the drug (or metal ion) binds to TCRαβ. TCR-induced downstream signaling via PLCγ and other pathways is indicated

might recognize these Ni-induced cryptic self-peptides, but not Ni itself (Griem et al. 1998).

3.1.8 Drugs Binding to TCRαβ

In principle drugs that bridge pMHC and TCRαβ (Fig. 3.2c, d, e) could have a higher affinity for pMHC or a higher affinity for TCRαβ, thus either first binding to pMHC or to TCRαβ before the pMHC-drug-TCRαβ complex forms. At first sight, it might seem irrelevant for the complex, and thus for T-cell activation, whether pMHC or TCRαβ binds first (Fig. 3.3c, d). However, the flexibility of the large TCRαβ hypervariable loops reduces the on-rate of the pMHC-TCR interaction and thus might act negatively on T-cell activation (Aleksic et al. 2010). If the drug binds

first to TCRαβ and thereby fixes the conformation of the hypervariable loops in the pMHC-permissive binding state, then the on-rate would be enhanced and T-cell activation would be favored. Thus, although hypothetical, drug binding to TCRαβ first might have different effects than binding to pMHC first.

The antibacterial sulfonamide sulfamethoxazole (SMX) is one of the drugs suggested to bind mainly or firstly to the TCR (in addition, to be a prohapten). TCRs from several SMX-specific T-cell clones isolated from patients with hypersensitivity to SMX reacted to SMX only in the presence of APCs indicating that the TCR stimulation by the drug was MHC dependent but antigen processing independent (Depta et al. 2004). Recent modeling of the drug-TCRαβ interaction suggested several candidate SMX binding sites on the CDR2 and CDR3 hypervariable loops of the TCRα and TCRβ chains (Pichler et al. 2011). Thus, SMX might be one example in which the drug binds stably to TCRαβ (Fig. 3.3d). Since the MHC-bound peptide did not affect the reactivity of SMX-specific T cells (Burkhart et al. 2002), the drug-TCRαβ complex might bind to the MHC molecule without the involvement of the peptide, thus resembling the way that superantigens use to bride TCRs to MHCs (Figs. 3.1c and 3.2e) with the difference that superantigens do not bind to the CDR loops of TCRαβ.

3.1.9 Drug-Induced MHC-Independent TCR Triggering

TCRs can be activated independently of pMHC, such as multivalent anti-TCRαβ and anti-CD3 antibodies (Chang et al. 1981; Kaye and Janeway 1984) or activation of a chimeric TCR by artificial ligands (Minguet et al. 2007). Even in vivo MHC-independent TCR activation was shown. In mice deficient for MHC class I, MHC class II, the coreceptors CD4 and CD8 thymocyte selection produced mature αβ T cells recognizing ligands independently of MHC (Van Laethem et al. 2007). The TCRs of two T-cell clones derived from these mice recognized glycosylation-dependent epitopes of the self-protein CD155 (Tikhonova et al. 2012). Thus, it is possible that drugs could bind to some endogenous proteins that are not pMHC, thereby creating new ligands for the TCRαβ or CD3 that could stimulate T cells in an MHC-independent manner.

3.1.10 The Second Signal of T-Cell Activation

The theory stipulating that an immune cell needs two signals to be activated was first developed in 1970 (Bretscher and Cohn 1970). TCR triggering is an important step in naïve T-cell activation, but it is usually not sufficient to release the T cells (αβ T cells) from their quiescent state. If TCR triggering (signal 1) is the only signal, the naïve cells usually become anergic and cannot be stimulated further (Jenkins et al. 1990; Schwartz 2003). To avoid the anergic, nonresponsive state,

naïve T cells require signal 2, provided by a costimulatory receptor such as CD28 (Rudd et al. 2009). In addition, other members of this family (CD2, ICOS) or of the tumor necrosis factor receptor family (including OX40 and 4-1BB) have been shown to be critical as stimulatory co-signals for T-cell activation (Sharpe 2009). So, a small chemical should require the presence of a costimulatory signal, in order to stimulate naïve T cells inducing proliferation and effector functions. Very often, in drug/metal hypersensitivities, the drug or metal also activates the innate immune system leading to the expression of costimulatory ligands by the APCs. Alternatively, the second signal could come from an ongoing infection or autoimmune reaction that takes place at the same time.

However, it is quite possible to imagine that some chemicals would directly stimulate effector or memory T cells where the requirement for a costimulatory signal is smaller (Boesteanu and Katsikis 2009; Kannan et al. 2012), thus overcoming the requirement for simultaneous stimulation of the innate immune system. In addition, effector or memory T cells have a lower affinity threshold for activation than naïve T cells (Bachmann et al. 1999; Cho et al. 1999; Iezzi et al. 1998; Kedl and Mescher 1998; Zimmermann et al. 1999) and respond to lower amounts of antigen than naïve T cells (Kimachi et al. 1997; London et al. 2000; Pihlgren et al. 1996; Rogers et al. 2000). The increased sensitivity to antigenic stimulation by effector and memory cells is partly caused by enhanced pre-clustering of the TCR (Kumar et al. 2011). This pre-clustering increases the avidity towards pMHC (Molnar et al. 2010, 2012). So an effector or memory T cell could be activated by a chemical without the need for a high affinity pMHC interaction with the TCR or even the need for costimulation.

3.2 Conclusion

The molecular mechanisms that small chemical compounds or metal ions use, in order to generate novel peptide-MHC surfaces that can bind with high affinity to TCRs, is an important topic in pharmacologic research. Beginning with the discovery that haptens can covalently bind to proteins whose peptides are presented by MHC in the last century, up to the recent demonstration that a drug altered the kind of self-peptides that are presented by a certain MHC haplotype, a number of different mechanisms have emerged. We believe that novel mechanisms will be discovered in the next years and decades. Detailed insight into these mechanisms might help in treating drug hypersensitivity reactions.

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Chapter 4
T Cell Responses to Contact Allergens

Hans Ulrich Weltzien, Stefan F. Martin, and Jean-François Nicolas

Abstract T lymphocytes are instrumental in the prevention of infections. With their antigen-specific T cell receptor (TCR), these cells recognize short peptides in the peptide-binding groove on MHC molecules of antigen-presenting cells. However, conventional T cells can also recognize non-peptide antigens including carbohydrates, phosphate groups, organic chemicals, and metal ions. The molecular basis of the interaction of TCR with these structures in the context of MHC has been partly solved. Organic chemicals and carbohydrates are recognized when bound to MHC-associated peptides, whereas metal ions are recognized due to their ability to form non-covalent coordination bonds with MHC molecules, bound peptides, and TCR. Peptide-independent metal ion recognition has also been described.

Abbreviations

ACD	Allergic contact dermatitis
APC	Antigen-presenting cell
CHS	Contact hypersensitivity
CMV	Cytomegalovirus
CSA	Cytokine secretion assay

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DNBS	2,4-Dinitrobenzene sulfonic acid
hTCPA	Human T cell priming assay
LLNA	Local lymph node assay
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cell
TCR	T cell receptor
TNBS	2,4,6-Trinitrobenzene sulfonic acid

Allergic contact dermatitis (ACD) is a delayed-type hypersensitivity response of the immune system to small, xenobiotic chemicals. These contact sensitizers or contact allergens activate initially the innate immune system to cause xenoinflammation (Martin 2012a, b). This essential inflammatory response in the skin eventually leads to the activation and migration of dendritic cells (DCs) from the skin to the draining lymph nodes. In the lymph nodes DCs that present the contact allergen in the context of MHC molecules activate naïve T cells expressing a contact allergen-specific T cell receptor (TCR) and drive their differentiation towards an effector T cell subset. The activated effector T cells can then enter the blood circulation. These events represent the sensitization phase of ACD. Encounter of the same contact allergen again induces skin inflammation which is required for the recruitment of the contact allergen-specific effector and memory T cells to the site of contact allergen exposure. The T cells then recognize their antigen on skin cells and exert cytotoxic effector functions and produce inflammatory mediators such as the cytokines IFN-γ, TNF-α, and IL-17. The result is the clinically evident eczematous skin reaction. This phase is called the elicitation phase of ACD.

Many studies have been performed in the mouse model of ACD, the contact hypersensitivity (CHS) model, in order to elucidate the immunological mechanisms underlying the activation of the innate and adaptive immune system by contact allergens (Martin 2013). Human in vitro studies have been conducted in parallel. The recent years have seen significant progress in our understanding of the molecular pathomechanisms that lead to xenoinflammation of the skin and activation of contact allergen-specific T cells (Martin 2012a, b; Martin et al. 2011).

4.1 T Cell Recognition of Organic Chemical Contact Allergens

The majority of contact allergens is represented by low molecular, protein-reactive chemicals. These may be of natural origin like urushiol from poison ivy or man-made synthetic compounds such as azo dyes, di- and trinitrochlorobenzene (DNCB, TNCB), or countless others. Contact sensitizers trigger a rather unspecific innate immune response via the generation of danger signals perceived, for example, by innate immune receptors and via effects mediated by induction of ROS

(Kaplan et al. 2012; Martin 2012a, b; Martin et al. 2011). The most specific response of the immune system to contact sensitizers is the T cell response. Antigen-presenting cells (APCs) can produce contact sensitizer-modified (haptenated) peptides from haptenated proteins and present these on MHC molecules. Alternatively direct modification of MHC-bound self-peptides on APCs can occur. The modified APCs then activate contact allergen-specific naïve T cells which then proliferate and differentiate to Tc1/Tc17 or Th1/Th17 effector cells of ACD.

The puzzling observation that the mammalian immune system is capable of specifically responding to such "unnatural" structures dates back to the first attempts to understand the molecular basis of immunological defense mechanisms in the early twentieth century.

Karl Landsteiner demonstrated the astonishing structural specificity of antibodies for low molecular chemicals coupled to autologous proteins (Landsteiner 1933) and introduced the term hapten (half antigen) for such molecules. He also was first to demonstrate hapten-specific skin sensitization in guinea pigs (Landsteiner and Jacobs 1935), a method still included in the methodology for risk assessment of potentially allergenic chemicals. The role of T cells and thymus in the adaptive immune response has been identified only much later (Gowans and Knight 1964; Osoba and Miller 1964) and in the following decade revealed the secret of MHC-restricted immune responses (Zinkernagel 1974).

It was then that contact hypersensitivity (CHS), or allergic contact dermatitis (ACD) as it was named by clinicians, was identified as a T cell-mediated, delayed-type hypersensitivity (DTH) reaction (Kimber and Dearman 2002). First studies in the mouse revealed that primary CHS to TNCB and oxazolone occurred 7 days after a single application of contact allergen. This response was transient. More efficient and long-lasting ear-swelling responses were observed after initial sensitization followed by ear challenge 7 days later. Passive CHS was established by the transfer of peritoneal exudate cells or lymph node cells from sensitized mice into naïve recipients that then received an ear painting with the contact allergen (Asherson and Ptak 1968). Consequently, hapten-reactive T cells were identified and shown to be MHC-restricted just like protein-specific ones (Shearer 1974) and, thus, to define the specificity of CHS.

In the verge of the identification of the T cell antigen receptor (TCR) (Haskins et al. 1984) and of MHC-bound peptides in class II (Buus et al. 1987) or class I MHC molecules (Falk et al. 1991) as TCR ligands, Ortmann et al. convincingly demonstrated that T cells recognized haptens when bound to MHC-associated peptides (Ortmann et al. 1992). Follow-up studies (Martin and Weltzien 1994) revealed that hapten-carrier peptides with allele-specific "anchoring motifs" (Rammensee 1995) served as high affinity anchors for haptens to specific MHC class I and II alleles (Martin et al. 1992, 1993a, b; Kohler et al. 1995). It was shown that antigen-presenting cells produced MHC class I-presented haptenated peptides from haptenated proteins delivered to the cytosol (Martin et al. 1993a, b) and that hapten-peptides extracted from class I MHC molecules of hapten-modified cells could sensitize target cells for lysis by hapten-specific cytotoxic T cell clones

(von Bonin et al. 1992). Studies with purified MHC molecules or cells deficient in MHC peptide loading excluded covalently hapten-modified MHC proteins ("altered self") as major TCR ligands (von Bonin et al. 1992, 1993). The in vivo relevance of these findings was shown by sensitization of mice with TNP-peptide-pulsed dendritic cells for allergic contact hyperreactivity to TNCB (Martin et al. 2000).

A contribution of the peptide carrier to antigenic specificity was negligible for haptens in central peptide positions, but was clearly demonstrated for haptens attached to peripheral amino acids. This was taken to indicate a possible role of haptens in the induction of autoimmunity (Martin et al. 1995).

The transferability of the hapten-peptide paradigm to human allergy has been evaluated in two systems, i.e., hyperreactivity to penicillins and allergic contact dermatitis to nickel (Ni). In perfect analogy to the mouse model, E. Padovan demonstrated that T cell clones from patients allergic to different penicillins reacted to penicilloyl peptides in an HLA-restricted, penicillin- and position-specific manner (Padovan et al. 1997).

4.2 T Cell Recognition of Metal Ions

The picture became more complex when T cell reactivities to metal ions such as nickel were investigated. Unlike chemically reactive haptens, Ni ions engage in non-covalent, i.e., reversible, coordination complexes predominantly with nitrogen residues in histidine or arginine. Thus, despite the existence of large collections of Ni-reactive T cell clones (Kapsenberg et al. 1987; Moulon et al. 1993; Sinigaglia et al. 1985), T cell antigenic nickel epitopes could only be identified upon the expression of nickel-specific human TCR in TCR-deficient mouse hybridoma cells (Vollmer et al. 1999). Besides resulting in long-lived, easy to culture cell lines, this system allowed the systematic mutation of the antigen-binding CDR3 regions in TCR α- and β-chains and, hence, the localization of contact sites for nickel in TCR and HLA protein.

These studies revealed two principally different ways of TCR interactions with Ni plus HLA: for one TCR, Lu et al, indeed, showed the combination of defined peptide and HLA haplotype to be essential for Ni recognition (Lu et al. 2003). However, a different TCR recognized nickel HLA haplotype specific but apparently independent of the nature of associated peptides (Gamerdinger et al. 2003). In this case histidine residues in defined positions of variable TCR and HLA regions were identified as coordination sites for a Ni-mediated, peptide-independent TCR-HLA complex, which effectively induced TCR signaling. Moreover, it was shown that Ni ions may be transferred from complexes with other proteins such as human serum albumin to coordination sites provided by TCR-HLA combinations (Thierse et al. 2004, 2005).

4.3 T Cell Receptor and Epitope Recognition

The exquisite antigen specificity of the T cell response to contact sensitizers has raised interest in the development of T cell-based assays for the in vitro identification of these chemicals (Martin et al. 2010). This specificity is illustrated by the ability of T cell clones to differentiate β-lactam antibiotics with different side chains and the ability to differentiate TNP- and DNP-modified peptides.

The antigen specificity of the T cell receptor is generated during T cell development in the thymus. In the developing T cells, somatic rearrangement of gene segments coding for variable (V) and joining (J) segments in the TCRa chain and additional D segments in the TCRb chain with constant (C) segments leads to VJC TCRα and VDJC TCRβ chains that are clonally distributed after having undergone positive and negative selection. These processes generate a broad repertoire of clonally distributed TCR containing about 10^{14} different antigen specificities. Interestingly, clonally distributed antigen-specific receptors have been identified in lymphocyte lineages of lamprey where so-called variable lymphocyte receptors (VLR) determine antigen specificity. Based on very different diversification mechanisms 10^{14}–10^{17}, different specificities may be generated which is similar to the diversity of TCR specificities (Boehm et al. 2012).

When foreign antigens are encountered, only T cell clones with specific receptors are selectively expanded from the highly diverse repertoire. The diversity of the antigen-selected repertoire is influenced by a variety of factors. These include the number of epitopes, the affinity of peptide binding to MHC and to the TCR.

Contact allergens turn self into foreign by the generation of chemically modified self-peptides. Moreover, it is also possible that altered processing of self-proteins occurs due to the modification of recognition sites for the proteasome and endolysosomal proteases that are involved in antigen processing for MHC class I and MHC class II presentation. Whether this may lead to the recognition of neoantigens, cryptic peptides that have not been presented to developing T cells in the thymus and thus have not contributed to thymic selection, is so far unknown. It is conceivable that most of the T cells involved in chemical-induced allergies recognize the chemical. However, it is possible that T cells primed by haptenated peptides may also react to the unmodified self-peptide. Such reactivities may underlie the vitiligo associated with melanoma therapy using the topical application of the contact sensitizers DNCB or DPCP. In that case the reactivity of cytotoxic T cells to unmodified self-peptides resulted in the killing of healthy melanoma cells resulting in depigmentations as a side effect observed in this therapy (Henderson and Ilchyshyn 1995). It remains to be determined whether the peptides recognized in these T cell responses are the non-haptenated carrier peptide sequences or peptide mimotopes of the haptenated peptides. A recent study has proven the existence of such peptide mimotopes (Yin et al. 2012). An HLA-DR52c-restricted nickel-reactive human T cell clone, ANi2.3, isolated from a Ni-allergic patient

(Lu et al. 2003), was used to screen engineered peptide libraries for peptides recognized in the presence of Ni. Surprisingly, only Ni-independent peptide mimotopes instead of a self-peptide complexed to nickel were recognized by the T cell clone. It was shown that the TCR Vβ CDR3 region interacted especially with a conserved lysine residue present in position 7 of the DR52c-binding nonameric peptides, and it was suggested that this lysine mimics Ni in the natural TCR ligand. The CDR3 loop extends into the region of the DR52c to interact with conserved residues in a panel of mimotope peptides and residues of the DR52c α1 and β1 helices. Interestingly, an aspartic acid residue was crucial for the peptide recognition and could not be replaced by a glutamic acid residue.

Biacore measurements and crystal structure analyses revealed that ANi2.3 TCR recognizes mimotope peptides with affinities in the range of normal peptide antigen recognition and that it was oriented in the typical diagonal orientation over the peptide-MHC complex. These data show that this Ni-specific TCR interacts with the positively charged Ni^{2+} complexed to a self- peptide and that Ni and maybe also other metals are most likely accommodated by an acidic pocket formed by the α1 and β1 chains of DR52c in the peripheral region of the peptide-binding groove where the conserved positively charged lysine residue in position 7 of the peptide mimotopes was located. Similarly, a recent study demonstrated the peptide-dependent recognition of beryllium by HLA-DP-restricted CD4+ T cells and identified peptide mimotopes with amino acid residues in defined positions that coordinate beryllium in cooperation with HLA-DP. HLA-DP tetramers loaded with such mimotopes plus beryllium as well as an endogenous peptide containing the core motif were used and revealed a high frequency of specific CD4+ T cells in patients with chronic beryllium disease (Falta et al. 2013).

The use of peptide libraries in combination with multimeric MHC molecules for the identification of unknown T cell epitopes is very attractive and may be done by the baculovirus/insect cell approach. A recent study reported the generation of plasmid-coded combinatorial peptide libraries co-transfected with MHC molecules into COS-7 cells for the identification of target antigens for CD8+ T cells (Siewert et al. 2012). The use of such approaches to also analyze the recognition of xenobiotic chemicals by T cells is of great interest and has a great potential as described for nickel and beryllium (Falta et al. 2013; Yin et al. 2012). It is likely that peptide mimotopes can be identified also for organic contact allergens and drugs mediating T cell-mediated adverse reactions. It may also be feasible to produce libraries of haptenated peptides that would provide a useful tool to dissect contact allergen-specific T cell responses. Such studies should help to understand the nature of the self-peptide-selected TCRs that react to such chemicals and to explain why they become chemical reactive.

4.4 Innate Immune Responses to Contact Allergens and Drugs

It is to be expected that T cell-mediated adverse drug reactions (ADR) follow similar basic mechanistic principles as T cell-mediated ACD. The culprit drugs must activate both the innate and the adaptive immune system. The nature of the danger signals generated remains to be determined, and tissue-specific effects have to be considered. Drugs are often given orally and end up in tissue microenvironments that are very different from the skin. Therefore, the innate immune responses in the gastrointestinal (GI) tract and the liver may be different than those triggered by contact sensitizers in the skin. However, in order to prime naive drug-specific T cells, danger signals will also be needed to eventually activate DCs that present the drugs to T cells in the local lymph nodes. The drug acetaminophen (APAP) can induce drug-induced liver injury (DILI). The mechanisms by which the inflammatory response is triggered are strikingly similar to the mechanisms triggered by contact allergens but involving in part other cell types, innate immune receptors, and danger signals (Martin 2012a, b). APAP triggers damage of liver cells resulting in the release of self-DNA which then acts as an endogenous danger signal (DAMP). This DAMP triggers TLR9 on sinusoidal liver endothelial cells which release ATP as another endogenous danger signal perceived via the P2X7R. P2X7R triggering then results in the activation of the NLRP3 inflammasome and formation of mature IL-1β and IL-18 (Hoque et al. 2012; Imaeda et al. 2009). Interestingly, up to now there is no evidence for the activation of conventional APAP specific T cells in this liver disease.

The fact that there are often skin manifestations after oral drug intake is very interesting and may indicate a transport of the drugs from the GI tract into the skin. Carrier molecules such as human serum albumin that is also found in the skin may play a role here. Whether the T cells responsible for the skin phenotype are primed in the skin-draining lymph node and thereby acquire a skin-homing receptor profile or whether they home to the skin due to the innate inflammatory response induced by the drugs is so far unknown.

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Chapter 5
Contact Hypersensitivity: Quantitative Aspects, Susceptibility and Risk Factors

Peter S. Friedmann and Christopher Pickard

Abstract The development of allergic sensitisation by environmental chemicals results in allergic contact dermatitis and highly undesirable morbidity and disability. This form of hypersensitivity is mediated by specific T lymphocytes that recognise the chemical sensitiser bound to self-proteins. Use of deliberate experimental contact sensitisation with dinitrochlorobenzene (DNCB) has been used to investigate the human immune system which exhibits dose-related responses. Many factors contribute to whether sensitisation occurs and the nature and magnitude of the immune response. Chemicals vary in sensitising potency, mainly reflecting their intrinsic protein-binding properties. The amount of sensitiser reaching the immune system is determined by many factors of which the concentration (dose per unit area), the relative lipid solubility and molecular weight are the most critical. Host-related factors contributing to the nature and magnitude of immune responses are mainly genetically determined including gender, age, the biochemical/physical integrity of the epidermal barrier and the quality of the innate and adaptive immune systems. The underlying mechanisms must be elucidated before it will be possible to make reliable predictions of whether a given individual will develop allergic sensitisation by a given chemical.

Keywords Contact hypersensitivity • Contact dermatitis • DNCB • Elicitation • Quantitative • Sensitisation • Susceptibility • Stratum corneum • Biochemical barrier

Allergic contact dermatitis is a common and unwanted response of the immune system to chemicals found in the human environment. Individuals can develop

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contact allergy to almost any chemical they come into contact with, whether in the form of dyes, preservatives or fragrances in personal products and clothing to substances in plants and foods. For many people, the strength of their reactivity is so severe that they suffer life-impairing morbidity which may necessitate major changes in lifestyle or occupation. The standard method for identifying substances to which individuals have become allergic is the epicutaneous patch test challenge in which suspected or potential substances are applied to the skin on purpose-made carrier chambers or patches. The allergic hypersensitivity is manifested over 24–48 h as an erythematous, oedematous and itchy reaction that reproduces the features of the naturally occurring contact dermatitis. Microscopic examination reveals heavy infiltration with lymphocytes and monocytes, and this so-called delayed hypersensitivity is now known to be mediated by specific T lymphocytes of both CD4+ and CD8+ types.

One of the central questions is why does the human immune system develop unwanted immune hypersensitivity to environmental chemicals (xenobiotics) that result in apparently valueless skin inflammation? Secondary questions relate to quantitative aspects of the immune system and its responses, for example, how low an exposure to an immunogen can the immune system detect and respond to? The human immune system functions mainly to protect us from harm by pathogens. In order to fulfil this function, it has to detect and respond to foreign substances appropriately—that is, it must generate an effective response when the foreign substance is perceived as dangerous, but the system is programmed not to respond to non-dangerous substances.

5.1 Factors Contributing to Sensitising Efficiency

The ultimate determinants of whether a chemical induces sensitisation are the resultant of a range of factors including the intrinsic sensitising potency of the chemical and the dose that reaches the cells of the immune system as well as host-related factors that influence susceptibility, which may be systemic or local.

5.1.1 Potency of Sensitisers

The details of how the immune response is activated to generate T lymphocytes specifically able to recognise contact sensitising chemicals have been covered in detail elsewhere. In order to be “seen” by the T cell, small molecules must bind to proteins, hence acting as haptens. Hapten-conjugated peptides can then be associated with the MHC groove making them recognisable by T cells with the appropriate cognate receptor. Some chemicals are intrinsically reactive; others may be rendered reactive through the actions of drug-metabolising and drug-detoxifying enzymes. It is thought that the more protein reactive a chemical, the more potent it

is as an immunogen/sensitiser. However, simply being "seen" by T cells via their specific receptors is not enough of an activation signal to induce them to proliferate. So a second property of contact sensitisers is the capacity to perturb the tissue environment and the antigen-presenting dendritic cells in ways that create a "sense of danger".

Some chemicals including dinitrochlorobenzene (DNCB), diphenylcyclopropenone (DPCP), oxazolone and squaric acid dibutylester (SADBE) are very potent immunogens, inducing contact sensitisation in 100 % of normal humans. Much work has gone into devising assays that can predict the sensitising potency of different chemicals. Thus, tests in animals include the guinea pig maximisation test, the Buehler test and the mouse ear swelling test (Patrick and Maibach 1995). Some of these experimental systems involve administering the chemical with an adjuvant which gives the "danger" signal and hence maximises the chance of the immune system responding. Implicit in this is that the chemical on its own may not be able to induce an immune response but requires the "danger signals" from the adjuvant. Kligman developed a modified human "maximisation" test which involved pretreating a skin area with 5 % sodium lauryl sulphate (SLS), a detergent with irritant properties. The agent under test was then applied to the area under occlusion for 48 h (Kligman 1966a). The local lymph node assay (LLNA), which involves quantification of proliferation of lymphoid cells in lymph nodes draining sites of topical application of putative sensitisers, has become widely adopted as a standard mouse-based screening test (Gerberick et al. 2000; Loveless et al. 1996).

Many chemicals found in everyday life are potential sensitisers; thus, Kligman showed 100 % sensitisation of human volunteers by paraphenylenediamine (PPD) using high concentrations of this substance (1 g of 10 % PPD in petroleum jelly to give 711 μg/cm^2 compared with up to 6 % in hair dyes) (Kligman 1966b). Indeed, in the LLNA it is more potent than DNCB (Warbrick et al. 1999). However, the doses in which most chemicals are encountered in the normal environment are either too low to activate a "positive" immune response manifesting as allergic hypersensitivity or they may even induce immunological tolerance, probably mediated by regulatory T cells. As indicated above, whether an active hypersensitivity or a state of nonreactivity is induced may be a reflection of how the chemical interacts with the innate immune system to produce danger signals. Most potent sensitisers are also irritants, and there is evidence that it is the irritant properties that exert the "adjuvant" effect of stimulating the innate immune response, hence potentiating the activation of the adaptive/acquired immune response. For example, Allenby showed that pretreatment with an irritant, SLS, potentiated allergic responses to nickel (Allenby and Basketter 1993). However, SLS is also a surfactant, and the possibility that it simply compromised the epidermal barrier and facilitated absorption of nickel is a possible alternative explanation. This "danger signal" effect resulting from the irritant properties of sensitisers is a major area of interest, but to date the relevant mechanisms are obscure.

Dose Dependence: What Determines the Dose that Reaches the Immune System?

Many factors affect the dose (number of molecules) of a chemical that reach the viable layers of the epidermis in which the dendritic cells are found. These include the concentration (quantity per unit area) applied to the surface, the duration for which it is applied, the molecular weight of the compound (larger than 500 Da do not penetrate into normal skin), the intactness of the stratum corneum barrier, occlusion (Menne 1994), the solubility characteristics (lipid-soluble molecules penetrate much better than water-soluble ones), the vehicle and the partition coefficient of the molecule in relation to the constituents of the vehicle and the stratum corneum. Additional factors may include metabolic processing of the compound which may either reduce its effective concentration or, alternatively, might generate an intermediate compound which is the actual sensitiser. These factors will all contribute at every encounter with a chemical—both during the initial sensitising exposure and also at subsequent elicitation/challenge exposure(s). Indeed, the diagnostic occluded patch test challenge deliberately maximises the exposure dose by using high concentrations applied for 48 h under occlusion. Additional factors may come into play when there is repeated exposure to a compound either through daily use or in a repeated challenge. These will be discussed below.

5.2 Dose–Response Relationships of Human Contact Sensitivity

Use of potent contact sensitisers such as DNCB has taught us much about the quantitative dose–response relationships of the human immune system. Apart from the chemical property of actual or potential reactivity with proteins, it is now clear that the major determinant of how the immune system responds to a sensitiser is the intensity of the sensitisation stimulus, that is, the dose of sensitiser in relation to the area of exposure—the dose per unit area. The experimental work that underpins this conclusion involved sensitising groups of healthy human volunteers with different doses of DNCB on an area of constant size and, in other groups, comparing responses evoked by varying the area of application of the sensitising dose. Thus, initially, 5 groups received 62.5, 125, 250, 500 or 1,000 μg of DNCB on a circle of forearm skin 3 cm in diameter (Friedmann et al. 1983). Since DNCB remains bound in the skin for some weeks, it is available for recognition as soon as a clone of memory T cells specific for DNCB enters the systemic circulation from the lymph node. The site of initial application of DNCB subsequently becomes inflamed with a contact dermatitis-like reaction—the so-called primary allergic response or delayed flare. This is an indicator not only that the individual is now clinically sensitised, but the time at which it occurs is a reflection of the time taken for the clonal expansion of specific T cells that occurs during the generation of clinical sensitisation. The proportion of individuals sensitised by increasing doses of DNCB showed a sigmoid log-dose–response curve, with 100 % being sensitised by 500 μg

DNCB and above. There was an inverse relationship between the sensitising dose and the time taken for the delayed flare to appear—thus it took a mean of 15.5 days to appear for those who received 62.5 or 125 μg but decreased for each group as the sensitising dose increased, so that those who received 1,000 μg showed the delayed flare after only 8 days (Friedmann 1991).

Four weeks after sensitisation, the degree of reactivity was assessed by quantifying responses to challenge with four simultaneous eliciting doses: 3.125, 6.25, 12.5 and 25 μg of DCNB. These challenges were applied to the other forearm on 1 cm paper discs (Al test, Hyrylia, Finland). The patches were left in place for 6 h and the responses were assessed at 48 h both clinically (grade 2 was an erythematous and palpably indurated response), and thickness was quantified by use of Harpenden skinfold callipers. The proportion of individuals in each sensitisation group that gave grade 2 or stronger (i.e. definitely sensitised) responses followed the same sigmoid curve with log-sensitising dose as was seen for the delayed flare. Thus, the proportions sensitised rose from 8 % by 62.5 μg through 62 % by 125 μg, 83 % by 250 μg to 100 % by 500 μg and above. This reveals that there is a normal distribution to the human immune response—some people being low/weak responders and others being high/strong responders. Within each sensitisation group, the normal distribution was evident—with some people being weakly or apparently unsensitised, others being moderately reactive and yet others being strongly reactive. The responses (increase in skinfold thickness) plotted against log-challenge dose formed the lower or central portions of sigmoid dose–response curves. When the linear segments of the family of challenge dose–response curves were analysed by a generalised linear interactive model (GLIM), the slopes of the curves were parallel (Friedmann et al. 1983) (Fig. 5.1). This revealed that whether individuals were strongly or weakly sensitised, increasing the challenge dose by a given increment (doubling) was associated with a constant factor of augmentation of the response—slope of dose–response curve. By extrapolation of the regression lines, it could be calculated/predicted that the lowest dose (threshold) of DNCB that each of the groups might respond to would be 3.9 μg for the group that received the lowest sensitising dose and 3.0, 2.0, 1.4 and 0.8 μg, respectively, for the other groups in increasing order of sensitising dose. These absolute doses applied on 1 cm diameter paper discs convert to 5, 3.8, 2.5, 1.8 and 1 $\mu g/cm^2$, respectively.

As mentioned above, one of the important variables determining elicitation responses is the duration of exposure—these DNCB challenge doses were applied only for 6 h, but in pilot work it was established that longer application periods were associated with much stronger responses. So it would be wrong to think that this represents an absolute lowest (threshold) dose which can detect the presence of sensitisation. Finally, it was possible to represent the relationship between increasing sensitising dose and degree of reactivity (strength of sensitisation) by plotting on a log scale the sensitising dose against the elicitation response for each group, represented as the skinfold thickness at the 12.5 μg challenge dose. This showed a log-linear increase in sensitisation with increasing sensitising dose (Fig. 5.2).

The same dose–response relationships were examined in individuals who spontaneously developed 3 or more unrelated contact allergies (detected in the patch test

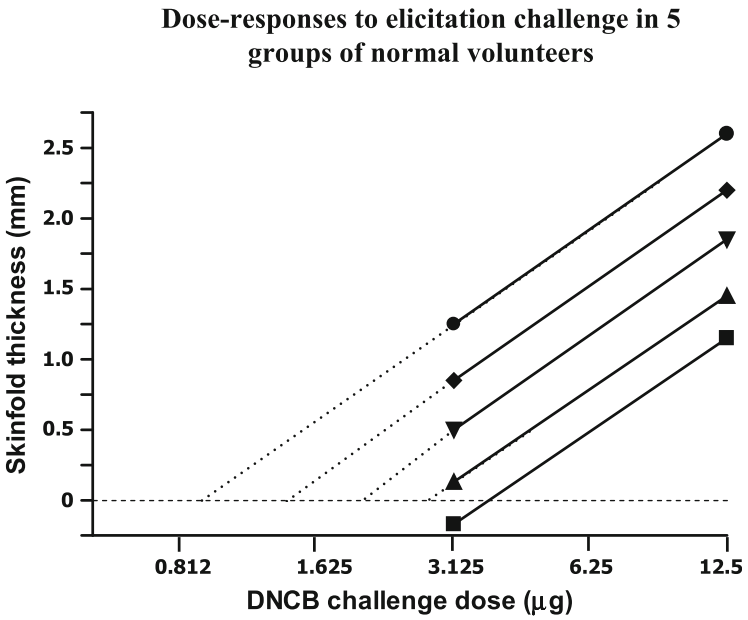

Fig. 5.1 Five groups of healthy volunteers received initial sensitising doses of DNCB of 1,000 µg (*filled circle*), 500 (*filled diamond*), 250 (*filled triangle*), 125 (*filled downward triangle*) or 62.5 µg (*filled Square*) of DNCB. Four weeks later, they were challenged with four doses of DNCB (from 3.125 to 25 µg) and responses measure as thickness with skinfold callipers. The regression lines of the linear portion of the dose–response curve are plotted as *solid lines*. The calculated extrapolation to $X = 0$ (theoretical threshold elicitation dose) is plotted as *dotted lines*

clinic). These individuals were shown to be "high responders" in that, as a group, they were sensitised to a much greater degree by a given dose of DNCB (Moss et al. 1985). This was shown to be a quantitative rather than a qualitative difference, namely, the high responder end of the normal distribution, since individuals with a single contact sensitivity were intermediate in their sensitisability.

5.2.1 Dose per Unit Area

The above set of dose–response relationships was the result of applying varying doses of sensitiser to a constant area of skin (3 cm diameter = 7.1 cm^2). Therefore, the sensitising doses can also be expressed as the dose per unit area—µg/cm^2 (Table 5.1). Next the effects were examined of varying the area of the sensitising application while maintaining a constant dose per unit area. Over a wide range of areas—from a circle 4.25 cm in diameter down to 1 cm—if the dose per unit area was kept constant, there was no effect of area, even though the total doses varied by up to tenfold (Table 5.1). It was only when the area was reduced to 3 mm

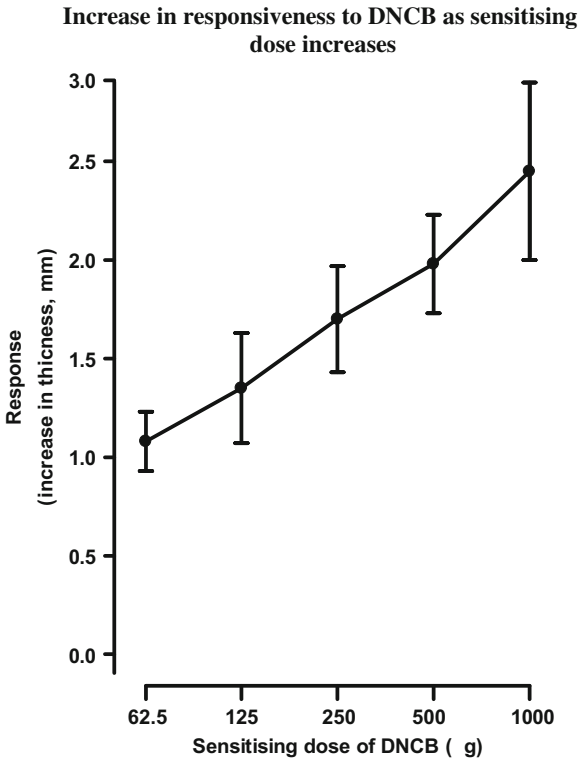

Fig. 5.2 Increase in responsiveness to DNCB as sensitising dose increases. Reactivity for each of the five groups shown as response at challenge with 12.5 μg (measured as skinfold thickness) plotted against initial sensitising dose. *Bars* are SEM (Reproduced from Friedmann 1991 with permission)

(by application of a 3 mm diameter paper disc impregnated with DNCB at the desired concentration) that area had an effect and only 26 % were sensitised compared with 93 % by the same dose per unit area on a 1 cm paper disc. Overall, these results can be interpreted in relation to Langerhans cell (LC) numbers. Since the mean density of LC in forearm skin is about 750/mm^2, an area 1 cm diameter (0.78 cm^2) contains about 59,000 LC (Ford et al. 1984). After application of DNCB, up to 20 % of LC migrate down into the dermis (Cumberbatch et al. 2005)—approximately 6–12,000 LC. This number of LC is sufficient to fully sensitise a human. Also, for a given number of molecules of DNCB, the potency of sensitisation is greater if few LC present many molecules per cell rather than having the same number of molecules presented by many more LC at fewer molecules per cell. Similar observations were made by Macatonia et al. in mice, and the relationship between sensitising dose and LC reaching the lymph node was defined in quantitative terms (Macatonia et al. 1986).

There have not been many other studies attempting to quantify induction of contact sensitisation in humans. Cardin examined the sensitising potency of Kathon (methylchloroisothiazolinone–methylisothiazolinone (MCI/MI)), applying the compound to normal volunteers in a range of concentrations from 5 to 20 parts per million (ppm) (=0.0005–0.002 %) (Cardin et al. 1986). However, the Kathon

Table 5.1 Relationship between proportions sensitised, area of application and sensitising dose of DNCB

	Application site		Sensitising dose			
Row	Diameter (cm)	Area (cm^2)	Total (μg)	Concentration (μg/cm^2)	Number of subjects	Percentage sensitised
1	3	7.1	1,000	142	24	100
2	3	7.1	500	71	40	100
3	3	7.1	250	35.4	30	83
4	3	7.1	125	17.7	30	63
5	3	7.1	62.5	8.8	24	8
6	1.5	1.8	62.5	35.4	7	86
7	2.1	3.5	58	16.4	22	55
8	3	7.1	116	16.4	34	50
9	4.25	14.2	232	16.4	15	66
10	1 cm paper	0.8	30	38	28	93
11	3 mm paper	0.08	3	38	15	26

Data are from several studies with DNCB (Friedmann et al. 1983, 1990; Rees et al. 1990b; White et al. 1986). The first five rows are the normal subjects from Figs. 5.1 and 5.2. Row 6 gives the same dose per unit area as row 3. In rows 7–9, the areas were half and double the standard 7.1 cm^2, but the dose per unit area was constant

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was applied on occluded patches at actual doses per unit area of 1.14 or 2.9 μg/cm^2 depending on which patch system was used. Moreover, repeated patch applications were made thrice weekly for nine applications. Although the authors reported the results as "20 ppm sensitised 2/45 volunteers," it is clear that a cumulative dose of 20 μg/cm^2 was applied under occlusion over the 3 weeks. In a large American study, 495 individuals were patch tested with a mix of MCI/MI at 250 ppm which is equivalent to 0.025 %. The material was applied on Finn chamber patches (7 mm) which were occluded for 48 h. While 13 individuals were already allergic, 3/495 were sensitised de novo by this concentration (Rietschel et al. 1990). Although no indication was given of the quantity of MCI/MI applied to each patch, it is possible to estimate the exposure. The normal quantity of allergen applied to a 7 mm Finn chamber is about 5–10 mg of white soft paraffin (see below) or 10–15 μl of a solution. Ten μl of a 0.025 % solution contains 2.5 μg of MCI/MI which, on a 7 mm Finn chamber, gives a dose per unit area of 6.4 μg/cm^2.

5.2.2 Effect of Repeated Exposure to Sensitiser

As discussed above, these dose–response relationships were determined by use of single exposures to the sensitiser. However, there is a variety of evidence of differing qualities showing that repeated exposure to low doses of sensitisers can

induce allergic contact sensitisation (Basketter et al. 2006; Kligman 1966b) and some of the evidence even suggests repeated low-dose exposure may be more potent than single higher-dose exposures (Paramasivan et al. 2010). Thus, Kligman observed the effect of increasing numbers of applications of several potential sensitisers including penicillin V, neomycin sulphate and ammoniated mercury (Kligman 1966b). He used rather high concentrations (10 %) but showed for these and others that as numbers of exposures increased to 15, sensitisation could be induced in healthy humans. Thus, 76 % were sensitised to penicillin V, 70 % to ammoniated mercury and 48 % to neomycin sulphate (Table 5.2). In a large prospective study, sensitisation by paraphenylenediamine (PPD) was assessed in groups of healthy human volunteers who either used a low concentration hair colourant applied frequently for short durations or a higher concentration hair dye applied for longer but at less frequent intervals (Basketter et al. 2006). Of the group which applied hair colourant (PPD final concentration of 0.48 %) for 5 min once a week for 6 months, 7.2 % were sensitised. In the comparator group which applied hair dye (PPD 3 %) for 30–40 min once a month for 6 months, only 1.3 % were sensitised. The present authors recently investigated the effect of repeated exposures to low doses of an experimental sensitiser (Paramasivan et al. 2010). Dinitrochlorobenzene (DNCB) was applied to one group of healthy human volunteers at 60 μg/cm^2, a dose which induced moderate sensitivity in 100 % of volunteers when quantitative elicitation doses were applied 4 weeks later (Pickard et al. 2007). The comparison group received three separate doses of 10 μg/cm^2, applied to the same site at weekly intervals before being challenged. All volunteers became sensitised, and their degree of reactivity to the challenges was identical to that of the group who received the single dose of 60 μg/cm^2. Thus, when applied as small increments, half the total dose (30 vs. 60 μg/cm^2) induced equal sensitisation. Taken together the above results suggest that when individuals become strongly sensitised to some of the ingredients in personal products which are present at very low concentrations, this is likely to be the result of repeated low-dose exposure (Friedmann 2008).

5.2.3 Responses to Elicitation Challenge

Factors affecting the response to elicitation challenges include the strength of sensitisation, whether the immune response to this antigen has stabilised or is still maturing; the effective dose received by the immune system; local tissue factors such as body site; and other chemical perturbing influences including irritancy and whether the challenge is encountered as a single or repeated contact.

1. The principle factor that determines the magnitude of responses to elicitation challenges with contact sensitisers is **the strength of sensitisation**—whether the individual is sensitised to a weak or strong degree. This has been dealt with above.

Table 5.2 Effects of increasing numbers of exposures on frequency of sensitisation

	Number of exposures			
Agent	3	5	10	15
Benzocaine (5 %)	0/23	1/22	3/25	6/25
	0[a]	**4.5**	**12**	**24**
Tetramethylthiuram disulphide (TMTD) (10 %)	0/25	0/25	2/22	6/18
	0	**0**	**9**	**33.3**
Neomycin sulphate (10 %)	0/24	1/25	4/23	10/21
	0	**4**	**17.4**	**48**
Penicillin G (10 %)	1/25	5/25	10/21	16/21
	4	**20**	**48**	**76**

[a] = % positive
Data modified from (Kligman 1966b)

2. **Boosting effect of repeated challenge**: During the early weeks and possibly months following initiation of sensitivity, repeated challenges with the sensitiser can boost the strength of sensitisation and hence the reactivity. We showed that individuals who received the 50 % effective sensitising dose of DNCB (116 μg applied to a 3 cm diameter circle), who failed to give positive responses to the first elicitation challenge, gave very strong positive responses when exposed to a second challenge some time later (Friedmann et al. 1990). This indicated that the first challenge had actually boosted the degree of sensitisation from a subclinical level to a strong degree of clinical allergic sensitivity. However, when people have been sensitised for a long time—as in the case of nickel sensitivity—repeated challenges are reproducibly the same, and there is no sign that repeated or continued exposures augment reactivity (Memon and Friedmann 1996). The effect of repeated exposures to low concentrations of potential sensitisers such as those in personal products/cosmetics has yet to be clearly defined, but there is clearly the possibility that repeated exposures will induce and boost sensitisation.
3. **The dose of antigen that penetrates the epidermis**: This is determined by the same variables as operated for the sensitising dose—the stratum corneum permeability barrier, the vehicle and the solubility and partition coefficient of the compound. In the same way as for the induction of sensitisation, the dose per unit area is a crucial determinant of response to elicitation challenge. Thus, when a series of DNCB elicitation challenge doses, as used by the author for the normal dose–response relationship studies above, were applied to pre-sensitised individuals on 1 cm diameter paper discs (Al test, Hyrylia, Finland) area 0.78 cm^2, absolute doses of 3.125, 6.25, 12.5 and 25 μg are actually 3.98, 7.96, 15.91 and 31.83 μg/cm^2. This yielded dose-related responses, with all four challenges eliciting positive responses in strongly sensitised individuals and the responses, measured as increase in thickness, showed a linear relationship with log of challenge dose (Friedmann et al. 1983; Moss et al. 1985; White et al. 1986) (Fig. 5.1). In those studies, the DNCB challenges were applied for only 6 h for two reasons: DNCB is a strong irritant, and if patch tests on DNCB

naïve individuals are left on for longer, then doses of 15.9 μg/cm^2 and above induce irritant responses which are erythematous but lack substantial oedema (thickness). Secondly, longer duration applications evoked significantly stronger responses which, at those concentrations, may blister and from which it is impossible to obtain meaningful calliper readings. There are data showing that as duration of application of elicitation challenges increases, there is a reciprocal drop in the concentrations required to elicit positive responses. This was clearly shown for paraphenylenediamine (Hextall et al. 2002; McFadden et al. 1998). Similarly, it has been shown for nickel that application of elicitation challenges for 48 h evokes a higher frequency of positive responses than application for 24 h (Kalimo and Lammintausta 1984). This observation reflects the fact that longer periods of application result in greater effective doses penetrating through the stratum corneum.

The crucial point about these observations is that the DNCB was applied in an acetone solution which, when applied directly onto skin, evaporates and deposits all the DNCB onto the skin or, when applied to a paper disc, the relative lipid solubility of DNCB ensures that it partitions out of the paper and into the stratum corneum. However, when DNCB or another lipid-soluble molecule is applied in a semi-solid base such as white soft paraffin (WSP), the position is quite different. This was examined by applying different total quantities of nickel sulphate in WSP on 7 mm Finn Chambers in nickel allergic individuals (Memon and Friedmann 1996). Thus, replicate chambers loaded with 2.5, 5, 10 and 15 mg of ointment containing 5 % nickel sulphate were applied to the back in duplicate, and the positive responses assessed at 48 h. Responses at chambers loaded with 2.5 mg were smaller and usually one grade weaker than those for chambers loaded with 5 mg. However, there were no differences in the intensity scores of reactions for chambers loaded with 5 mg or more, although the area of the response for chambers loaded with 15 mg was sometimes greater. The probable explanations for these observations are as follows. Firstly, 2.5 mg is not sufficient to cover the chamber so the reaction is smaller. Five and 10 mg are contained by the chamber as a "column" of ointment of different thicknesses or heights. Fifteen mg filled the chambers to the brim and even spilled over producing slightly larger areas of application than the size of the chambers, but the thickness/height of the column of ointment may also have been greater than for the other loading quantities. However, the crucial consideration is what is happening at the skin surface? A number of molecules of the antigen will diffuse into the stratum corneum, the number being determined by the concentration in the vehicle and the partition coefficients and relative solubilities of the substance in the vehicle and the stratum corneum. The loss of substance from the vehicle into the epidermis will lower the concentration of the substance in the vehicle in a narrow zone where it is in contact with the skin, but a steady state will be reached leaving much of the substance still in solution in the vehicle. So although 5, 10 or 15 mg of a 5 % concentration were applied in columns of increasing heights, the same number of molecules penetrate into the epidermis from each column—hence the reactions of equal intensity. Whereas, if all the

nickel in 5, 10 or 15 mg had been deposited onto the skin surface in a volatile solvent that left all the solute on the surface, then the effect would have been very different and would have given a dose–response curve. The above observation is of critical importance in the patch test clinic as it shows that as long as an adequate quantity of the ointment containing the antigen is applied, reproducible results will be obtained. The corollary is that workers attempting to demonstrate dose–response relationships should not apply the compound under investigation in a non-volatile vehicle as, although dose–response curves can be obtained, the actual amounts delivered to the skin surface are uncertain.

4. **Body site**: There are clear variations in reactivity at different body sites. Thus, forearm skin is significantly less responsive to inflammatory challenges including contact hypersensitivity elicitation with nickel (Memon and Friedmann 1996) (Fig. 5.3) or ultraviolet-induced erythema (Rhodes and Friedmann 1992; Waterston et al. 2004). Also, measurement of irritant responses induced by dithranol showed significant site variations between the medial and lateral sides of the forearm (Lawrence et al. 1986). When body sites were compared by repeated open application, lower arm was less responsive than upper arm while back skin was most reactive (Hannuksela 1991). It is also important to note that recent exposure to ultraviolet radiation or the use of topical immunosuppressive agents on any body site can reduce responses at that site. These points all mean that the performance of challenges on the forearm is less likely to yield positive results compared with other, more reactive sites.
5. **Augmentation of tissue responses either through irritant effects or by the presence of other sensitisers**: In proportion to their potency as irritants or sensitisers, xenobiotics applied to the skin activate innate immune responses with induction of a wide range of cytokines, adhesion molecules and activation of DCs, all important for maximising the efficiency of immune surveillance mechanisms (Friedmann et al. 1993). This tissue response to chemical perturbation primes the tissues so they can respond to lower concentrations of antigen. Thus, McLelland observed that when a concentration of a contact sensitiser too low to elicit a clinical response was applied to sensitised recipients in combination with a sub-irritant concentration of an irritant such as sodium lauryl sulphate (SLS), positive patch test responses could be evoked (McLelland et al. 1991); as mentioned above, SLS is not only an irritant but also a detergent and so will be likely to alter the absorption of the sensitiser. Similarly, if two allergens are mixed at concentrations below their elicitation thresholds and applied to individuals allergic to both allergens, the mixture elicited positive responses (McLelland and Shuster 1990)—there was clearly a synergistic effect when the two allergens were combined. This has particular relevance to challenges performed with patients' own products, either in patch tests or in repeated open application (ROAT) tests (see below). Grabbe and colleagues performed similar experiments in mice and confirmed the potentiation by irritancy. One hapten was applied at sub-eliciting doses in the presence of irritant concentrations of a different hapten to which the mice had not been sensitised (Grabbe

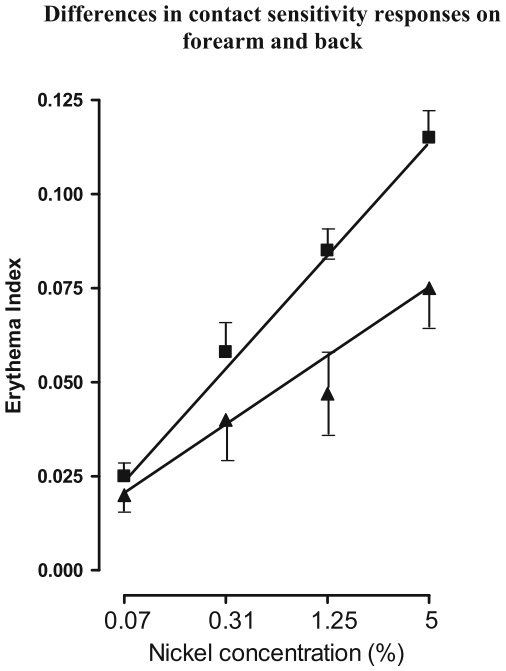

Fig. 5.3 Differences in contact sensitivity responses on forearm and back. Erythema responses as measured with an erythema metre in response to challenge with increasing concentrations of nickel sulphate applied 48 h before. *Filled Square* = responses on back *filled triangle* = responses on forearm; *Bars* are SEM. (Figure reproduced from Memon and Friedmann 1996 with permission)

et al. 1996), thus allowing separation of irritant effects from possible detergent effects.

5.3 Host-Related Factors Determining Individual Susceptibility

Individual susceptibility to contact sensitisation can be determined at several levels between the skin and the immune system. These include:

1. The physical integrity of the epidermal barrier (filaggrin gene, lipid types)
2. The integrity and quality of biochemical defences including antioxidant defences and drug-detoxifying/drug-metabolising enzyme systems
3. The innate immune responses of skin
4. The adaptive immune response:

   (a) Immune response reflected in MHC-linked ability to present immunogens.
   (b) T cell receptor recognition of immunogens.
   (c) Immune response controls balancing between immunological tolerance and active expression of hypersensitivity (Tregs: Teffectors).

In considering the human host factor(s), two approaches will be taken here. The first is to examine how discrete modifying factors such as genes, age and sex can affect overall susceptibility to develop contact sensitivities. Secondly, although it has been traditional to focus on the adaptive immune response which generates the specific T cell-mediated responses to immunogenic chemicals/haptens, there is now a growing realisation that the skin is in fact the major determinant of whether the adaptive immune system becomes activated. It does this partly by determining the dose of chemical that reaches the adaptive immune system and partly by contributing activating signals to DC that augment the chance they will initiate T cell activation. Many elements of skin function are variable as a result of genetic, environmental, hormonal, nutritional and even age-related factors. Many of these functional elements were previously regarded as separate or independent but are now recognised as being closely interdependent.

The key functions include:

- Stratum corneum integrity/impermeability
- Innate immune defences
  - Tissue defences against microbial invasion
  - Tissue defences against chemical perturbation
  - Responses to physical, microbial or chemical perturbation

5.3.1 Discrete Factors Modifying Susceptibility

5.3.1.1 Race

The evidence on whether race is a significant factor in susceptibility to contact sensitisation is weak. There are studies of susceptibility to irritants which have claimed that Asians may be more susceptible than Caucasians. However, the main report collected data from nine studies performed at three different centres in the USA (Robinson 2002). A total of 384 individuals aged 18–74 had been exposed to a number of irritants including SLS (all) acetic acid, 1-decanol and octanoic acid. Patches were applied to the upper outer arm for various times up to 4 h, and responses were assessed by clinical scoring at 24 and 48 h. The Asians developed irritant responses to SLS after shorter durations of patch application. A problem with the results from this report is the degree of human variability in responses to SLS. All subjects were tested with SLS as a "positive control" in each study, and across the nine studies, the percent of people giving positive responses to a 4 h challenge with 20 % SLS varied from 60 to 100 %.

Some evidence on racial differences in susceptibility to allergic contact sensitisation was obtained by Kligman. He sensitised groups of white and black volunteers (25 per group) with a number of sensitisers (Kligman 1966b). Lower frequencies of sensitisation were observed in black subjects with four of five allergens tested: monobenzyl ether of hydroquinone, nickel sulphate, penicillin G and neomycin

sulphate (Kligman 1966b). There is no evidence on possible reasons, but differences in stratum corneum thickness and possibly increased cutaneous antioxidant defences may contribute.

5.3.1.2 Age

Age has little overall effect during childhood and adult life. However, infants are clearly less sensitisable. Cassimos used a high dose (1 %) of DNCB to sensitise groups of neonates at 1, 3 and 9 months of age (Cassimos et al. 1980). It was shown that over the first 9 months of age, the proportions sensitised (as reflected by a positive response to challenge with 0.1 %) rose from 6.7 % in the first 15 days of life, through 26 % at 1 month, 63 % at 3 months to 91 % by 9 months. Even though the methodology used was not well quantified and the results are only expressed as proportion sensitised, it is a clear result. There is clinical evidence that immune function declines in old age as reflected by the increase in viral infections and tumours, but the evidence on cutaneous immune reactivity shows some interesting divergence. Thus, T cell-mediated responses including recall responses to intradermal challenge with tuberculin, streptokinase/streptodornase or *Candida albicans* are reduced after the age of 65 (Girard et al. 1977). That study also used rather "heavy-handed" methodology to assess induction of contact sensitivity with DNCB (10 %) and found significantly fewer individuals older than 65 years could be sensitised. The present author (PSF) has used quantitative methods to measure sensitisation by DNCB in a wide range of adults including the very old (the late 80s) and found that responsiveness does not diminish until after about 80 years of age (personal observation) (Friedmann and Pickard 2010). As clear evidence that susceptibility to irritants may be different from susceptibility to contact sensitisation, there is significant evidence of a decline in skin irritation responses with age. Thus, in the studies described in relation to race, Robinson also looked at effects of age. A significant decline in responses to SLS and octanoic acid was observed after 56 years of age (Robinson 1999). This may be related to the recent demonstration that in older people, there is a failure of production of TNF-α by dermal macrophages, which results in impaired cellular recruitment through interaction with dermal microvascular endothelium (Agius et al. 2009).

5.3.1.3 Gender

There is still uncertainty about the importance of gender in determining susceptibility to contact sensitisation. Some aspects of the immune and inflammatory responses are clearly influenced by gender. Thus, most autoimmune diseases are commoner in females; certain diseases such as urticarias and atopic dermatitis often exacerbate in relation to the menstrual cycle. Vascular responses following degranulation of mast cells have a distinct variation with the menstrual cycle (Kalogeromitros et al. 1995). The magnitude of weal and flare reactions following

prick test challenge with morphine or histamine was measured at different phases of the menstrual cycle, and it was shown that greatest responses occurred at days 12–16 of the cycle, corresponding to maximal oestrogen levels. However, T cell-mediated responses such as contact sensitisation and delayed-type hypersensitivity have not been well studied in relation to the gender differences or menstrual cycle. One study used DNCB to quantify contact sensitisation and observed significantly greater responses in females (Rees et al. 1989). However, in that study, no attention was given to the phase of the menstrual cycle of the volunteers, but a more recent study which also measured sensitisation by DNCB compared males and females but carefully avoided the start of the menstrual cycle ±5 days (Morrissey et al. 2008). A small but significantly greater reactivity was detected in males. Also, Robinson reported that males gave significantly greater responses to irritants such as SLS and octanoic acid (Robinson 2002).

This is clearly an area where careful investigation of gender differences and the effects of menstrual cycle are required.

5.3.1.4 Genetic Factors

There will be genetic contributions to all the steps in the pathway between the intact epidermal barrier, the epidermal defences including biochemical (metabolic and redox-sensitive pathways), innate immunity and the adaptive immune response which generates hapten-specific T cells including effector and regulatory cells. This could be the subject of a large review on its own. Therefore, only selected and pertinent genetic components will be touched on here.

"Epidermal" integrity is complex and clearly important. Mutations in the filaggrin gene have been identified as causing the inherited dry skin of ichthyosis vulgaris. Similar mutations resulting in filaggrin deficiency are closely involved in the pathogenesis of atopic eczema, and up to 10 % of the normal population carry at least one copy of these mutations (Palmer et al. 2006). The filaggrin mutations lead to a more water-permeable stratum corneum, but it is not known whether the permeability for lipophilic chemicals is altered. Atopic eczema sufferers are more susceptible to skin irritation by surfactants which may be a reflection of greater penetration of the surfactant molecules which is associated with further barrier disruption and activation of the inflammatory response. Interestingly, there is no clear evidence that the increased water permeability also applies to lipid-soluble agents. We have evidence that DNCB penetrates into atopic epidermis equally (Newell et al. 2013) and, paradoxically, atopic eczema sufferers are sensitised less strongly by DNCB than non-atopic controls (Newell et al. 2013; Rees et al. 1990a).

A number of diseases characterised by ichthyosis (dry skin) have been shown to have genetic mutations of the formation of the intra-epidermal lipids. There are many examples, but one of the best known is recessive X-linked ichthyosis in which the enzyme steroid sulphatase is deficient (Elias et al. 2008). It is presumed that there are disturbances of the epidermal barrier function in these conditions, but whether it is the water-soluble or lipid-soluble constituents of the environment that

are affected has yet to be defined. Similarly, the susceptibility to irritation or contact sensitisation of individuals with these rare conditions has yet to be determined.

5.4 Integrated Skin Responses to Physical or Chemical Perturbation

5.4.1 Interrelationships Between Epidermal Barrier Integrity and Innate Defences

The impermeability and integrity of the stratum corneum barrier is the major determinant of the penetration not only of microbes but also of water-soluble molecules. Susceptibility to irritation by sodium lauryl sulphate (SLS), a water-soluble surfactant, is proportional to the amount that penetrates (de Jongh et al. 2006), but how/why it generates an inflammatory reaction is not clear. However, there is now evidence that, following disruption of the stratum corneum barrier either with SLS or by tape stripping, a set of protective/restorative responses is activated. Thus, rapid repair of the stratum corneum is initiated involving a wave of pseudo-apoptosis of upper epidermal keratinocytes to generate new corneocytes (Demerjian et al. 2008; Grubauer et al. 1989; Hachem et al. 2006). Also, there is a burst of synthesis and secretion of lamellar bodies to restore the intercellular lipid layers (Menon et al. 1992). Although it has not yet been shown, it is likely that elements of the innate immune response will also be activated in preparedness for defence against microbial invasion through the disrupted physical defences. This will involve expression of antimicrobial peptides, pro-inflammatory cytokines and activation of dendritic cells.

Many factors including genetic and environmental agents may result in impairment of the water permeability barrier of the stratum corneum. This impairment is reflected by increased trans-epidermal water loss and clearly has implications for susceptibility to penetration of water-soluble chemicals as outlined above. However, regarding the penetration of lipid-soluble molecules, the general view is that it is a passive diffusion process, the rate of which is concentration dependent in accordance with Fick's laws but which is also determined by the relative solubility (partitioning) of the compound between the vehicle and the epidermis. This is reflected by the log P, where P is the octanol-water partition coefficient. If there is passive and easy entry for lipid-soluble agents, the question that arises is "what form of innate defence is present to prevent chemical perturbation by many lipid-soluble compounds—such as chemical sensitisers"? Within the stratum corneum is a layer of what was previously described as "sulphur-rich" proteins, thought to be breakdown products of filaggrin or other structural proteins. We have now shown that this layer is in fact extremely rich in sulphydryl groups that can bind thiol-reactive chemicals, thereby impeding their penetration into the viable layers of the epidermis (Pickard et al. 2009) (Fig. 5.4). This discovery provides the explanation

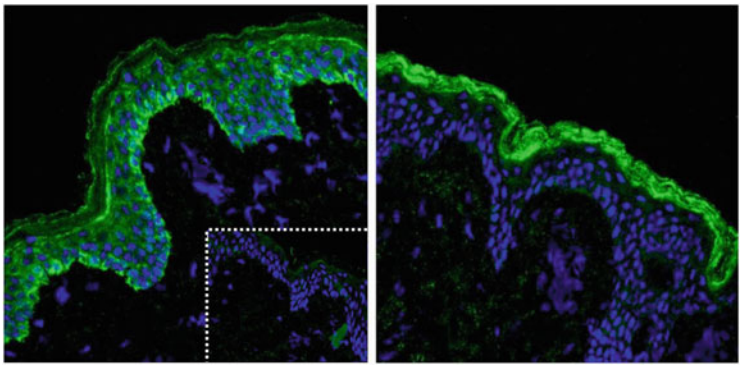

Fig. 5.4 The differential penetration of human skin by DNCB and DNTB. Normal human breast skin, mounted in Franz's diffusion chambers, was challenged with vehicle alone (*inset*), DNCB (*left panel*), DNTB (*right panel*) for 24 h. Cryosections were obtained and stained with an anti-DNP Ab to detect protein dinitrophenylation (shown in *green*). Nuclei were stained with To-Pro-3 (shown in *blue*); the *scale bar* represents 50 nm (Reproduced from Pickard et al. 2009 with permission)

for why there is a big difference in the skin sensitising potencies of 2,4-dinitrochlorobenzene (DNCB) and 2,4-dinitrothiocyanobenzene (DNTB) at equimolar concentrations. DNCB is a potent sensitiser in humans, while at comparable doses, DNTB is almost a non-sensitiser. However, DNTB is much more reactive with thiols, as in glutathione, and readily binds to the thiol-rich layer in the stratum corneum. It requires a much greater concentration of DNTB to saturate the thiol-rich layer and to penetrate into the epidermis in similar quantities to DNCB (Pickard et al. 2009). This clearly adds an additional element to the innate barrier defences. While the reactive thiol groups may be components such as cysteine residues in molecules such as filaggrin, overall, the finding points to the high likelihood of a more substantial redox-based defence system that will be important in defending against tissue damage by reactive chemicals. Other contributors to the redox-based defences include xenobiotic-metabolising enzymes such as glutathione-S transferase and members of the cytochrome P450 superfamily.

5.5 Conclusion

Contact allergy to environmental chemicals is a significant social and medical problem, but by using the immune responses to define chemical sensitisers, it has been possible to learn a great deal about the immune system at the cellular and physiological level of the whole organism. Whether and how the immune response is activated by chemicals is the resultant of the properties of the chemical itself together with a range of host-related factors that determine susceptibility. We are at

a rather early stage of characterising these factors, but one day in the future, it should become possible to explain exactly why of two individuals given identical exposure to a defined dose of DNCB, one will develop significant contact allergy to it and the other will show no clinical evidence of allergic reactivity. Whereas it was widely thought this was all determined by the cells of the adaptive immune system, it is clear now that the contribution of the innate immune system in the skin itself is likely to be critical in determining these clinical outcomes.

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Chapter 6
Tools and Methods for Identification and Analysis of Rare Antigen-Specific T Lymphocytes

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Abstract T lymphocytes are essential as effector and memory cells for immune defense against infections and as regulatory T cells in the establishment and maintenance of immune tolerance. However, they are also involved in immune pathology being effectors in autoimmune and allergic diseases or suppressors of immunity in cancer, and they often cause problems in transplantation. Therefore, strategies are being developed that allow the in vivo amplification or isolation, in vitro expansion and genetic manipulation of beneficial T cells for adoptive cell therapies or for the tolerization, or elimination of pathogenic T cells. The major goal is to make use of the exquisite antigen specificity of T cells to develop targeted strategies and to develop techniques that allow for the identification and depletion or enrichment of very often rare antigen-specific naïve as well as effector and memory T cells. Such techniques are very useful for immune monitoring of T cell responses in diagnostics and vaccination and for the development of T cell-based assays for the replacement of animal testing in immunotoxicology to identify contact allergens and drugs that cause adverse reactions.

Abbreviations

ACD	Allergic contact dermatitis
APC	Antigen-presenting cell
CHS	Contact hypersensitivity
CMV	Cytomegalovirus

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CSA	Cytokine secretion assay
DNBS	2,4-Dinitrobenzene sulfonic acid
hTCPA	human T cell priming assay
LLNA	Local lymph node assay
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cell
TCR	T cell receptor
TNBS	2,4,6-Trinitrobenzene sulfonic acid

6.1 T Cells in Immune Defense and Immune Pathology

T lymphocytes are highly specific immune cells that together with B lymphocytes constitute the main cell types of the adaptive immune system. Due to their antigen-specific T cell (TCR) and B cell receptor (BCR), respectively, these cells detect antigens with exquisite specificity and efficiently mount primary immune responses and generate immunologic memory. T lymphocytes are among the most studied cell types of the immune system as important effector cells in the defense against pathogens and tumors, but also in the establishment of acute and chronic diseases.

T cells are central players initiating and maintaining adaptive immune responses, eventually forming memory T cells. With a broad repertoire of cytokines, cytotoxic substances, and other mediators, they execute and control immune reactions. Therefore, the diverse $CD4^+$ helper T cell (Th) subsets and the $CD8^+$ cytotoxic T cells (Tc) specifically recognizing and reacting towards defined antigens are analyzed to understand their generation, differentiation, functions, and dynamics in the various types of immune responses. Moreover, the analysis of T cell counts and especially of antigen-specific T cell responses has proven useful to monitor the course of diseases or of therapeutic interventions as done, for example, in HIV infections (Davenport et al. 2007; McMichael et al. 2010; Boyle et al. 2012; Makedonas and Betts 2011) or cancer vaccine therapy (Nagorsen et al. 2004). Furthermore, these studies have encouraged work on prophylactic and therapeutic intervention strategies to initiate or amplify effective T cell responses, to generate T cell memory, and to interfere with unfavorable T cell responses. Adoptive cell therapies (ACT) using T cells are being used in which antigen-specific T cells isolated from a patient are reinfused after in vitro expansion (Restifo et al. 2012; Lizee et al. 2013). One example is the infusion of tumor-infiltrating lymphocytes (TILs) after in vitro expansion for melanoma therapy (Rosenberg and Dudley 2009; Lee and Margolin 2012; Wu et al. 2012; Wang et al. 2012). TCR gene therapy involves the introduction of tumor antigen-specific TCR genes into patient cells (Jorritsma et al. 2011; Linnemann et al. 2011). In some cases T cells with engineered antigen-specific TCRs, chimeric antigen receptors (CARs), are exploited in this type of immunotherapy (Cheadle et al. 2012; Maher and Wilkie 2009; Ramos and Dotti 2011; Shirasu and Kuroki 2012; Gilham et al. 2012; Park et al. 2011; Turtle et al. 2012). Inefficient T cell responses, for example, in tumor

therapy, can be amplified by blocking negative regulatory molecules such as PD1 or CTLA-4 by antibodies in vivo (Bour-Jordan et al. 2011; Quezada et al. 2011; Drake 2012) or by interfering with regulatory T cells (Treg) (Byrne et al. 2011).

In the case of autoimmune diseases or transplantation-associated complications such as graft versus host disease (GvHD), strategies are being used and developed that interfere with pathogenic T cell function and T cell migration (Getts et al. 2011; Sallusto et al. 2012). Alternatively, immunosuppressive Treg are being exploited for therapeutic approaches (Battaglia and Roncarolo 2011; Lan et al. 2012; Daniel and von Boehmer 2011), e.g., in GvHD (Tang et al. 2012).

6.2 Exploiting the Antigen Specificity of T Cells

A great advantage of T cell-mediated immune responses is their antigen specificity. Knowledge of the T cell epitope and of the MHC allele presenting it allows specific T cell targeting to defined antigens such as tumor antigens. Many approaches to suppress pathogenic T cell responses usually lack specificity and, thus, target T cells in general. Therefore, detection and specific therapeutic targeting of the antigen-specific pathogenic T cells are challenges that have to be met in the future. Strategies are being developed to identify T cell epitopes and to induce antigen-specific T cell tolerance, for example, in the specific immunotherapy (SIT) of allergies (Allam and Novak 2011; Jahn-Schmid et al. 2011; Holgate 2012; Maggi et al. 2012; Schulten et al. 2013). However, the methodological challenge is the reliable detection of antigen-specific T cells which are often rare.

6.3 T Cells in Immunotoxicology

T cells have gained significant interest as tools in immunotoxicology. Many chemicals such as contact allergens and drugs trigger chemical-specific T cell responses and thereby cause T cell-mediated adverse reactions leading to allergic contact dermatitis (ACD) or adverse drug reactions (ADR) such as bullous eczematous skin reactions or drug-induced liver injury (DILI). Therefore, T cell-based assays have been developed for diagnostic purposes and for hazard identification in immunotoxicology by testing chemicals for their ability to induce a primary T cell response to contact allergens and drugs (Martin et al. 2010). Moreover, monitoring drug-specific T cell responses can be used for diagnostic purposes and serves to prevent ADR and unwanted cross-reactivities of drug-specific T cells as in the case of β-lactam antibiotics.

The lymphocyte transformation test (LTT) is used to detect drug-specific effector and memory T cells in the blood of patients with drug hypersensitivity reactions (DHR) (Nyfeler and Pichler 1997; Pichler and Tilch 2004). This test measures T cell proliferation in response to the drug of interest. DHR is often associated with

cytotoxic T cell responses. It has been demonstrated that such responses can be monitored efficiently in the blood of patients using an ELISPOT assay that detects the cytotoxic T cell molecule granzyme B (Zawodniak et al. 2010). Interestingly, this approach is also working in DHR patients with only weak drug-specific proliferative responses and may thereby be a useful supplement to the LTT. The patch test is used as an in vivo assay to detect T cell reactions to chemical contact allergens including metals (Dickel et al. 2011) and to drugs in the skin (Barbaud 2009). It is based on the application of the test substances to the skin and, in case of an existing sensitization, the T cell-mediated reddening and eczema formation of the test area of the skin.

6.4 Technologies for the Detection and Isolation of Rare Antigen-Specific T Cells

Of great importance in the T cell field is the analysis of antigen-specific T cells in the naïve, effector, and memory T cell pools. For example, the identification and enumeration of antigen-specific naïve T cell precursors is of interest with the aim to predict the chance to induce adaptive immunity as it is described in Chaps. 7, 8, 9, and 10. The analysis of the precursor T cell pool size and the TCR repertoire diversity may provide information regarding the strength of a T cell response and the efficiency of T cell memory formation (Jenkins and Moon 2012; Kimber et al. 2012; Moon et al. 2009).

Several methods for the identification of T cell epitopes have been developed using protein digests, synthesis of overlapping peptides, prediction algorithms based on the identification of MHC allele-specific binding motifs for mixtures of peptides eluted from affinity-purified MHC molecules subjected to pool sequencing, and biochemical and proteomic approaches for epitope identification (Gilchuk et al. 2013; Hillen and Stevanovic 2006; Rammensee et al. 1999; Wolk et al. 2012). Moreover, genetic approaches (Britten et al. 2005; Schuler et al. 2005) and the identification of peptide mimotopes (Crawford et al. 2006; Siewert et al. 2012; Yin et al. 2012) help to identify natural epitopes that are extremely useful in the analysis of T cell function and specificity as well as for the in vitro and in vivo expansion and use of antigen-specific T cells in ACT. Large data bases are generated that allow screening, for example, for T cell epitopes from allergens (Schulten et al. 2013; Vaughan et al. 2012; Salimi et al. 2012). Another important factor is the knowledge of the MHC restriction as simplified, for example, by the generation of a broad panel of human MHC allele transfectants (McKinney et al. 2013).

Identification of T cell epitopes and MHC restriction elements facilitates the use of T cells as tools in immunology and immunotoxicology. Appropriate methods for the detection and immunophenotyping of antigen-specific T cells ex vivo are needed. Various isolation and enrichment methods for antigen-specific T cells have been developed in the last years that allow isolating even very rare precursor

cells. Basically two strategies can be distinguished. The first strategy is based on the direct identification of antigen-specific T cells by staining the specific TCR with MHC/peptide multimers such as dimers, tetramers, or pentamers. These are multimerized MHC molecule containing proteins that contain the antigenic peptide of interest. Alternatively, chimeric molecules consisting of an antibody backbone and two MHC molecule peptide binding domains loaded with the antigenic peptide are used (Greten and Schneck 2002; Oelke and Schneck 2010; Sims et al. 2010; Davis et al. 2011; Nepom 2012). These technologies are especially powerful to enumerate $CD8^+$ T cells recognizing a well-known epitope independent of their functional properties. Thereby frequencies of antigen-specific T cells can be determined in the naïve T cell repertoire and also in the effector/memory T cell pool. MHC multimers also allow to isolate antigen-specific T cells by magnetic bead or flow cytometric separation techniques and to detect them in tissues in vitro and in vivo by in situ staining, e.g., with MHC tetramers and 2-photon microscopy (Skinner and Haase 2002, 2005; Hong et al. 2009; Andersen et al. 2012; Coppieters et al. 2012; Haanen et al. 2000; Hadrup et al. 2009; Vyth-Dreese et al. 2006). Sophisticated multiplex methods have been developed that allow the analysis of complex T cell responses using peptide-MHC multimers displaying large peptide arrays (Toebes et al. 2006, 2009; Hadrup et al. 2009). These multiplex approaches enable T cell epitope identification and may be useful for T cell frequency analyses in diagnostics. The second strategy is the analysis of T cell effector functions such as proliferation, cytokine production, expression of activation markers, and cytotoxicity.

6.5 Functional Assays for Antigen-Specific T Cell Analysis

Functional analysis of antigen-specific T cells which might be combined with MHC/peptide multimer analysis is based on the analysis of effector molecules (cytokines, chemokines, cytotoxic molecules, activation markers) and cellular functions (proliferation, cytotoxicity) upon ex vivo stimulation. Human peripheral blood T cells can be stimulated in vitro with their respective antigen in the form of peptides, proteins, and cellular or pathogen lysates added to PBMC or loaded on antigen-presenting cells (APCs) or by using infected cells. The presence and functional properties of antigen-specific T cells can be measured on the single cell level or on the level of a population.

Several assays allow the measurement of cytokines, chemokines, and other mediators either as secreted products in cell culture supernatants (ELISA, flow cytometric bead-based assays, or multiplex analyses) (Lash and Pinto 2010; Maecker et al. 2012). Detection on the single cell level can be done by ELISpot (Lehmann and Zhang 2012) or by flow cytometric analysis of T cells after intracellular staining or by using the cytokine secretion assay (CSA) technology (Thiel et al. 2004; Campbell et al. 2011). All of these techniques make use of monoclonal antibodies directed against the molecule of interest. Furthermore the activation

marker CD40L/CD154, which is upregulated within hours after stimulation of a T cell, allows the flow cytometric detection of antigen-activated $CD4^+$ T cells. In combination with detection of cytokines, CD154 is very powerful for a comprehensive functional assessment of an antigen-specific $CD4^+$ T cell population. T cell expansion can be measured by radioactive methods due to incorporation of ^3H thymidine into newly synthesized cellular DNA or by flow cytometry using the dilution of fluorescent dyes such as CFSE (Lyons 1999, 2000; Thiel et al. 2004). Cytotoxic activity of T cells can be measured by radioactive methods such the ^{51}chromium release assay (Brunner et al. 1968) or ^3H thymidine-based JAM assay (Atarashi et al. 2000; Bohm et al. 1998; Hoves et al. 2003; Matzinger 1991) or nonradioactive flow cytometric-based methods using a combination of CFSE and PI for target cell labeling (Godoy-Ramirez et al. 2005; Marcusson-Stahl and Cederbrant 2003). Alternatively, the surface expression of the intracellular lysosomal-associated membrane protein 1 (LAMP-1/CD107a) upon degranulation of cytotoxic T cells is measured by flow cytometry (Rubio et al. 2003; Betts et al. 2003). Combinations of the different readouts in multiparametric analyses are now well-established strategies.

6.6 Magnetic Bead Isolation and Multiparametric Flow Cytometry

One of the major challenges for the T cell analysis is the low frequency and absolute number of T cells specifically recognizing a certain antigen. Other challenges are the kind, the availability, and the size of the cellular source. Blood samples are the best available material, but T cells in the PBMC do not necessarily reflect the T cells at the site of the immune reaction. Skin biopsies are usually very small, and cell numbers isolated from these samples are limited. A further complication is that T cell subset composition and function in patient samples may be altered due to therapy.

Multiparametric flow cytometric approaches are able to identify >1 cells in 10^4–10^5 total cells and are performed to detect specific T cells directly ex vivo, e.g., using MHC/peptide multimers or expression of cytokines or activation markers upon short-term in vitro activation (Bacher and Scheffold 2013; Brosterhus et al. 1999; Frentsch et al. 2005; Pittet et al. 2001). Detection of, e.g., virus-specific T cells like human cytomegalovirus (CMV)-specific T cells is in most cases simple, because these are present in frequencies of 0.1 up to 1 % among T cells or even more in CMV-seropositive donors, as the example shows (Fig. 6.1). Specific CD8+ T cells are detected, for example, by combined anaylsis of MHC class I/peptide tetramer$^+$ staining and their IFN-γ secretion after one hour stimulation of PBMC from a seropositive donor either with a single CMV peptide or with a pool of overlapping peptides spanning the complete sequence of the pp65 antigen. The steps of the procedure are as follows: (1) isolation of PBMC from

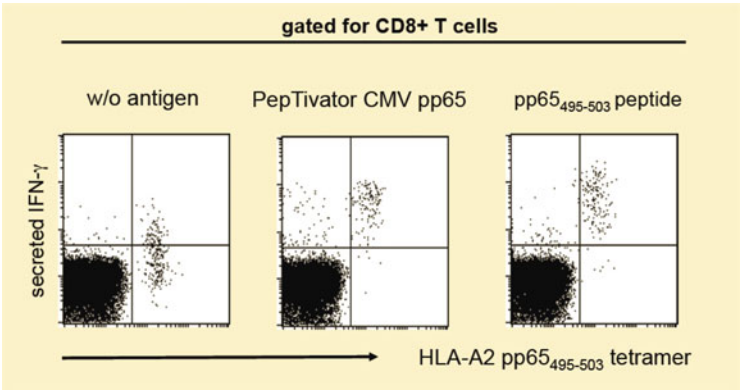

Fig. 6.1 Flow cytometric analysis of cytomegalovirus CMV pp65-specific CD8+ T cells by combined detection of MHC/peptide multimers and IFN-γ expression. PBMC from a CMV seropositive HLA-A2-positive donor were isolated and stained with a CMV $pp65_{495–503}$ peptide loaded HLA-A2 tetramer for 30 min at 2–8 °C. Afterwards the cells were cultured in the presence of the HLA-A2-restricted $pp65_{495–503}$ peptide, with a pool of overlapping peptides spanning the complete sequence of the pp65 antigen, or for control without addition of antigen for 1 h. Subsequently, the IFN-γ secreting CD8+ T cells were detected by performing a cytokine secretion assay

CMV-seropositive donor, (2) stimulation for 1 h with antigen, (3) staining with CMV peptide-loaded MHC class I tetramers for 30 min, and (4) flow cytometric detection of IFN-γ secreting T cells by cytokine secretion assay (CSA).

In this type of approach, even frequencies below 0.1 % are detectable by flow cytometry, as depicted for PBMC from a healthy donor stimulated o/n with a *Candida albicans* lysate (Fig. 6.2). In order to make sure that the detected events faithfully represent antigen-specific T cells, the following precautions should be taken: enough positive cells have to be measured to detect a difference in the number and the frequency between the unstimulated control and the stimulated sample, and using at least two parameters for the readout improves the reliability of the measurement.

In order to identify very rare T cells, for example, 1 in 10^5 up to 1 in 10^7, including naive T cells having a certain antigen specificity, for example, for TCR repertoire analysis and immunotoxicologic prediction assays, there are two options: (1) amplification and (2) pre-enrichment of rare T cells.

1. Amplification can be achieved by in vitro cell expansion after antigenic stimulation; often multiple rounds of stimulation are used. Another method to amplify rare T cells is the polyclonal expansion and subsequent interrogation of the expanded precursor pool by stimulation with protein antigens (Geiger et al. 2009). However, this method has not been successful so far when using chemical antigens such as contact sensitizers. To assess rare contact sensitizer-specific T cells within the naive T cell compartment in order to predict antigenicity of chemicals human naive T cells can be primed with chemicals (Martin

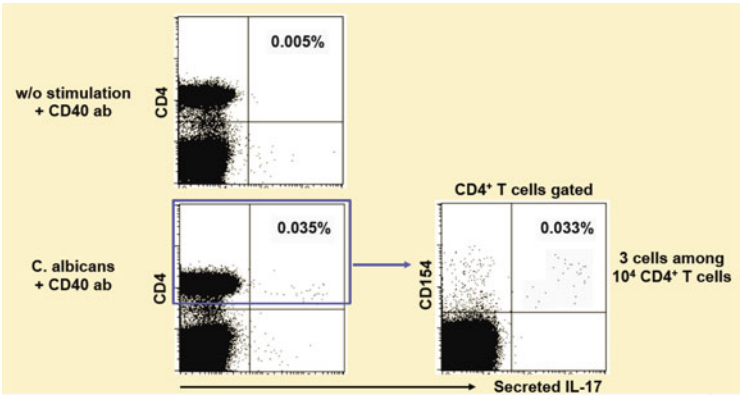

Fig. 6.2 Flow cytometric analysis of rare *Candida albicans*-specific CD4+ T cells by combined detection of CD154 and IL-17 expression. PBMC from a healthy donor were isolated and stimulated with or without *Candida albicans* lysate for 4 h. Anti-CD40 antibody was added to the culture to prevent downregulation of cell surface expression of CD154, which is induced upon antigen stimulation on CD4+ T cells. Subsequently, an IL-17 secretion assay was performed together with a co-staining for CD154. IL-17 secreting CD154+ *Candida albicans*-specific CD4+ T cells were detected with a frequency of 0.033 % among the total CD4+ T cell compartment

et al. 2010). Together with partners in the EU project Sens-it-iv, we have developed an in vitro human T cell priming assay (hTCPA) (Dietz et al. 2010; Richter et al. 2013). A very similar assay has been developed by Vocanson et al. (2008). Based on these protocols, the induction of primary T cell responses to drugs can also be investigated (Faulkner et al. 2012). The hTCPA is used for the identification of contact allergens for the purpose of hazard identification in immunotoxicology. It should be a useful and, due to the exquisite antigen specificity of T cells, would be in fact the most specific element in an integrated testing strategy to replace animal testing in the local lymph node assay (Adler et al. 2011; Jowsey et al. 2006).

In this context we have also established a cell culture system to allow primary activation of naive CMV-specific T cells from CMV noninfected, i.e., seronegative individuals and detection of the primed cells after 9 days of in vitro culture (Fig. 6.3). First, naive T cells are isolated and cocultured with autologous monocyte-derived DCs pulsed with antigen. During the culture feeder cells, CD28 antibody to ensure co-stimulation signals and cytokines for T cell proliferation and maintenance are added. On day 9 cells are reactivated with antigen-pulsed T cell-depleted PBMC and effector functions are analyzed. As shown in Fig. 6.4, during the 9 days of culture, T cells proliferated, demonstrated by the dilution of CFSE and a small fraction of the highly proliferated T cell-secreted IFN-γ upon restimulation with the pp65 antigen, but not without reactivation.

These data demonstrate that this assay allows identifying rare antigen-specific T cells within the naive compartment. As outlined in Chap. 7, we have successfully translated this protocol for the detection of contact sensitizer-specific T

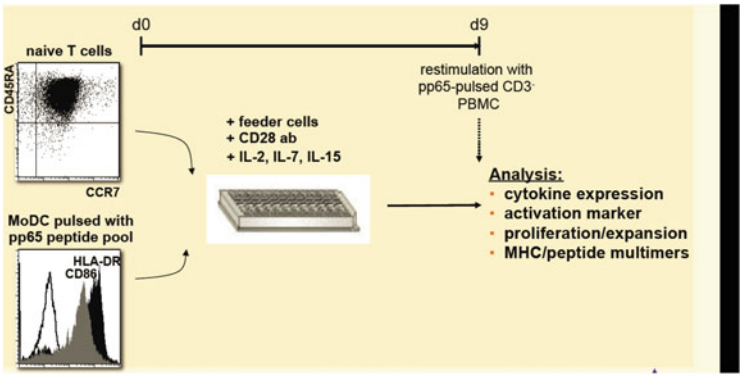

Fig. 6.3 Cell culture system for identification of naïve antigen (pp65)-specific T cells. Naïve T cells and monocytes were magnetically enriched from PBMC of a healthy CMV non-infected donor. Monocytes were differentiated and matured to dendritic cells (MoDC) and pulsed with a pool of overlapping peptides spanning the whole pp65 protein. MoDC and naïve T cells were cultured in the presence of feeder cells, co-stimulatory anti-CD28 antibody, and IL-2, IL-7, and IL-15 in microcultures of a 96-well plate. On day 9 after onset of the cultures, antigen(pp65)-specific T cells were identified and analyzed using MHC/peptide multimers or after a short-term restimulation by detection of cytokine or activation marker expression, or proliferation and expansion

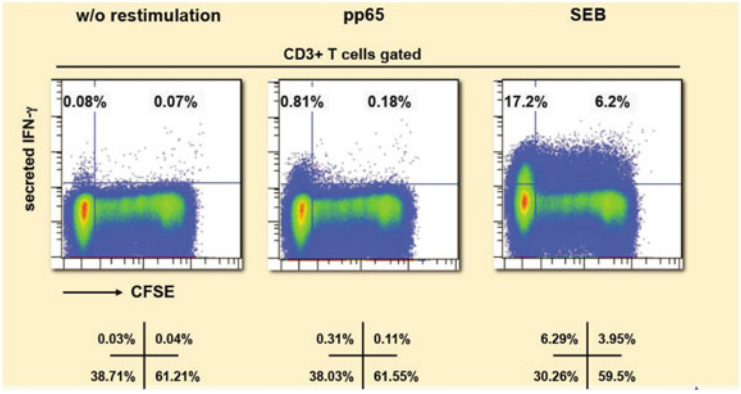

Fig. 6.4 Identification of pp65-specific cells within the naïve T cell compartment by detection of proliferation in combination with cytokine secretion upon antigen stimulation. Enriched and CFSE-labeled naïve T cells from a CMV noninfected donor were primarily activated with pp65 as described in Fig. 6.3. On day 9 cells were restimulated with the cognate pp65 antigen or for control with the polyclonal stimulus SEB or left without antigen for 4 h. Subsequently, an IFN-γ secretion assay was performed to detect the rare IFN-γ+ pp65-reactive T cells, which are present with a frequency of 0.31 % in the T cell compartment and represent 0.81 % within the highly proliferated, $CFSE^{dim}$ cell fraction

cells. Instead of a conventional pulsing of DCs with antigen, we used a chemical modification of immature DCs with DNBS and TNBS to generate DNP and TNP epitopes, respectively, a protocol that has been adapted from work with contact allergen-specific mouse T cells (Martin 2004; Martin et al. 2010). Using this assay we can clearly identify DNP- and TNP-specific CD4+ as well as CD8+ T cells, derived from the naive compartment (Dietz et al. 2010; Richter et al. 2013). The standard operating procedure and the discussion of advantages and limitations of the hTCPA are presented elsewhere (Richter et al. 2013).

2. Pre-enrichment of rare T cells directly prior to the analysis is another strategy to detect very rare antigen-specific T cells. Substantial improvement of the sensitivity for detection of such highly rare T cells by a magnetic pre-enrichment of specific T cells prior to flow cytometric analysis can be achieved (<10 cells in 10^8 total cells) (Alanio et al. 2010; Bacher et al. 2013; Brosterhus et al. 1999; Campbell et al. 2010, 2011; Pittet et al. 2001).

For many years now strategies for the magnetic isolation of rare cells, including hematopoietic stem cells (HSC), have been developed, and this technique is being used for preparative applications in research and for cellular therapy but also for analytical applications, like enumeration of endothelial progenitor cells (EPC), circulating tumor cells, and antigen-specific T cells. New technical developments now allow combining both magnetic enrichment and flow cytometric detection in a single instrument.

6.7 Obstacles in Rare Cell Analysis

As expected there are a number of problems associated with the analysis of rare antigen-specific T cells. There is often a high background of false-positive cells in flow cytometric analysis due to abundant nontarget cells. This can be significantly reduced by magnetic pre-enrichment. Rare cell analysis is also limited by low cell numbers, and often the calculation of the number of events required for a given precision is necessary. Here, magnetic pre-enrichment is also helpful since it allows the rapid analysis of high cell numbers for optimal precision. As an example, without pre-enrichment 10^6 total cells are measured in the flow cytometer in 2–20 min. Rare cells can be 0.01 % or 0.001 % of total cells corresponding to 100 target cells and ten target cells, respectively. Magnetic pre-enrichment of (target) cells + flow cytometric analysis allows for the analysis of 10^8–10^9 total cells in 20 min.

The characterization of subpopulations of rare cells is often of great interest. Magnetic pre-enrichment is also used here to reduce the background (false-positives) and to increase the sensitivity of flow cytometric analysis, e.g., of signature cytokines of T cell subsets (Bacher et al. 2013). Using an automated combined magnetic enrichment and flow cytometric analysis allows the fast and sensitive detection of rare cell populations, e.g., antigen-specific T cells, stem cells,

circulating tumor cells, and the detailed characterization, e.g., phenotype and cytokine profile. The limit of the sensitivity depends on starting cell number. Less than ten cells per 10^8 cells can be detected.

6.8 Summary

- Direct ex vivo antigen-specific T cell analysis is possible (>1 cells in 10^4–10^5 total cells).
- Increased reliability of rare cell detection is achieved by multiparametric analyses.
- The human in vitro T cell priming assay allows the identification of naïve precursors and subsequent T cell receptor repertoire analysis. The hTCPA can be used as a prediction assay for the antigenicity of chemicals including sensitizers and drugs. Therefore, the assay is useful for hazard identification in immunotoxicology. Modifications of the assay can also be used to analyze contact sensitizer or drug-specific effector and memory T cells and can thus be a useful diagnostic tool (see also Chaps. 9 and 10).
- Magnetic pre-enrichment improves the detection of rare cells (<10 cells in 10^8 total cells):
  - Increased sensitivity of flow cytometric analysis
  - Reduced background (false-positives)
  - Characterization of subpopulations of rare cells

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Chapter 7
Human T Cell Priming Assay: Depletion of Peripheral Blood Lymphocytes in $CD25^+$ Cells Improves the In Vitro Detection of Weak Allergen-Specific T Cells

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Abstract To develop an in vitro assay that recapitulates the key event of allergic contact dermatitis (ACD), that is the priming of effector T cells by hapten-presenting dendritic cells, and then allows for the sensitive detection of chemical allergens represents a major challenge. Classical human T cell priming assays (hTCPA) that have been developed in the past, using hapten-loaded monocyte-derived dendritic cells (MDDCs) as antigen-presenting cells and peripheral blood lymphocytes (PBLs) as responding cells, were not efficient to prime T cells to common allergens with moderate/weak sensitizing properties. Recent progress in the understanding of the effector and regulatory mechanisms of ACD have shown that T cell priming requires efficient uptake of allergens by immunogenic DCs and that it is controlled by several subsets of regulatory cells including $CD25^+$ Tregs. We therefore analyzed various parameters involved in allergen-specific T cell activation in vitro and showed that priming of allergen-specific T cells is hampered by several subsets of immune cells comprising $CD1a^{neg}$ DCs, $CD25^+$ T cells, and $CD56^+$ regulatory cells.

$CD4^+CD25^+FoxP3^+$ Tregs prevented the in vitro T cell priming to moderate/weak allergens, and depletion of human PBLs in $CD25^+$ cells significantly increased specific T cell proliferation and IFN-γ secretion. $CD56^+$ cells exerted an additional control of T cell priming since co-depletion of both $CD56^+$ and $CD25^+$ cells improved the magnitude of chemical-specific T cell activation. Finally, $CD1a^{low}$ MDDCs were

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able to inhibit T cell activation obtained by allergen-pulsed CD1a^{high} MDDC. Moreover, we showed that uptake by DC of allergen-encapsulated nanoparticles significantly increased their activation status and their ability to prompt specific T cell activation. Hence, by combining the different strategies, i.e., depletion of CD25^{+} and CD56^{+} cells, use of CD1a^{high} MDDC, and nanoparticle encapsulation of allergens, it was possible to induce T cell priming to most of the moderate/weak allergens, including lipophilic molecules highly insoluble in culture media. Therefore, the present optimized in vitro human T cell priming assay is a valuable method to detect the sensitizing properties of chemical allergens.

Abbreviations

ACD	Allergic contact dermatitis
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNCB	2,4-Dinitrochlorobenzene
FITC	Fluorescein isothiocyanate
HCA	a-Hexylcinnamaldehyde
hTCPA	human T cell priming assay
ISs	Immunological synapses
mAb	monoclonal antibody
MDDCs	Monocyte-derived dendritic cells
MDLR	Mixed dendritic cell lymphocyte reaction
PBLs	Peripheral blood lymphocytes
PBMCs	Peripheral blood mononuclear cells
SLS	Sodium lauryl sulfate
TNBS	2,4,6-Trinitrobenzene sulfonic acid

7.1 Introduction

Allergic contact dermatitis (ACD) is a frequent skin inflammatory disease that develops upon repeated skin exposure of individuals to nonprotein chemicals, called haptens (Honda et al. 2013; Peiser et al. 2012; Vocanson et al. 2009). The priming of specific cytotoxic CD8^{+} T cells by hapten-presenting dendritic cells is regarded as the key event of the allergic process and the onset of the pathology (Vocanson et al. 2009; Akiba et al. 2002; Martin et al. 2000; Kehren et al. 1999).

T cell priming ensues from highly complex molecular and cellular processes that originate in the skin with chemical penetration and its fixation on extra- or intracellular proteins (Peiser et al. 2012) and lead to the emigration of skin resident (Kaplan et al. 2012; Bursch et al. 2007) or newly recruited DCs (Le Borgne et al. 2006), processing haptenated proteins, toward the draining lymph nodes (LNs). T cell priming is then initiated with the formation of transient but stable junctions (also referred to as immunological synapses (ISs)) (Dustin 2010;

Henrickson et al. 2008) at the interface between dendritic cells presenting haptenated-peptide MHC (hpMHC) complexes and naive T cell precursors, bearing T cell receptors (TCRs) with sufficient affinity/avidity for cognate hpMHCs.

Hence, throughout the travel of a hapten in the organism, a plethora of physico-chemical, biochemical, or immunological factors is susceptible to impact and finely tune the activation and differentiation fate of hapten-specific T precursors. A non-exhaustive list of the main immunological factors that can shape T cell priming and differentiation is as follows: (1) the duration and the strength of antigenic stimulus, which are directly correlated with the number of skin antigen-presenting cells (APCs) emigrating to LNs (Gorbachev and Fairchild 2010; Martin et al. 2003); (2) the density of pMHC per APCs (Henrickson et al. 2008), the nature of APCs (Gomez et al. 2012; Kaplan et al. 2012), and their functional state (Martin et al. 2008; Ring et al. 2010); (3) the antigen sensitivity of T cell precursors that pertain to TCR affinity/avidity for pMHC molecules (Speiser et al. 2008); and (4) finally, as T cell priming must not be envision as a bilateral dialogue between APCs and effector T cells, the collateral activation of T helper (Feau et al. 2012; Semmling et al. 2010) and/or regulatory T cells (Tregs) (Vocanson et al. 2010; Ring et al. 2010), as well as non-$\alpha\beta TCR^+$ lymphocytes such as $\gamma\delta$T cells (Macleod and Havran 2011), NKT cells (Goubier et al. 2013; Semmling et al. 2010; Mattarollo et al. 2011), NK cells (Soderquest et al. 2011; Iyori et al. 2010), and even B cells (Nakashima et al. 2010), that trigger crucial loops of amplification or regulation on the functions of APCs and/or T cells.

Understanding the interplay between all these diverse parameters then represents a major challenge to develop a minimal in vitro assay that recapitulates the key event of the allergic process and allows for the characterization of chemicals endowed with sensitizing properties.

7.2 Difficulty in Obtaining Chemical-Specific T Cell Priming in Classical Autologous Mixed Dendritic Cells/Lymphocyte Reaction

In the past 2 decades, several groups have tested for the ability of haptens to prime naive human peripheral blood T cells in protocols, referred to as human T cell priming assays (hTCPA) (Martin et al. 2010). Approaches in these assays mainly consist of an autologous mixed DC-lymphocyte reaction (MDLR) using hapten-loaded DC as antigen-presenting cells and normal human peripheral blood leucocytes (PBL) as responding cells. The in vitro priming of hapten-specific T cells was usually assessed by cell proliferation and/or T cell cytokine production at the end of the 5–7 day culture. Although some strong experimental haptens were able to activate T cells in these primary sensitization assays, the magnitude of the T cell response was routinely low and the results were poorly reproducible, especially when clinically relevant molecules endowed with moderate/weak sensitizing

properties were evaluated (Moed et al. 2005; Rougier et al. 2000; Rustemeyer et al. 1999; Krasteva et al. 1996).

Possible reasons for the poor sensitivity of these assays are multiple: (1) the low uptake by DCs of molecules that are for the most part highly hydrophobic and therefore very difficult to dissolve in conventional culture media, (2) the inability of weak haptens to generate sufficient "danger signals" when delivered to DCs and to prompt their activation/maturation (Martin et al. 2011), and (3) the low frequency or affinity/avidity of hapten-specific T cell precursors in normal individuals (see the Chap. 8).

7.3 Depletion of Human Peripheral Blood Lymphocytes in $CD25^+$ T Regulatory Cells Improves the In Vitro Priming of Chemical-Specific T Cells and Allows for the Development of a First-Generation hTCPA Able to Detect the Sensitizing Properties of Chemicals

Another explanation to these results is that in vitro priming of T cells may be repressed by $CD4^+CD25^+FoxP3^+$ Tregs (that comprise up to 5 % of the peripheral $CD4^+$ T cell pool), which are known to exert profound effects on inhibiting proliferation of naive T cells (Ohkura and Sakaguchi 2010). In this respect, studies in contact hypersensitivity reaction (CHS, the murine equivalent of ACD) to both strong and weak haptens have shown that hapten painting leads to a rapid and robust expansion of a highly suppressive Treg subset (that coexpressed $FoxP3^+$ and Inducible Costimulator (ICOS)) that specifically suppresses hapten-reactive $CD8^+$ T cells both in vivo and ex vivo (Vocanson et al. (2010) and data not shown). Alternatively, acute depletion of $CD4^+$ T cells, $CD25^+$ T cells, or of $FoxP3^+$ Tregs in wild-type mice exposed to large variety of strong or weak sensitizers (such as perfumes, dyes, drugs, arnica tinctures) results in a dramatic increase in the number of hapten-specific effector $CD8^+$ T cells (producing IFN-γ) in the LNs draining the sensitization site and exacerbation or development of skin inflammation (Rozieres et al. 2010; Lass et al. 2008; Vocanson et al. 2006a) and data not shown).

Therefore, we postulated that hTCPA to haptens might be more efficient if Tregs were absent from the responding T cell population and tested in a standard autologous MDLR, whether depletion of the responding PBLs in $CD25^+$ cells could increase the magnitude of hapten-specific T cell sensitization in vitro.

Monocyte-derived DC (MDDC) were incubated for 10 min with the strong/moderate haptens TNBS (2,4,6-trinitrobenzene sulfonic acid) and FITC (fluorescein isothiocyanate), with the clinically relevant weak fragrance allergen HCA (a-hexylcinnamaldehyde), or with the non-sensitizers DMSO (dimethyl sulfoxide) and SLS (sodium lauryl sulfate). After washing, haptenated DC were cocultured for 5–7 days with either undepleted or $CD25^+$ cell-depleted PBLs from normal human donors. Depletion of PBLs in $CD25^+$ T cells was achieved using anti-CD25

mAb-coated microbeads and negative selection columns (Myltenyi Biotech, Bergish Gladbach, Germany) and routinely resulted in 95 % depletion of $CD25^+$ T cells (and in consequence >95 % depletion of $FoxP3^+$ Tregs). Priming of hapten-specific T cells during the MDLR was determined by (1) analysis of cell proliferation following addition of ^3HT for the last 8 h of culture and (2) titration of IFN-γ production in culture supernatants after addition of a sub-mitogenic dose of PHA for the last 15 h of culture, as previously described (Vocanson et al. 2006b; Cohen et al. 1974). Results were expressed as a stimulation index (SI) corresponding to the ratio of values recorded with hapten-pulsed versus unpulsed MDDC. Results (Fig. 7.1, Vocanson et al. 2008) showed that depletion of naive PBLs in $CD25^+$ T cells improves the sensitivity of the primary sensitization assay and allows for detecting of the sensitizing properties of chemicals with moderate (FITC)/weak (HCA) allergenic properties. Indeed, classical primary autologous MDLR, i.e., using undepleted PBLs as responding cells, resulted in (1) a moderate proliferative response to TNBS (in 6/11 experiments) with IFN−γ production in 2/11 exp. only, (2) no proliferative response and IFN−γ production to FITC, and (3) proliferative response to HCA in 1/6 exp. only without IFN−γ production. In contrast, depletion of naive PBLs in $CD25^+$ T cells increased the sensitivity of the MDLR inasmuch as (1) a strong T cell proliferation and IFN−γ production in response to TNBS was observed in almost all (12/13) experiments; (2) mild but significant proliferative responses to FITC and HCA were obtained in, respectively, 2/3 exp. (with IFN−γ production in 1/3 exp.) for FITC and 4/8 exp. (with IFN−γ production in 1/7 exp.) for HCA; and (3) the non-sensitizers DMSO and SLS did not induce any PBL proliferation or IFN−γ production.

Thus, depletion of PBLs in $CD25^+$ T cells allows for improved in vitro priming of T cells against contact sensitizers but not against xenobiotics devoid of sensitizing properties (DMSO, SLS), confirming our primary hypothesis that regulatory cells prevent the priming/expansion of specific T cell precursors.

To date, the mechanisms by which Treg depletion boosts in vitro priming of hapten-specific T cells in primary sensitization assays remain to clarify. $CD4^+CD25^+FoxP3^+$ Tregs harbor a wide arsenal of immunomodulatory mediators (Yamaguchi et al. 2011). Depending on the experimental model studied and their environment (lymphoid organs or peripheral tissues, inflammatory or steady-state conditions), Tregs could impair T cell responses through a large set of mode of action (Wing and Sakaguchi 2012) and modulate both the functions of antigen-presenting cells or the expansion program of T cells (Tang and Bluestone 2008). Hence, Tregs deliver inhibitory signals to DCs or T cells via their high expression of negative costimulatory molecules such as CTLA-4, the secretion of an inflammatory cytokines (IL-10, TGF-β, IL-35), the generation of immunosuppressive adenosine, or through cell-directed cytotoxicity and granzyme/perforin mechanisms (Yamaguchi et al. 2011). Of note, another prominent mechanism for Treg suppression, inherent to their high expression of the alpha chain of the IL-2 receptor (CD25), also consists in IL-2 deprivation in the pericellular environment of proliferating T effector cells (Pandiyan et al. 2007). Interestingly, in vivo imaging revealed that Treg depletion considerably affects DC behavior and profoundly

Chemical	Experiment number	Cell Proliferation						Cytokine secretion					
		Undepleted MDLR			CD25+ T cell-depleted MDLR			Undepleted MDLR			CD25+ T cell-depleted MDLR		
		^{3}HThymidine incorporation (cpm +/- SD)		Stimulation Index	^{3}HThymidine incorporation (cpm +/- SD)		Stimulation Index	IFNγ secretion		Stimulation Index	IFNγ secretion		Stimulation Index
		Hapten-pulsed MDDC	Unpulsed MDDC		Hapten-pulsed MDDC	Unpulsed MDDC		Hapten-pulsed MDDC	Unpulsed MDDC		Hapten-pulsed MDDC	Unpulsed MDDC	
TNBS (1000µg/ml)	1	1337 +/- 1707	2561 +/- 860	0,5	756 +/- 359	90 +/- 43	**8,4**	24	29	0,8	41	4	**10,1**
	2	1163 +/- 620	368 +/- 11	**3,2**	2192 +/- 894	502 +/- 216	**4,4**	6	6	1,0	37	7	**5,3**
	3	12447 +/- 2204	4760 +/- 586	**2,6**	17786 +/- 3066	1385 +/- 1418	**12,8**	76 +/- 2	50 +/- 22	1,5	397 +/- 62	89 +/- 48	**4,5**
	4	2137 +/- 733	1963 +/- 337	1,6	6424 +/- 264	740 +/- 529	**8,7**	43 +/- 6	13 +/- 4	**3,3**	72 +/- 22	16 +/- 6	**4,5**
	5	1063 +/- 755	706 +/- 134	**2,6**	8123 +/- 390	338 +/- 157	**24**	102 +/- 8	89 +/- 17	1,1	269 +/- 51	73 +/- 22	**3,6**
	6	8643 +/- 708	3133 +/- 486	**2,8**	6186 +/- 1002	1630 +/- 307	**3,8**	41	31	1,3	197	32	**6,2**
	7	1495 +/- 611	1245 +/- 355	1,2	7560 +/- 2126	315 +/- 102	**24**	10 +/- 3	9 +/- 11	1	22 +/- 15	7 +/- 3	**3**
	8	6769 +/- 1208	4195 +/- 649	1,6	6070 +/- 1845	2722 +/- 723	**2,2**	13	16	0,9	225	11	**20,5**
	9	4655 +/- 1242	2253 +/- 787	**2,1**	7449 +/- 1178	2068 +/- 1272	**3,6**	137	68	**2**	89	70	1,3
	10	1141 +/- 464	363 +/- 78	**3,1**	9798 +/- 262	3297 +/- 1246	**3**	29 +/- 7	23 +/- 17	1,3	519 +/- 188	130 +/- 4	**4**
	11	1125 +/- 315	1086 +/- 262	1	744 +/- 452	432 +/- 418	1,7	144	155	0,9	19	14	1,4
	12	nd		-	1049 +/- 902	102 +/- 43	**10,3**	nd		-	137	44	**3,1**
	13	nd		-	5653 +/- 3381	1193 +/- 547	**4,7**	nd		-	222	20	**11,1**
FITC (300µg/ml)	5	650 +/- 218	706 +/- 134	0,9	1036 +/- 488	338 +/- 157	**3,7**	87 +/- 33	117 +/- 47	0,7	222 +/- 29	202	1,1
	6	2930 +/- 853	3133 +/- 486	0,9	2836 +/- 350	1630 +/- 307	1,7	16	31	0,5	52	32	1,6
	8	5859 +/- 2035	4195 +/- 649	1,4	6890 +/- 2034	2722 +/- 63	**2,5**	9	16	0,6	41	11	**3,7**
	1	663 +/- 228	2561 +/- 860	0,25	499 +/- 71	90 +/- 43	**5,5**	nd		-	nd		-
	2	407 +/- 152	368 +/- 11	1,1	570 +/- 227	502 +/- 216	1,1	5	6	0,8	3	7	0,4
	7	1871 +/- 615	1245 +/- 355	1,5	275 +/- 71	315 +/- 102	0,9	10 +/- 1	9 +/- 11	1	7	7 +/- 3	1
HCA (130-400 µg/ml)	9	2335 +/- 514	2253 +/- 787	1	4202 +/- 1181	2068 +/- 1272	**2**	88	68	1,3	153	70	**2,2**
	10	706 +/- 64	364 +/- 78	**2,1**	11237 +/- 3951	3287 +/- 1246	**3,4**	25 +/- 29	23 +/- 17	1,1	218 +/- 91	130 +/- 4	1,7
	11	1219 +/- 349	1086 +/- 263	1,1	851 +/- 626	432 +/- 418	**2**	136	155	0,9	12	14	0,8
	12	nd		-	156 +/- 43	102 +/- 43	1,5	nd		-	31	44	0,7
	13	nd		-	1501 +/- 964	1193 +/- 547	1,3	nd		-	18	20	0,9
DMSO (1000µg/ml)	5	666 +/- 149	706 +/- 134	0,9	248 +/- 57	338 +/- 157	0,7	100 +/- 21	119 +/- 47	0,8	203 +/- 68	202	1
	6	3052 +/- 383	3133 +/- 486	1	1733 +/- 485	1630 +/- 307	1,1	10	31	0,3	22	32	0,7
	8	5347 +/- 2381	4195 +/- 649	1,3	2650 +/- 347	2722 +/- 63	1	14	16	0,9	5	11	0,5
SLS (4-40µg/ml)	1	1091 +/- 125	2561 +/- 860	0,4	172 +/- 109	90 +/- 43	1,9		nd	-		nd	-
	2	366 +/- 201	368 +/- 11	1	939 +/- 230	502 +/- 216	1,9		nd	-		nd	-
	12	nd		-	125 +/- 34	102 +/- 43	1,2		nd	-	17	44	0,4

Fig. 7.1 In vitro T cell priming to contact sensitizers is improved in the absence of CD25$^+$ cells. Dendritic cells were generated from 6-day cultures of monocytes (isolated from peripheral blood of healthy donors) in complete RPMI medium supplemented with 10 % FCS and recombinant human GM-CSF and IL-4. For each experiment, monocyte-derived dendritic cells (MDDC) were then haptenized with 1000 µg/ml TNBS, 300 µg/ml FITC, 130–400 µg/ml HCA, 1,000 µg/ml DMSO, or 4–40 µg/ml SLS for 10 min at 37 °C. After extensive washing, 4–8 × 10^5 hapten-pulsed and unpulsed MDDCs were cultured for 5–7 days with 2 × 10^5 autologous lymphocytes (depleted or not in CD25$^+$ cells using anti-CD25 mAb-coated microbeads and negative selection columns (Myltenyi Biotech, Bergish Gladbach, Germany)). The proliferative responses were assayed by ^{3}HThymidine incorporation (1 µCi/well) for the last 8 h of culture. INFγ secretion was measured by ELISA assay in the supernatant of cultures restimulated for the last 15 h with 2 µg/ml of PHA. Lymphoproliferation and INFγ secretion induced by hapten-pulsed MDDC were compared to that obtained with unpulsed MDDC, and results are expressed as stimulation index (SI): response recorded for hapten-pulsed MDDC/response recorded for unpulsed MDDC. Results are considered positive for SI >2 (underlined in the figure). *nd* not determined

increases their ability to initiate long-standing and stable ISs with naive T cells (Tang and Krummel 2006). Similarly, others studies reported that DCs, collected in the LNs of animals depleted by an anti-CD25$^+$ mAb, display a much more mature phenotype (increased levels of the costimulatory CD86 marker and/or decreased level of the inhibitory B7-H3 molecule) (Schildknecht et al. 2010; Mahnke et al. 2007). Those two observations then suggest that DCs operating in Treg-depleted environment are intrinsically more prone to activate naive T cells and stimulate their proliferation/differentiation. Alternatively, we cannot exclude that additional inhibitory signals are brought into cultures of undepleted PBLs by a potent hapten-induced activation of Tregs. Indeed, we detected a significant enlargement in the percentage of CD25$^+$FoxP3^{high} Tregs expressing the cell cycle protein Ki67 in cultures of undepleted PBLs stimulated with TNBS-pulsed MDDCs versus unpulsed cultures (data not shown). Are the proliferating CD25$^+$FoxP3^{high} Tregs operate in an antigen-dependent manner, similarly to our previous results in mice (Vocanson et al. 2010), or are they polyclonal Tregs that are activated by the inflammatory signals delivered by haptens? Both antigen-specific and polyclonal Tregs (encompassing a very low number of specific cells and mainly cells dedicated

to respond to self-antigen presentation) were reported to modulate immune responses to exogenous antigens and CHS to hapten (Vocanson et al. 2010; Ring et al. 2006). Nevertheless, the two subpopulations would not mediate their biological functions using the same mechanisms. Antigen-specific Tregs operate early in the immune response and completely inhibit T effector cell expansion and differentiation via inhibition of DC functions, while polyclonal Tregs operate in later stage and appear to modulate differentiation and trafficking of effector T cells (Shevach 2011).

7.4 Obstacles to Overcome in Order to Increase the Efficiency of In Vitro T Cell Priming to Contact Sensitizers

While we succeeded in detecting significant proliferative responses to moderate contact sensitizers (FITC or HCA) in the first-generation hTCPA where $CD25^+$ Tregs are absent from the responding PBL population, our assay failed to give positive results in about one out of two donors tested (similar data were recently recorded for a large panel of moderate/weak contact sensitizers) and was not sensitive enough to activate weak sensitizer-specific T cells. Several hypotheses could explain the difficulty in obtaining an optimal and consistent T cell activation in primary MDLR: (1) the frequency or affinity/avidity of hapten-specific T cell precursors may be too low in some individuals to be detected in the assay, (2) regulatory mechanisms other than $CD25^+$ Tregs may control specific T cell priming, and (3) the delivery of chemicals to DCs may not be optimal, the more so since most of contact sensitizers are hydrophobic and cannot be easily dissolved in culture medium.

In order to improve the efficiency of in vitro T cell priming, we tested the two latter hypothesis and obtained significant improvement in the ability to detect the sensitizing properties of chemicals.

7.5 Second-Generation hTCPA: In Vitro Priming of Allergen-Specific T Cells Requires Allergen Uptake by $CD1a^+$ Dendritic Cells and Is Hampered by Both $CD25^+$ and $CD56^+$ Regulatory Cells

7.5.1 $CD56^+$ Cells Prevent T Cell Priming

We demonstrated that along with $CD4^+CD25^+FoxP3^+$ Tregs, $CD56^+$ cells (that comprise both NK and T cell subsets) present in responding PBLs exert a

supplementary control on T cell responses. Indeed, $CD56^+$ cell depletion in responding PBLs modestly increased T cell priming to haptens, but when cells were included in $CD25^+$ cell-depleted cultures, they actively restrained hapten-induced T cell proliferation and IFN−γ-secretion, notably because those cells (and respective NK and T cell subsets) efficiently kill chemical-pulsed MDDCs. Hence, double $CD25^+/CD56^+$ cell depletion in responding PBLs enhanced very significantly T cell responses and detection of a large panel of standard contact sensitizers (data not shown).

7.5.2 CD1a Positive DC Is the Most Efficient DC Subset for In Vitro T Cell Priming

Next, we also showed that T cell priming critically depends on the phenotype/functions of MDDCs used in the MDLR. Indeed, we observed that, depending on the donors, MDDCs generated from blood monocyte fractions varied substantially for their expression of CD1a marker (with distinction between $CD1a^{neg}$ and $CD1a^{pos}$ subsets). Respective $CD1a^{neg}$ MDDC subset displayed less mature phenotype (with persistent expression of the $CD14^+$ monocyte marker) and poor stimulatory capabilities compared to their $CD1a^{pos}$ counterpart. Importantly, addition of $CD1a^{neg}$ MDDC to $CD1a^{pos}$-stimulated cultures abrogated TNBS proliferation and type-1 differentiation (data not shown), demonstrating that precious attention must be brought to the type of MDDCs that is used in T cell priming assays.

7.5.3 Efficient Uptake of Hydrophobic Chemicals by DC Increases Their Ability to Prime T Cells

Finally, we also demonstrated that T cell priming to hydrophobic molecules such as HCA or DNCB is significantly hampered by their DC uptake. Indeed, the use of encapsulated chemicals in nanoparticles to pulse MDDCs significantly increased their activation state (as measured by their increased expression of the maturation markers CD86, CD83, HLA-DR), as well as their ability to prompt T cell responses to those two chemicals (but not to the non-sensitizer methyl salicylate).

7.5.4 Toward a Second-Generation hTCPA

Of great importance, combining depletion of cells endowed of regulatory functions (both $CD25^+$ and $CD56^+$) and nanoparticle strategies to increase the allergen uptake

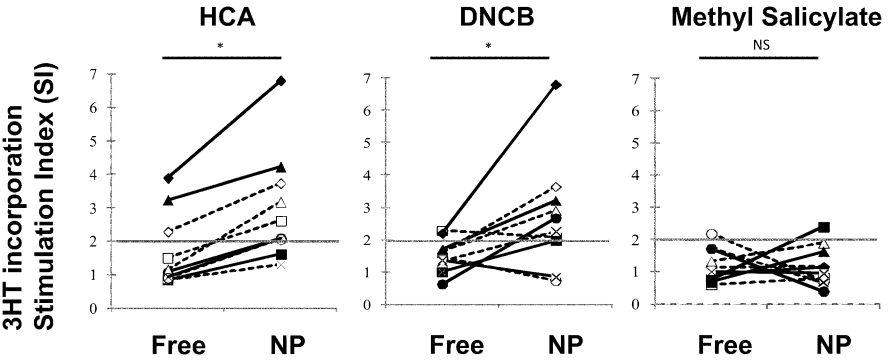

Fig. 7.2 Combining depletion of cells endowed with suppressive activity ($CD25^+/CD56^+$) and nanoparticle encapsulation of chemicals allows for sensitive screening of contact sensitizers in quasi-all the donors tested (A). MDDCs were pulsed for 15 min with various concentrations of free- or nanoparticle-encapsulated chemicals (20 (_), 40 (...) μg/ml HCA; 10–20 (_), 40–50 (...) μg/ml HCA; 10 (_), 50 (...) μg/ml methyl salicylate). MDDCs pulsed with RPMI medium or empty nanoparticles were used as controls. 16×10^4 DC were then irradiated and cultured for 5 days with 2×10^5 autologous PBLs depleted in $CD25^+/CD56^+$ cells. Proliferative responses were assayed by ^{3}HThymidine incorporation for the last 8 h of culture. Responses induced by chemical-pulsed MDDCs were compared to that obtained with control MDDCs and results were expressed as stimulation index (SI): response recorded for chemical-pulsed MDDCs/response recorded for control MDDCs. Results were considered positive for SI > 2. Responses recorded for five different donors (*round, square, diamond, triangle, cross*) per chemicals are shown

by DC allowed the detection of specific T cell priming to HCA and DNCB (but not to the non-sensitizers methyl salicylate) in about all the donors tested (4 out 5) (Fig. 7.2). Therefore, the second-generation hTCPA can be a valuable method to detect the sensitizing properties of chemicals.

7.6 Conclusions

So far, primary T cell sensitization assays had proved poorly robust for the screening of weak contact sensitizers, suggesting that some important signals relative to ACD response are missing in these assays. We report that T cell priming to haptens, notably to some weak sensitizers, can be significantly improved by eliminating from PBLs several cell subsets that suppress T cell proliferation and differentiation. Hence, we demonstrated that $CD4^+CD25^+FoxP3^+$ Tregs (as expected) but also $CD56^+$ cells (comprising both NK and T cell subpopulations) and $CD1a^{neg}$ MDDCs deliver potent inhibitory signals that prevent or limit T cell expansion. Thus, hTCPA protocols using naive T cells that are depleted of both $CD4^+CD25^+FoxP3^+$ and $CD56^+$ cells will undoubtedly provide progress for the development of these assays (Martin et al. 2010; Richter et al. 2013; Vocanson, manuscript in preparation). Nevertheless, it appears crucial to combine such

strategy to the use of MDDCs endowed with potent stimulatory capacities and, more importantly, to the use of synthetic vectors that can boost chemical uptake (notably the less soluble in conventional culture media) and MMDC activation. That positive hTCPA responses to haptens, such as HCA or DNCB, were obtained for quasi-all the donors tested indicates that such simple immunological assay represents undeniably a good alternative to test for the potential immunogenic properties of haptens, especially those endowed with weak sensitizing properties.

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Chapter 8
Correlation of Contact Sensitizer Potency with T Cell Frequency and TCR Repertoire Diversity

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Abstract Allergic contact dermatitis is a T cell-mediated skin disease. Many hundreds of organic chemicals and some metal ions are contact sensitizers. They induce an innate inflammatory immune response in the skin that results in the priming of contact sensitizer-specific T cells by dendritic cells in the draining lymph nodes. The factors that determine the strength of this T cell response and thereby define the potency of a contact sensitizer are largely unknown. This chapter highlights different variables such as precursor frequency of antigen-specific T cells, possible bystander activation, and T cell receptor diversity or avidity of the TCR/peptide-MHC interactions, which might impact the quality and strength of T cell responses to contact sensitizers. In addition, different methods available to determine both the frequency of antigen-specific T cells and T cell receptor repertoires are discussed. Identification of the factors determining potency may allow for the development of suitable in vitro assays for potency assessment of contact sensitizers.

Abbreviations

ACD	Allergic contact dermatitis
APC	Antigen-presenting cell
BCR	B cell receptor
CD	Cluster of differentiation

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CDR	Complementarity-determining region
CHS	Contact hypersensitivity
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
LLNA	Local lymph node assay
MAMP	Microbe-associated molecular pattern
MHC	Major histocompatibility complex
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PRR	Pattern recognition receptor
SSCP	Single-stranded conformational polymorphism
TCR	T cell receptor

8.1 Introduction

The innate immune system utilizes germline-encoded pattern recognition receptors (PRRs) to identify compounds that might be harmful to the body (Janeway 1989; Medzhitov 2009). However, this strategy can only accommodate a limited number of different molecular structures, designated as pathogen–/microbe-associated molecular patterns (PAMPs/MAMPs) or endogenous damage-associated molecular patterns (DAMPs), which are recognized by the respective PRRs. In general, PRR ligands represent danger signals that trigger innate immune responses and, for example, via activation of dendritic cells (DCs) and their migration to lymph nodes, allow for the priming of naive T cells. The innate immune system induces a fast and efficient response to many potential pathogens and to endogenous PRR ligands that may result in sterile inflammation and trauma. However, it fails in the detection of foreign proteins that do not contain such common molecular patterns per se or of self-proteins that have been altered, for example, by hapten modification as caused by contact sensitizers (Martin 2012).

Therefore, to be able to identify potential pathogens and harmful compounds with high specificity, the adaptive immune system has evolved several strategies that result in the generation of a highly polymorphic, and hence highly specific, T (TCR) and B cell receptor (BCR) repertoire, allowing for the recognition of a vast array of antigenic structures. One of the hallmarks of adaptive immunity is the formation of memory cells that protect against previously encountered pathogens. Interestingly, T and B cells also express PRR (Green and Marshak-Rothstein 2011; Mills 2011), allowing for a direct interaction between innate and adaptive immune responses. Here, we will focus on the identification of antigens by the T cell receptor.

8.2 Antigen Recognition by T Cells: The T Cell Receptor

Antigen recognition by T cells is reviewed in great detail in Chaps. 2 and 3. So far, current models claim that T cells can be activated only by antigens that are bound to—and presented on—transmembrane proteins encoded by the major histocompatibility complex (MHC) (MHC restriction). Therefore, antigens have to be presented on MHC-I or MHC-II molecules (Davis and Bjorkman 1988) by antigen-presenting cells (APCs) such as DCs, B cells, or macrophages in combination with co-stimulatory molecules. The exact reason for the MHC restriction was unclear. This question was recently addressed by van Laethem et al. who showed in an elegant study that mice lacking CD4 and CD8 co-receptor as well as MHC expression (quad-deficient mice) still generate α/β T cells (Van Laethem et al. 2007). These T cells are able to recognize antigens in an MHC-independent manner, claiming that the MHC specificity is imposed on T cells during thymic selection by sequestration of the T cell activating tyrosine kinase Lck by the co-receptors CD4 and CD8 (Van Laethem et al. 2007, 2012). TCR of T cells from such mice that have not undergone thymic selection have antibody-like specificities (Tikhonova et al. 2012). In addition, Thomas et al. showed that during viral infections also non-hematopoietic cells are able to amplify effector CD8 T cell expansion (Thomas et al. 2007). Interestingly, here the extent to which these cells enhance CD8 proliferation is pathogen dependent (Thomas et al. 2007). However, the MHC-independent activation of T cells and potential contribution of non-hematopoietic cells in the context of contact sensitizers is still an open question. Interestingly, TCR-dependent but MHC-independent TNP recognition of the cell surface ectonucleotidase CD39 by a human cytotoxic T cell line has been described (Stockl et al. 2001). This reactivity required the modification of the antigen-presenting cells with high concentrations of TNBS (5 mM). At low concentrations (10 μM) TNP recognition was entirely HLA-A1 restricted.

8.3 T Cell Receptor Diversity

The third complementarity-determining region (CDR3) is the only non-germline-encoded region of the TCR and is responsible for the fine tuning of antigen recognition within the TCR (for the structure of the TCR see Chap. 2). The hypervariability of this region is primarily a function of the process of somatic recombination, where the noncontiguous Vα and Jα segments of the α-chain and the variable (Vβ), diversity (Dβ), and joining (Jβ) gene segments of the β-chain locus are re-organized (Hughes et al. 2003). The underlying structural and biophysical determinants of αβ T cell antigen recognition have recently been reviewed extensively (Bridgeman et al. 2011, see also Chaps. 2 and 3).

This process provides for a very large number of different CDR3 sequences to be assembled. In addition, variability within this region is enhanced further by

template-independent addition and deletion of nucleotides at the Vβ-Dβ, Dβ-Jβ, and Vα-Jα junctions, thereby enabling junctional diversity. Thus, 10^{15}–10^{20} unique αβ TCR structures (Davis and Bjorkman 1988; Lieber 1991; Shortman et al. 1990) can be generated that allow for the recognition of many different antigens. However, each individual does not produce all of these different structures, but a considerably lower number of approximately 2.5×10^8 different TCRs in man (Robins et al. 2009) and 2×10^6 in mice (Casrouge et al. 2000; Doherty et al. 2000; Jenkins et al. 2010; Morris and Allen 2012).

Nevertheless, the ability to recognize a vast number of antigens comes at a price. The drawback of this broad receptor repertoire is that only a very small number of cells express each of the highly specific receptors able to recognize a given single antigen. Therefore, a prolonged period of time is necessary to allow for an antigen-specific expansion of these initially rare cells before efficient immune responses can be mounted. This clonal expansion is reduced in the case of antigens that have been previously encountered and that have caused immunological priming resulting in the generation of a memory T cell pool that is readily available to induce accelerated antigen-specific recall responses. However, if a new antigen variant due to antigenic drift—as typical for seasonal influenza epidemics—is presented by APCs to one of the rare naive T cells expressing the matching TCR, a new, primary T cell response must be activated and the rare T cells must proliferate in order to provide sufficient numbers of T lymphocytes that are able to mount an effective immune response. A recent review article draws together further conclusions from the bias in αβ TCR repertoire for disease pathogenesis and vaccination (Miles et al. 2011).

8.4 Impact of Antigen-Specific T Cell Frequency and T Cell Receptor Diversity on Immune Responses

The frequency of cells carrying a specific TCR or BCR plays an important role in the efficiency and eventually in the strength of adaptive immune responses. For viral infections, the question of what mechanisms drive virus-induced T cell responses has been a matter of contention for some time. In theory, these responses could be due to antigen-driven expansion of specific T cells, cytokine-mediated bystander activation, T cell proliferation induced by cross-reactive antigens, or a combination of one or more of these parameters. While initial studies supported the bystander theory, a strong support for the antigen-driven expansion was provided since 1998, when the first studies using tetramer staining (Murali-Krishna et al. 1998) or ELISpot analysis (Butz and Bevan 1998) were reported. Analysis of the frequency of activated T cells in response to LCMV infection by tetramers of MHC class I molecules containing viral peptides and single-cell IFN-γ staining revealed that at the peak of primary and secondary responses, between 50 and 70 % of the activated CD8 T cells were LCMV specific (Murali-Krishna et al. 1998). An even higher percentage (80–95 %) of LCMV-specific CD8 T cells was observed

in a follow-up study analyzing granzyme B, 1B11, CD62L, CD11a, and CD127 expression (Masopust et al. 2007). A similar observation was made for acute antiviral CD8 T cell responses to smallpox and yellow fever vaccines in humans using HLA-DR and CD38 as phenotypic markers (Miller et al. 2008). In addition, a study by Kotturi et al. analyzing CD4 and CD8 T cell responses in three human donors accidentally infected with LCMV showed that disease outcome correlates with both the magnitude of the memory CD8 T cell response and the phenotypes of CD4 and CD8 LCMV-specific memory T cells (Kotturi et al. 2011).

8.5 Identification of Antigen-Specific T Cell Activation

Recently, T cell activation and proliferation are being measured in several approaches to develop in vitro assays for the identification of skin sensitizing chemicals (see also Chaps. 6, 7, and 9) (Martin et al. 2010), where low frequencies of antigen-specific T cells occurring in the naive T cell pool are expanded by antigen-specific priming and restimulation (Dietz et al. 2010; Vocanson et al. 2008; Richter et al. 2013). Another strategy is used for the detection of protein allergens wherein initial polyclonal amplification is followed by antigen-specific stimulation (Geiger et al. 2009). These T cell assays can be used to test the hypothesis that the magnitude of induced T cell responses as measured by T cell proliferation largely reflects the clonal expansion of allergen-reactive T lymphocytes (Kimber et al. 2012a) and thereby the ability of the contact sensitizers and protein allergens to induce immune responses in vivo. Another method, allowing for the measurement of antigen-specific T cell frequencies in either primed T cell cultures or in fresh PBMC purified from patient blood samples, is the detection of cytokine-secreting cells using ELISPOT or intracellular cytokine detection methods (Hobeika et al. 2005). For this purpose, naive T cells are primed and restimulated with specific contact sensitizers. The activated T cells release cytokines responsible for the induction of a proinflammatory micromilieu in vivo. Analysis is performed by multiparametric flow cytometry. The advantage of this approach compared with using proliferation as a readout is the ability to correlate directly the frequency of cytokine-producing T cells with the frequency of T cells displaying an antigen-specific TCR. In addition, this approach provides the opportunity to broaden the spectrum of parameters that are analyzed, for example, by addition of further activation markers such as CD137 or CD154, or the identification of cytotoxic T lymphocytes via detection of granzyme B release (Dietz et al. 2010; Martin 2010). Moreover, assessment of cytotoxic activity by analysis of the surface expression of CD107a, an integral membrane protein in cytolytic granules of T cells, might provide a valuable new marker for the identification of antigen-specific T cell activation in the context of skin sensitization. CD107a has already been shown to provide for reliable detection of virus-activated, antigen-specific CD8 T cells by flow cytometry (Betts et al. 2003) and has been used, for

example, to identify tumor-cytolytic T cells isolated directly from patient blood samples (Rubio et al. 2003).

8.6 Allergic Contact Dermatitis: A T Cell-Mediated Skin Disease

Although immune responses are, in general, intended to protect the body from harm, recognition of antigens (especially in the case of autoantigens) can also lead to unwanted, adverse reactions. Allergic contact dermatitis (ACD) is a T cell-mediated skin disease that is caused by the inappropriate and unnecessary recognition of chemicals (metal ions and organic chemicals) encountered at skin surfaces being presented on MHC class I or II molecules by APCs to T cells. According to the current models, CD8 cytotoxic effector T cells play a major role in the induction of the skin inflammation in mice (Martin et al. 2004; Vocanson et al. 2009) and probably also as initiator cells in human ACD. Such a role for CD8 T cells was recently shown for atopy (Hennino et al. 2011). Studies in the mouse model for ACD, the contact hypersensitivity (CHS) model, have shown that strong contact sensitizers induce a rather polyclonal T cell response with an unusually high frequency of CD8 effector T cells of the Tc1 phenotype (Martin et al. 2003). Promiscuous recognition of contact sensitizers bound in a specific position to a large variety of MHC-binding carrier peptides by hapten-specific TCR may explain these findings (Martin et al. 1992, 1993; Martin 2004). Due to such a limited contribution of the carrier peptide sequence to T cell recognition of the chemical, T cells encounter an unusual high density of cross-reactive hapten epitopes. Thus, the potency of a contact sensitizer as usually determined by the magnitude of the proliferative response of lymph node cells in the Local Lymph Node Assay (LLNA) (Loveless et al. 2010) may correlate with its chemical reactivity with proteins or peptides (Fig. 8.1) (Gerberick et al. 2007). According to our current hypothesis, this chemical reactivity most likely determines the number of different proteins that become haptenated and the number of modifications on a single protein. This is then translated into the number of T cell epitopes. In the case of strong chemical reactivity, a high number of (cross-reactive) T cell epitopes should be generated and also low-affinity T cells should become activated. As a consequence the size and TCR repertoire diversity of the T cell pool should be higher as compared to the T cell pool activated by weakly reactive chemicals (Figs. 8.1 and 8.2). These parameters may thus determine the size and diversity of the primary T cell response. Recent evidence supports this hypothesis (Kotturi et al. 2008; Moon et al. 2007). Thereby, the reactivities of different contact sensitizers could play an important role in the outcome of T cell priming and subsequent ACD responses.

Another important determinant of contact sensitizer potency which may depend on the chemical reactivity must be the magnitude of the innate inflammatory response caused by contact sensitizers. This may be directly translated into the

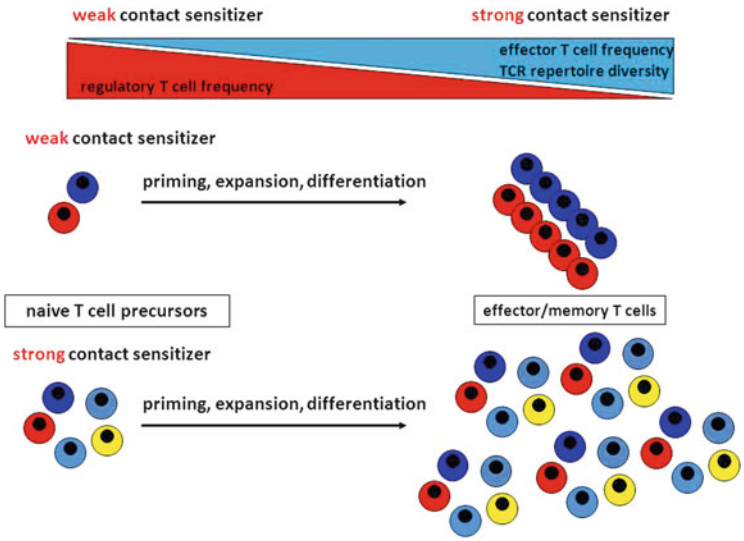

Fig. 8.1 Correlation of contact allergen potency with effector T cell and Treg pool size and TCR repertoire diversity. Differences in the size of the naive T cell precursor pool may be reflected in the size of the effector/memory T cell pool and the diversity of the corresponding TCR repertoire. The balance between regulatory T cells and effector/memory T cells is likely a critical determinant of contact allergen potency

efficiency of overcoming constitutive immune regulatory mechanisms and into the number of DCs that become activated in the skin and migrate to draining lymph nodes to prime contact sensitizer-specific naive T cells. The number of DCs that arrive in draining lymph nodes as well as the number of (cross-reactive) T cell epitopes could then be translated into the size of the contact sensitizer-specific effector and memory T cell pool and its TCR repertoire diversity (Figs. 8.1 and 8.2) (Kimber et al. 2012b; Kotturi et al. 2008; MartIn-Fontecha et al. 2003; Moon et al. 2007).

8.7 T Cell Receptor Repertoire

One example illustrating that the mode of T cell activation determines the TCR repertoire was provided by Currier et al. (1996). The authors analyzed the influence of PBMC stimulation by mitogen (PHA), superantigen (TSST-1), or recall antigen (tetanus toxoid), on TCR CDR3 heterogeneity. For their approach they combined CDR3 spectratyping analysis with single-stranded conformational polymorphism (SSCP) analysis and showed that both freshly isolated and mitogen-activated PBMC displayed a normally distributed (Gaussian-like) spectrum of CDR3 lengths. In contrast, tetanus toxoid-stimulated PBMC resulted in oligoclonal samples with a

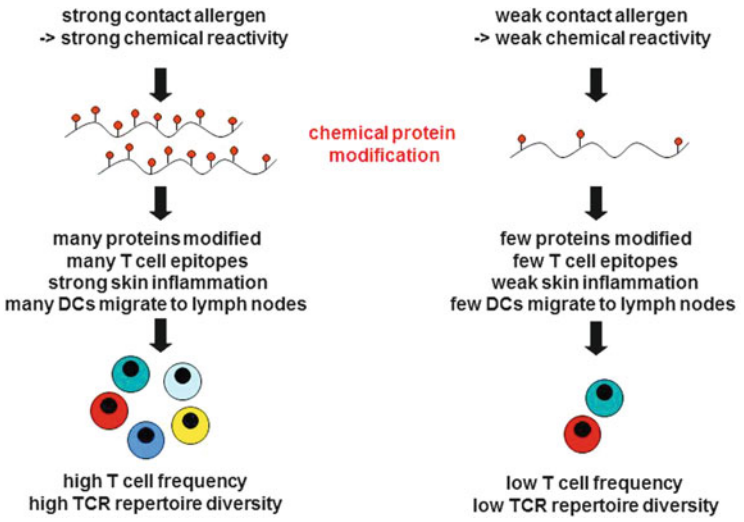

Fig. 8.2 Determinants of contact sensitizer potency. Chemical reactivity may be a crucial determinant of contact sensitizer potency. The extent of chemical protein modification should be translated into the strength of the innate inflammatory response that determines the number of contact allergen modified dendritic cells migrating from the inflamed skin to the draining lymph nodes. There, the DC number correlates with the number of naive T cells that are primed. In addition, chemical reactivity should also determine the extent of protein modification and thus the number of T cell epitopes which in turn correlates with the size, and most likely also with the TCR repertoire diversity, of the effector T cell pool

restricted profile (i.e., one peak accounting for more than 50 % of the total profile) for most TCR Vβ families after 7 days. The stimulation with superantigen resulted in unique patterns of diversity that included polyclonal expansion of specific TCR Vβ families, as well as oligoclonal expansion of most other TCR Vβ families, indicating that the superantigen-binding site is located outside of the CDR3 region. Taken together, these studies indicate that the activation of T cells via different stimuli induces different responses regarding TCR diversification. Recently, Sainz-Perez et al. have shown that the TCR repertoire of tumor-associated Treg and Teff cells in non-TCR transgenic mice was skewed towards public sequences shared by cells derived from different mice in two different tumor models. Nevertheless, the TCR repertoire observed in cells derived from either model compared with cells from the other model differed completely (Sainz-Perez et al. 2012). A puzzling question was if a broad TCR repertoire expression could be maintained under conditions where the expression of one specific TCR chain becomes preferred, as for example the expression of the TCRBV29 chain in influenza A virus infected C57BL/6 mice. One possible explanation was provided recently by Day et al. (2011). In their study, they analyzed the ternary structure of a canonical D^bPA_{224}-specific TCR in complex with cognate pMHC complex. They observed that while the CDR3α loop made peptide contact via the amino acid side chains, the CDR3β loop made main-chain interactions and here the backbone, but not the

specific amino acid side chains, made contact with the bound peptide. In addition, fixed expression of a single D^bPA_{224}-specific TCRα-chain resulted in preferred TCRVB29 (as observed during influenza A virus infection) expression, although a high level of TCR diversity was still maintained. TCR sequence diversity can still be maintained within the CDR3 loop despite preferred Vα and Vβ use in virus-specific TCR repertoires (Day et al. 2011). It is not yet clear whether these observations hold true for other non-viral primed TCR repertoires such as in the case of contact sensitizer-mediated TCR repertoire shifts. Interestingly, it has been shown that clonal expansion and receptor diversity even in young, antigen-inexperienced, and genetically identical mice can be quite different from mouse to mouse (Ahmed et al. 2009; Yager et al. 2008). On the other hand, there is evidence for a surprising interindividual bias or identity of TCRs selected in response to certain antigens resulting in public T cell responses (Li et al. 2012; Turner et al. 2006; Venturi et al. 2008). Moreover, clonal expansion and TCR diversity is markedly reduced in the naive CD8 T cell repertoire of aged mice (Ahmed et al. 2009; Yager et al. 2008). This finding indicates that age might be a factor determining susceptibility to sensitization. This observation adds another layer of interest and complexity to the determination of the TCR repertoire after stimulation of T cells from donors of different ages with different contact sensitizers.

8.8 T Cell Receptor Spectratyping

The methodology used for determination of the TCR repertoire has been improved within the last years. Rapid results can be obtained using fluorescent monoclonal antibodies directed against the variable region of the TCR β-chain as has been done, for example, for the detection of tumor-specific CD8 T cells within melanoma lesions or in blood samples following therapeutic vaccination in melanoma patients (Valmori et al. 2002). Though this approach provides rapid results, it does not reveal whether the T cell response is monoclonal or polyclonal in nature. This complexity can be integrated in another approach, known as the "immunoscope" or CDR3 spectratyping method that was described initially by Pannetier et al. (1995). This method is based on two steps. In the first step, a PCR is run to saturation with unlabeled Vβ- and Cβ- or Jβ-specific primers. This product is then visualized in a second step by performing a so-called run-off reaction that includes one additional fluorescent primer. The products of this run can then be separated on sequencing gels and analyzed by an automated sequencer (Pannetier et al. 1995). This method has been used successfully to monitor T cell responses after vaccination with peptides derived, for example, from Melan-A/MART-1 in cancer therapy. Jager et al. observed that the TCR repertoire reactive with Melan-A/MART-1 peptide epitopes was broadened during vaccination (Jager et al. 2002).

Recently, the spectratyping methodology has been improved regarding time- and cost- efficiency using a multiplex PCR approach (Mariani et al. 2009). This

capillary-based sequencing method has been improved further by employing, for example, an Illumina Genome Analyzer (Robins et al. 2009). Here, a library of template molecules, carrying universal PCR adapter sequences at each end, is hybridized to a lawn of complementary oligonucleotides immobilized on a solid surface. Solid-phase PCR is then used to amplify the hybridized library with subsequent use of reversible dye-termination chemistry to sequence a 30- to 54-nucleotide interval in the molecules in each cluster (Robins et al. 2009).

A further step to enable deep TCR profiling is the use of next-generation sequencers like the leading platforms Roche454, Illumina, and Ion Torrent. The underlying technology of the different platforms and methods used to isolate starting material has been recently described by Benichou et al. (Benichou et al. 2012). Using next-generation sequencing, the sequence of millions of receptor clones representing the entire immune repertoire can be analyzed routinely. In addition, a recent publication by Bolotin et al. provides advanced platform-specific correction algorithms, thereby enabling the correction of errors that are likely to occur during such analyses (Bolotin et al. 2012). This approach might help to overcome the current technical challenges of deep individual TCR profiling (Baum et al. 2012).

Immunoscope technology is now being used to address the question of whether or not T cell receptor repertoires are influenced by stimulation with contact sensitizers or irritants. Initial results show that naive sorted human T cells exhibit a Gaussian-like distribution, whereas T cells analyzed after priming and antigen-specific restimulation with the strong contact sensitizer TNCB show a shift to oligoclonal Vβ distributions.

The same holds true for murine T cells isolated from spleens of naive mice which show a Gaussian CDR3 length distribution pattern, whereas T cells isolated from spleens of mice that were previously treated with TNCB for 5 days show a shift to oligoclonal Vβ distribution indicating an antigen-selected shaping of the TCR repertoire. Interestingly, initial results using relatively weak sensitizers like eugenol or cinnamic aldehyde indicate that the shift to oligoclonal distribution is induced under these conditions as well, although some Vβ families can no longer be detected. This indicates that contact sensitizer driven clonal selection is efficient within this short culture period and results in the narrowing of the TCR repertoire. In contrast to strong sensitizers, the overall diversity of the TCR repertoire able to react to a given hapten seems to be further reduced after priming with a weak sensitizer. Contact sensitizers form T cell epitopes and are therefore able to change the TCR repertoire while non-sensitizing chemicals lack this property. It might be the case that the changes in the TCR repertoire and T cell pool size reflect skin sensitizing potency (Kimber et al. 2012a, b) However, other mechanisms such as the reactivity of the chemicals with proteins that may correlate with the number of immunogenic epitopes generated, the precursor frequency of antigen-specific T cells, the generation/availability of sensitizer-specific regulatory T cells, or the ability of a sensitizer to activate innate immune responses (that are known to be an important first step aiding successful subsequent T cell activation) may play a

more prominent role in determining relative skin sensitizing potency (Figs. 8.1 and 8.2).

8.9 Conclusion

Identification of the mechanisms influencing the vigor of T cell responses and thus identifying mechanisms that might explain the strength of ACD reactions to weak, moderate, strong, and extreme sensitizers is a challenge still to be solved. Although it has been used to characterize shifts in T cell receptor repertoires in response to different diseases involving T cell activation like viral infections or tumor development, the approach of T cell receptor Vβ usage and CDR3 length analysis has not been used to answer the question whether or not changes in the T cell receptor repertoire can be correlated to the severity of allergic contact dermatitis reactions. Approaches that attempt to correlate changes induced by contact sensitizers of different potency with changes in the T cell pool size and receptor repertoire diversity as a means to assess the sensitizing potency of unknown chemicals or drugs are just emerging, and further results have to show the validity and usability of such methods that aim to replace animal testing.

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Chapter 9
On the Role of Co-inhibitory Molecules in Dendritic Cell: T Helper Cell Coculture Assays Aimed to Detect Chemical-Induced Contact Allergy

Matthias Peiser, Manuel Hitzler, and Andreas Luch

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_{reg}	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-γ, 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}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. Groundbreaking 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 $CD8^+$ 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 ($CD4^+$) cells in ACD remained ambiguous. Studies using mouse models and depletion of $CD8^+$ cells suggested that distinct $CD4^+$ 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 $CD4^+$ 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-γ, IL-2, TNF-α, 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; Duhen 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-γ 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, $CD4^+$ 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-γ, 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_{reg}) cell-derived anti-inflammatory cytokines such as IL-10 and TGF-β (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 (Pandiyan 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$^+$ 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_{regs} are present in the eczematous lesions, albeit CD25 at intermediate expression may also be detectable on activated T cells different from T_{regs}. Further evidence for the participation of T_{regs} in ACD was delivered through the detection of FoxP3$^+$ 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_{regs}, measured by an increased ear swelling in rechallenged mice. Elevated expression of CTLA-4 in a subpopulation of T_{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_{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_{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-γ, TNF-α, 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-γ, TNF-α, 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-γ 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-γ were found in a distinct subpopulation of T_{reg} cells (Vocanson et al. 2010). These $ICOS^+$ T_{reg} cells displayed a strong suppressive potential in vitro and in vivo by inhibiting $CD8^+$ T cell proliferation and the release of IFN-γ. The question whether these $ICOS^+$ T_{reg} 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 $CD3^+$ 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$^+$ LC-like cells led to a decrease in the proliferation of total and CD8$^+$ 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$^+$ 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-γ by CD8$^+$ and CD4$^+$ 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$^+$ 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 MIP1α 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$^+$ T cells, blockage of PD-L1 induced the release of high amounts of TNF-α and IL-22 (Hitzler et al. 2012). Upregulation of IL-17 secretion via blocking of PD-L1, however, was restricted to CCR6$^+$/CCR4$^+$ 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$^+$ monocytes isolated from peripheral blood or CD34$^+$ hematopoietic progenitor cells obtained from cord blood (Caux et al. 1992; Meierhoff et al. 1998). However, due to their ready availability, CD14$^+$ 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 $CD34^+$ 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-γ, 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 $CD4^+CD25^+$ regulatory T cells. Congruently the depletion of such $CD4^+CD25^+$ cells in test systems for contact sensitizers resulted in significantly pronounced effects regarding T cell proliferation and IFN-γ 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 $CD4^+$ T cells and in an increased secretion of IFN-γ 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_{regs} were removed and CTLA-4 was blocked by specific antibodies. Interestingly the removal of T_{regs} 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 $CD4^+$ 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_{reg} 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 $CD4^+$ T cells stimulated with anti-CD3 antibodies, the addition of soluble PD-L1 resulted in a decrease of proliferation and impaired secretion of IFN-γ and IL-10 (Freeman et al. 2000). On the other hand, after blockade of PD-L1/PD-1 interactions in anti-CD3-stimulated naïve $CD4^+$ T cells co-stimulated with macrophages, the in vitro production of IL-2 and IFN-γ was found increased although the proliferation of T cells was reduced (Yamazaki et al. 2005); similarly in $CD4^+$ T cells cocultured with MoDCs, the blockade of PD-L1 with specific antibodies increased the amount of T

cell-derived IFN-γ (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. $CD34^{+}CD14^{-}CD11b^{-}$ cells constitute the proliferative compartment of the cell line with the ability to differentiate through a $CD34^{-}CD14^{-}CD11b^{+}$ stage to give rise to a morphologically large, nonproliferating $CD14^{+}CD11b^{hi}$ progeny. The addition of a distinct cytokine cocktail to these $CD14^{+}CD11b^{hi}$ 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 $CD4^{+}$ 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 $CD3^+$ T cells after cocultivation with THP-1 cells (Janic et al. 2008), whereas Cho and colleagues were able to detect increased IFN-γ levels in $CD8^+$ 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-1β 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_{regs}, modulate inflammatory responses to a large extent by the inhibitory mediators IL-10 and TGF-β. 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 (EC3) 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_{reg}, 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_{reg} cells, thereby executing an inhibitory role on T effector cell responses. The functional relevance of ICOS in hapten-induced ACD was demonstrated in $ICOS^+$ T_{reg} cells that secreted IL-10, IL-17, and IFN-γ. 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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Chapter 10
T Cell Responses to Drugs and Drug Metabolites

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Abstract Understanding the chemical mechanisms by which drugs and drug metabolites interact with cells of the immune system is pivotal to our knowledge of drug hypersensitivity as a whole.

In this chapter, we will discuss the currently accepted mechanisms where there is scientific and clinical evidence to support the ways in which drugs and their metabolites interact with T cells. We will also discuss bioanalytical platforms, such as mass spectrometry, and in vitro test assays such as the lymphocyte transformation test that can be used to study drug hypersensitivity; the combination of such techniques can be used to relate the chemistry of drug antigen formation to immune function. Ab initio T cell priming assays are also discussed with respect to predicting the potential of a drug to cause hypersensitivity reactions in humans in relation to the chemistry of the drug and its ability to form haptens, antigens and immunogens in patients.

Keywords Drug hypersensitivity • In vitro tests • Mass spectrometry • T cells • Peptide antigens

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Abbreviations

ADH	Alcohol dehydrogenase
ADR	Adverse drug reaction
APC	Antigen-presenting cell
CFSE	Carboxyfluorescein succinimidyl ester
DC	Dendritic cell
DHR	Delayed hypersensitivity reaction
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
FACS	Fluorescence-activated cell sorting
HHV-6	Human herpes virus-6
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSA	Human serum albumin
IFN	Interferon
IL	Interleukin
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LTT	Lymphocyte transformation test
MHC	Major histocompatibility complex
MRM	Multiple reaction monitoring
PBMC	Peripheral blood mononuclear cell
RCM	Radiocontrast media
REDOX	Reduction-oxidation
SI	Stimulation Index
SJS	Stevens–Johnson syndrome
TCR	T cell receptor
TEN	Toxic epidermal necrolysis
T_H	T helper
TNF	Tumour necrosis factor
β-lactam	Beta-lactam

10.1 Introduction

Drug safety is a very complex area of science, and although in recent years our greater understanding of immunopathological mechanisms have led to improvements in drug safety science, there is still much to be done to elucidate the chemical, immunological and genetic components that may contribute to drug-induced adverse reactions.

Adverse drug reactions (ADRs) are a major cause of patient morbidity and mortality; they are also a key reason for drug attrition in drug development

(Lazarou et al. 1998; Pirmohamed et al. 2004; Kola and Landis 2004). Some ADRs appear to be independent of the dose administered and do not directly correlate with the known pharmacological actions of the drug. Due to the idiosyncratic and unpredictable nature of these reactions, the exact pathogenesis remains unknown, despite decades of research. These reactions are often referred to as off-target toxicity, or type B reactions, and are usually of immunological aetiology.

Drug hypersensitivity was classified in the 1960s by Coombs and Gell into four main types: I, II, III and IV (Coombs and Gell 1968). Types I, II and III are mostly antibody mediated, in contrast to type IV (delayed hypersensitivity) that is T cell mediated. This was later revised, and type IV reactions were further subclassified into types a, b, c and d in order to reflect the scientific knowledge gained over the last four decades, particularly in the role of T cells in immune responses (Pichler 2003). Type IVa reactions involve T helper 1 (T_H1) cells that secrete IFN-γ leading to macrophage activation. Other cytokines such as TNF-α and IL-18 may also be implicated in this type of reaction. An example of a type IVa response is a reaction to tuberculin. Type IVb reactions are characterised by the secretion of IL-4, IL-13 and IL-5 by T helper 2 (T_H2) cells, leading to eosinophilic inflammatory responses; this type of reaction can lead to a clinical phenotype of maculopapular exanthema with eosinophilia, chronic asthma and rhinitis. Type IVc reactions are cytotoxic reactions that are mediated by CD4+ and CD8+ cells. The effector cells in such reactions are cytotoxic T cells that secrete perforin, granzyme B and FasL leading to clinical phenotypes of maculopapular and bullous exanthema and hepatitis amongst others (Pichler et al. 2010). Type IVd reactions are characterised by the secretion of high levels of CXCL8/GM-CSF produced by T cells and correspond to a neutrophilic inflammatory response leading to drug-induced acute generalised pustulosis and Behçet's disease (Britschgi et al. 2001; Pichler et al. 2010).

Indeed, the heterogeneity in the clinical phenotype of drug-induced hypersensitivity reactions is attributed to differences in T cell functions in the skin and in other organs (Pichler 2003; Roujeau 2005). Skin reactions are a common clinical manifestation associated with drug-hypersensitivity reactions: it is thought that skin-related hypersensitivity reactions account for 2–3 % of hospitalised patients (Bigby et al. 1986; Hunziker et al. 1997). Furthermore, sensitised T cells are found in the blood of patients with cutaneous hypersensitivity reactions. Other organs such as the liver may also be involved resulting in hepatotoxic events (Castell and Castell 2006); however, the ability of drug-specific T cells to cause liver injury in man has not yet been fully defined. Drug interaction with T cells can lead to tolerance or in the presence of co-stimulatory molecules can result in a hypersensitivity reaction via the induction of drug-specific effector T cells (Rozieres et al. 2009b).

Clinical diagnosis of drug-induced hypersensitivity reactions is not straight forward as drugs can cause different types of adverse reactions in susceptible patients, the drug-derived antigen may be unknown, and patients are often on multiple drug regimens thus making it difficult to elucidate the culprit drug that has elicited the immunological response. Identification of the culprit drug is important to prevent the patient from being re-exposed to it in the future. A clinical

diagnosis of drug hypersensitivity is based on patient history, skin tests, in vitro tests and less commonly culprit drug provocation tests. Skin tests such as patch, prick and intracutaneous testing lack efficacy as positive responses from patients with a well-documented history of drug-hypersensitivity reactions are not always obtained (Torres et al. 2003; Romano et al. 2004; Padial et al. 2008).

Drugs are thought to interact with the immune system in many ways and as such can be classified as haptens, antigens, immunogens or co-stimulatory agents (Naisbitt et al. 2000a; Pichler 2003; Pichler et al. 2011).

Hapten: A low-molecular-weight, chemically reactive compound such as a chemically reactive drug that can covalently bind to protein. Hapten-protein conjugates may or may not stimulate an immune response.

Antigen: A substance that can interact directly and bind to immunological receptors.

Immunogen: A substance that is able to stimulate a cellular or humoral immune response.

Co-stimulatory agent: A substance that interacts with dendritic cells, driving dendritic cell maturation.

Drugs may possess different combinations of the aforementioned properties, for example, not all antigens are immunogens, but nevertheless all immunogens can function as antigens.

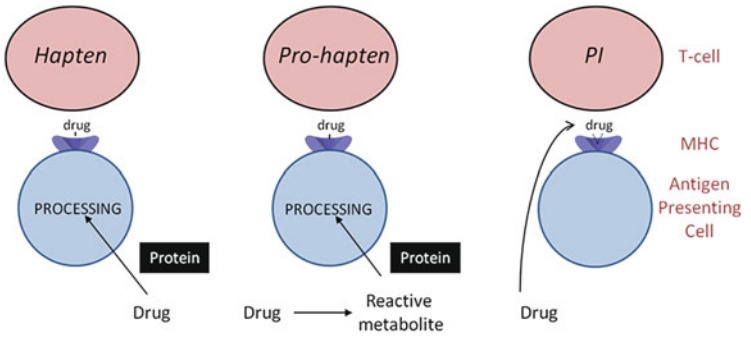
In this chapter, we will discuss the theories underpinning our current understanding of drug-hypersensitivity reactions in relation to T cell responses using paradigm drugs that are frequently implicated with hypersensitivity reactions.

10.2 Chemical Mechanisms of Drug Hypersensitivity

There are two main theories that are currently used to explore the mechanisms by which drugs can stimulate T cells based on both clinical and scientific evidence: the hapten/prohapten hypothesis and the p-i concept (Fig. 10.1). In addition to this, the danger hypothesis was put forward to complement the aforementioned theories by accounting for the co-stimulatory signalling that is thought to be an important factor in facilitating immunologically mediated, idiosyncratic drug reactions.

10.2.1 Hapten Hypothesis

Pioneering studies by Landsteiner and Jacobs (1935, 1936) marked the beginning of the hapten hypothesis which states that chemically reactive, low-molecular-weight compounds (typically under 1,000 Da) are too small to be immunogenic per se and must first bind to a macromolecular carrier, for example, an endogenous protein, in order to stimulate an immune response. These hapten-carrier complexes are then



Metabolism	No	Yes	No
Processing	Yes	Yes	No
Binding	Covalent	Covalent	Non-covalent

Fig. 10.1 A schematic diagram to summarise the pathways (hapten, prohapten and PI) of drug antigen presentation to T cells

taken up by antigen-presenting cells (APCs) and are processed and presented on major histocompatibility complexes (MHC) leading to T cell sensitisation. The ability of a drug to behave as a hapten is related to its chemical reactivity or that of a metabolite.

Beta-lactams (β-lactams) are widely prescribed therapeutic agents, and their use is commonly associated with drug-induced hypersensitivity reactions: it is because of this that they have been extensively investigated and serve as a good paradigm to investigate aspects of the hapten hypothesis. Penicillins form part of the β-lactam class of compounds, with their structure comprising of a β-lactam ring fused to a thiazolidine ring. Penicillin is a chemically reactive drug that is associated with a high incidence of hypersensitivity reactions. A critical factor in the pathogenesis of penicillin ADRs is thought to be neoantigen formation arising through the covalent binding of penicillin to endogenous proteins via the hapten mechanism. It is well reported that penicillin covalently modifies protein, such as human serum albumin (HSA); this occurs by the nucleophilic attack of the β-lactam ring by free amino groups, causing the ring to open and the penicilloyl group to bind. This covalently linked penicilloyl group is often referred to as the major antigenic determinant for antibodies and T cells. This is the established mechanism underpinning antigen generation following treatment with penicillin (Levine 1960; Levine and Ovary 1961; Batchelor et al. 1965; Lafaye and Lapresle 1988; Brander et al. 1995; Jenkins et al. 2009; Meng et al. 2011; Whitaker et al. 2011). In solution, degradation products of penicillin may be formed; these include penicillenic and penicilloic acid products that are able to form disulphide bonds with sulfhydryl groups on cysteine residues forming penicillenyl and penicillamine conjugates, respectively: these are often referred to as the minor antigenic determinants. Although these are formed at low levels, it is possible that they contribute to the induction of a

hypersensitivity reaction. In the context of the penicillins, HSA has often been used as a model protein to investigate drug binding owing to the fact that it accounts for approximately 90 % of serum bound-penicilloyl group in patients treated with benzylpenicillin (Lafaye and Lapresle 1988).

Another important class of antibiotics are the cephalosporins. Cephalosporins are semi-synthetic antibacterial agents used for the treatment of a variety of bacterial infections and in surgical prophylaxis. They form part of the β-lactam family of antibiotics that are structurally and pharmacologically related to the penicillins (Perez-Inestrosa et al. 2005); however, much less is known about how they react post-administration (Romano et al. 2005). Skin reactions such as maculopapular exanthema and urticaria are reported to occur in 1–3% of patients treated with cephalosporins (Norrby 1987).

The β-lactam ring is the common structural feature to both the penicillins and cephalosporins; in the penicillins, it is fused to a five-membered thiazolidine ring, whereas in the cephalosporins, it is fused to a six-membered dihydrothiazine ring. In contrast to the penicillins, cephalosporins possess up to two chemical side chains, giving rise to a greater heterogeneity of allergenic determinants (Moreno et al. 2008). Up until relatively recently, the emphasis was on penicillin-induced hypersensitivity reactions, and it has long been the assumption of many researchers that the cephalosporins react with endogenous proteins in the same way (Perez-Inestrosa et al. 2005; Moreno et al. 2008), although there is little scientific evidence to support this. Consequently, cephalosporins have been investigated very much in the context of the penicillins, and it is for this reason that the antigenic determinants remain unknown. This lack of understanding has hindered the clinical interpretation of such reactions and the development of diagnostic tests.

10.2.1.1 Mass Spectrometric Methods to Define Modified Peptide Antigens In Vitro and In Vivo

In recent years, technological advancements particularly in the field of mass spectrometry have allowed us to further probe the hapten hypothesis and investigate this hypothesis in the context of immunopharmacology. Using proteomic-based techniques such as mass spectrometry, it is possible to explore the relationship between protein biochemistry and immunology, particularly in the characterisation of epitope formation.

In order to elucidate the chemical mechanisms of protein haptenation, drugs are usually incubated with drug-binding proteins such as HSA. Using this approach, drug-protein conjugate formation can be investigated in both a concentration and time-dependent manner. Following a suitable incubation period, the protein is precipitated, reduced, alkylated and digested with a protease such as trypsin, thus generating a complex mixture of unmodified and drug-modified peptides. These peptides can then be separated using liquid chromatography prior to analysis by tandem mass spectrometry (LC-MS/MS). These discovery-phase experiments enable the chemistry of drug-protein conjugate formation to be determined, and

once the masses of the drug-modified peptides and diagnostic fragment ions have been elucidated, highly sensitive multiple reaction monitoring (MRM) methods can be developed (Kitteringham et al. 2009; Amacher 2010). MRM-based methods can detect modified peptides with enhanced specificity and sensitivity as the mass spectrometer is focussed only on peptides with the predefined parent ion and diagnostic fragment ion masses: these are referred to as transitions. The development of such methodologies that exhibit high sensitivity and specificity is particularly important for the analysis of patient samples owing to the low abundance of some drug-modified peptides. Once the chemistry of drug-protein conjugate formation has been defined, synthetic, physiologically relevant drug–protein conjugates can be prepared and used as a reagent in T cell assays (Whitaker et al. 2011; Meng et al. 2011). This permits the nature of the drug-derived epitopes to be investigated further and related back to antigenicity with respect to T cell stimulation. The haptenic nature of minor degradation/rearrangement products of benzylpenicillin has been studied using mass spectrometry, and it was revealed that benzylpenicillin and its rearrangement product, benzylpenicillenic acid, haptenate lysine residues of HSA. These drug–protein conjugates have been detected in plasma isolated from patients undergoing benzylpenicillin therapy. Physiologically relevant benzylpenicillin-albumin synthetic conjugates were prepared and were shown to stimulate T cells derived from benzylpencillin-hypersensitive patients in lymphocyte transformation test (LTT) assays (Meng et al. 2011).

A mass spectrometric approach was also used to detect piperacillin-specific T cell responses ex vivo from cells isolated from hypersensitive patients in a cystic fibrotic patient cohort (Whitaker et al. 2011). This patient population rely on high-dose, long-duration antibiotic therapy for the treatment of recurrent chest infections; consequently many of these patients (26–50 %) suffer hypersensitivity reactions to β-lactam antibiotics (Koch et al. 1991; Pleasants et al. 1994; Burrows et al. 2007). Whitaker et al. reported that the frequency of drug-specific T cells was greater than 75 %. A synthetic piperacillin-albumin conjugate was produced that reflected physiological conditions, and this was used as a reagent in in vitro assays for diagnosis, and was shown to stimulate T cells (Whitaker et al. 2011).

Drug–protein conjugates formed by flucloxacillin with HSA both in vitro and in vivo have also been reported (Jenkins et al. 2009). In vitro experiments revealed that drug–protein conjugate formation was a function of concentration and time, and mass spectrometric analysis of patient plasma samples showed that up to nine lysine residues in HSA are modified by the drug. In addition to this, it was revealed for the first time that the 5-hydroxymethyl metabolite of flucloxacillin can also modify the same lysine residues.

More recently, amoxicillin protein haptenation has been studied in vitro by incubating amoxicillin with human serum and using mass spectrometry to elucidate the proteins that are modified (Ariza et al. 2012). Studies such as this that define the chemistry can provide a panel of candidate peptides that can be used in T cell assays. Although binding of penicillin to an endogenous protein such as HSA is thought to be a prerequisite for the induction of an immune response, it is important to acknowledge that drug–protein conjugates have also been detected in

non-hypersensitive patients. This suggests other factors are required to stimulate the immune system: these may include the immunological state of the individual, the route of drug administration, dose and duration of treatment, and immunogenetic predispositions.

10.2.1.2 T Cell Recognition of Modified Peptide Antigens

Chemically reactive drugs can bind to endogenous proteins, which may induce conformational changes in the structure of the protein. Proteins are then subjected to proteasomal processing by antigen-presenting cells resulting in proteolysis and the generation of a peptide pool: some of these peptides may act as immunogenic epitopes by binding noncovalently to the groove of MHC molecules and being presented to T cells. Peptides presented on MHC class I stimulate CD8+ T cells and peptides that are presented on MHC class II stimulate CD4+ T cells. Studies have shown that peptides loaded in the MHC molecule could consecutively trigger T cell receptors (TCRs) (Chakraborty 2002). Designer peptides that were covalently modified with penicillin G on specific lysine residues were shown to stimulate TCRs (Padovan et al. 1997). Whilst it is possible to generate designer peptides that contain the drug modification of interest, it is important to stress that the synthesis of such peptides is extremely challenging and time consuming. This is because the drug modification must reflect the antigen that is formed in vivo. This requires complex chemistry in order to ensure that the drug modification is intact, in the correct conformation and in the correct position on the peptide. At present, many immunologists are doing such experiments blindly without first defining the chemistry of the synthetic reagents they have produced.

10.2.2 Prohapten Hypothesis

Immunological reactions to drugs that are not chemically reactive are explained by the prohapten hypothesis. Prohaptens are chemically inert drugs that when metabolised are bioactivated to form chemically reactive metabolites that are then able to covalently modify protein. Many drugs that are commonly associated with inducing adverse drug reactions are capable of generating toxic metabolites upon drug metabolism. These reactive metabolites may be formed in such small quantities that they fall below the limit of detection of routinely used bioanalytical techniques and thus cannot be detected; however, it is important to recognise that they could still be implicated in the immunological response. Studies investigating the immunological threshold at which T cells are stimulated have found that they require as little as a few hundred receptor ligands in order to become activated (Schodin et al. 1996). Although the liver is the main site of drug metabolism, other organs such as the skin express drug-metabolising enzymes. Liver-generated metabolites are unlikely to stimulate T cell-mediated reactions unless they are

able to avoid the detoxification process and are sufficiently stable to enter the circulation.

The sulphonamides represent a class of compounds that are intrinsically chemically unreactive and require cellular metabolism to generate reactive intermediates. Sulfamethoxazole is known to cause rare but serious cutaneous reactions and serves as a good example of a prohapten. After undergoing intracellular metabolism by CYP2C9 or peroxidases, sulfamethoxazole hydroxylamine is generated (Vanderven et al. 1994; Cribb et al. 1995; Mitra et al. 1996) that in turn can then react with molecular oxygen generating nitroso sulfamethoxazole (Cribb et al. 1991; Naisbitt et al. 1996). Nitroso sulfamethoxazole can react with sulfamethoxazole hydroxylamine to form azo and azoxy dimers (Naisbitt et al. 2002). Further oxidation of nitroso sulfamethoxazole results in the formation of nitro sulfamethoxazole. Nitroso sulfamethoxazole can be reduced by non-protein thiols (such as glutathione), ascorbate or enzymatically (Cribb et al. 1991). Sulfamethoxazole is commonly used, in combination with trimethoprim, for the treatment of *Pneumocystis carinii* pneumonia in AIDS patients (Pirmohamed and Park 2001). These patients often have deficiencies in ascorbate and thiols (Walmsley et al. 1997; Naisbitt et al. 2000b; Trepanier et al. 2004); thus, this patient cohort may not be able to reduce as much nitroso sulfamethoxazole causing enhanced cellular toxicity giving rise to co-stimulatory signalling and a greater risk of having a hypersensitivity reaction (Pirmohamed and Park 2001).

Using animal models, it has been demonstrated that nitroso sulfamethoxazole is a potent immunogen via protein conjugate formation and stimulation of CD4+ and CD8+ T cells (Naisbitt et al. 2001, 2002). In order to explore the antigenicity of sulfamethoxazole in man, T cell cloning experiments have been performed using T cells isolated from hypersensitive patients. Up until recently, patient studies investigating sulfamethoxazole hypersensitivity have revealed that T cell clones responded to nitroso sulfamethoxazole in a binding-dependent but processing-independent way. This may be due to nitroso sulfamethoxazole binding directly to the MHC-peptide complex (Schnyder et al. 2000). However, it has since been shown that T cell clones also respond to nitroso sulfamethoxazole in a processing-dependent manner (Castrejon et al. 2010; Elsheikh et al. 2011). Furthermore, it has been shown that antigen-presenting cells that have been cultured with sulfamethoxazole are able to metabolise the drug. Subsequent haptenation of intracellular protein generates a functional T cell antigen. This demonstrated a prohapten mechanism whereby the antigen generated was able to stimulate T cells from sensitised mice and from lymphocytes and T cell clones obtained from hypersensitive patients (Elsheikh et al. 2010).

10.2.3 Pharmacological Interaction with Immune Receptors (P-I Hypothesis)

The hapten/prohapten model certainly gives a very elegant and well-documented explanation as to how a small molecule, such as a drug, can activate the immune system.

However, it has been shown by Pichler and his group that there is an alternative mechanism whereby a drug is able to stimulate the immune system. This relatively new model was termed the p-i concept which stands for 'pharmacological interaction of drugs with immune receptors' (Schnyder and Pichler 1997). According to this concept, chemically inert drugs that are unable to form hapten-protein complexes are able to act as antigens by directly binding to TCRs or MHCs with high enough affinity to induce activation of certain T cells. The metabolism and processing of the drug or drug–protein complexes are not required, and stimulation only depends on the structural features of a drug that enable it to fit into the TCR or human leukocyte antigen (HLA) molecules thereby becoming immunogenic (Schnyder et al. 1997; Zanni et al. 1998a). There is significant clinical and immunological evidence in support of the p-i model. Aldehyde-fixed APCs are unable to process a drug but are still able to activate specific T cell clones, and washing glutaraldehyde-fixed APCs to remove a drug prevents the reactivity (opposite to haptens) (Schnyder et al. 1997; Depta et al. 2004). Furthermore, chemically inert drugs such as carbamazepine, lamotrigine, sulfamethoxazole, mepivacaine, lidocaine, p-phenylenediamine and radiocontrast media (RCM) are able to interact with TCRs with sufficient affinity and specificity to cause cytokine secretion, proliferation and cytotoxicity in the reactive T cell clones (Cribb and Spielberg 1992; Zanni et al. 1997; Christiansen et al. 2000; Sieben et al. 2002; Naisbitt et al. 2003). Another argument arises from TCR downregulation caused by drug-induced Ca^{2+} influx that occurs within seconds after the addition of the drug and so cannot involve drug processing (Zanni et al. 1998b). This body of evidence implies that p-i-stimulated T cells arise from previously primed effector and memory T cells. In vivo, p-i-activated T cells expand and subsequently infiltrate the skin and the other organs resulting in T cell orchestration of an inflammatory response. Recently, the p-i concept has been further dissected into two subforms: (a) The drug first binds to the HLA-molecule. This seems to be the case in well-documented HLA allele-associated drug-hypersensitivity reactions such as HLA-B*15:02 and carbamazepine allergy, and HLA-B*57:01 allele and abacavir allergy. In these cases, the drug binds in a non-covalent way to a specific HLA molecule, whilst other HLA alleles fail to provide such a preferential drug binding pocket. The HLA-drug complex elicits an immune response which is mostly CD8+ and cytotoxic (Adam et al. 2011, unpublished data). (b) Many drug allergies are not associated with a particular HLA allele in which case the drug may bind to the TCR directly (Ko et al. 2011). The p-i concept explains the way in which drugs such as carbamazepine stimulate T cells in hypersensitive patients. Carbamazepine, first introduced in the 1960s, is an anticonvulsant and mood-stabilising drug that is widely used for the treatment of

epilepsy, bipolar disorder and trigeminal neuralgia. Carbamazepine is associated with a high incidence of hypersensitivity reactions, typically delayed-type hypersensitivity reactions such as cutaneous manifestations and eosinophilia. Although it is extensively metabolised, it has been found that both the parent drug and some of its stable metabolites can induce an immunological response without the need for the more conventional antigen processing and presentation. The drug is metabolised by hepatic microsomal enzymes and has been reported to form many metabolites, some of which are stable and some that are unstable (Lertratanangkoon and Horning 1982). The stable metabolites include carbamazepine-10, 11-epoxide and 10-hydroxy carbamazepine: these have been shown to stimulate T cell proliferation by directly interacting in a non-covalent manner with MHC molecules and drug-specific T cell receptors leading to the secretion of IFN-γ and perforin as demonstrated in T cell cloning experiments (Naisbitt et al. 2003). It is reported that that approximately 70 % of carbamazepine-specific clones were found only to proliferate to the stable carbamazepine metabolites and not the parent drug, thus indicating the variety of T cells found within peripheral blood mononuclear cells (PBMCs) isolated from hypersensitive patients (Wu et al. 2006).

Investigations into carbamazepine-induced SJS show that cytotoxic T cell-mediated death may arise through the non-covalent interaction between carbamazepine and the HLA complex (Yang et al. 2007). More recently, it has been revealed using cells isolated from HLA-B*15:02-positive patients that there is a strong association with the activation of TCR Vβ-11-ISGSY: in vitro analyses revealed that patients that expressed this TCR phenotype reacted to carbamazepine, whereas carbamazepine-tolerant patients did not express this TCR phenotype. Therefore, it was concluded from this study that the defining feature that is necessary to elicit T cell responses to carbamazepine between tolerant and hypersensitive subjects is the TCR clonotype (Ko et al. 2011).

Sulfamethoxazole can conform to both the prohapten and p-i concept. It has been demonstrated that T cell clones can also interact with sulfamethoxazole without the need for protein haptenation and processing (Schnyder et al. 1997; Zanni et al. 1998b). Peptide elution experiments have shown that sulfamethoxazole does not always react with an embedded peptide; instead, it can react directly with the MHC molecule giving further weight to the p-i concept (Burkhart et al. 2002). This serves to inform us that drug-hypersensitivity reactions are not as simplistic as previously thought and sometimes cannot be classified according to a single hypothesis.

10.2.4 Danger Hypothesis

First proposed by Matzinger (1994), the danger hypothesis states that if antigen presentation occurs without any danger signals, then tolerance ensues. Conversely, if antigen presentation occurs in the presence of danger signals, a full immune-mediated reaction will result. Curtsinger and co-workers proposed that three

signals are a prerequisite for an immunological response to be mounted (Curtsinger et al. 1999; Curtsinger and Mescher 2010). These are as follows: signal 1, MHC-restricted antigen and T cell receptor interactions; signal 2, interactions with co-stimulatory molecules and receptors leading to the generation of pro-inflammatory cytokines such as IL-2, TNF-α and IFN-γ and signal 3, polarising cytokines that directly interact with T cells generating either a T_H1 or T_H2 immune response. In the context of drug hypersensitivity, a chemically reactive drug and/or metabolite can stimulate the first signal by acting as an antigen and subsequently signals 2 and 3 by causing cellular damage leading to the production of endogenous danger signals (Pirmohamed et al. 2002). At present, not enough is known about how drugs and/or metabolites interact to activate signalling pathways that are involved in co-stimulation.

Drug hypersensitivity is more common in people who have concomitant viral infections such as Epstein-Barr virus (EBV), human immunodeficiency virus (HIV) and human herpes virus-6 (HHV-6) (Pirmohamed and Park 2001; Sullivan and Shear 2001; Nishio et al. 2007). This may be due to the presence of viral inflammatory signals, such as increased levels of cytokines, HLA expression and co-stimulatory signalling leading to a potential hyperactivation of the immunological response in the presence of the drug (Pirmohamed et al. 2002).

10.3 Predisposing Factors to T Cell-Mediated Drug-Hypersensitivity Reactions

Genetic and environmental factors can predispose individuals to adverse drug reactions.

Environmental factors such as underlying viral infections can predispose an individual to an adverse drug reaction. Patients that have Epstein-Barr virus-induced infectious mononucleosis may develop a rash when treated with amoxicillin: the incidence of such a reaction is reported to be increased by a factor of 58 (van der Linden et al. 1998). Patients with chronic diseases such as HIV experience a high prevalence of drug-hypersensitivity reactions, often due to the high doses and long duration of drug therapy in combination with the underlying viral infection (Pirmohamed and Park 2001).

However, some individuals may have a genetic predisposition to T cell-mediated drug-hypersensitivity reactions. Abacavir serves as a paradigm for investigating genetic predispositions to drug-hypersensitivity reactions in an HIV patient cohort. Abacavir is an HIV-1 reverse transcriptase inhibitor that is metabolised by alcohol dehydrogenase (ADH) (Walsh et al. 2002). Approximately 5 % of patients develop systemic, delayed hypersensitivity reactions with a mean onset of 11 days (Hetherington et al. 2001); the clinical phenotype ranges from fever and rash to respiratory and musculoskeletal symptoms (Clay et al. 2000). Using T cells isolated from abacavir-hypersensitive patients, it has been shown that in the presence of the

parent drug and/or of an ADH-metabolising system, they proliferate. This shows that T cells can respond either to the parent drug or a metabolite (Dodd et al. 2003). It was deduced using flow cytometry that these T cells were CD8+ cells (Phillips et al. 2005). In recent years, studies have been performed to investigate the association between HLA restriction and drug-hypersensitivity reactions (Table 10.1). One such example of this is described by Mallal et al. where they investigated the relationship between MHC alleles and abacavir hypersensitivity (Mallal et al. 2002). They concluded that by not prescribing abacavir for patients that express the MHC alleles HLA-B*57:01, HLA-DR7 and HLA-DQ3, the overall instance of abacavir-hypersensitivity reactions could be reduced from approximately 9 % to 2.5 % (Mallal et al. 2002). Subsequently, T cell responses to abacavir were found to be exclusively HLA-B*57:01 restricted. Similar responses are detectable in hypersensitive patients and drug-naive volunteers carrying the HLA risk allele. It has also been suggested that T cells from naive volunteers were primed in vitro with abacavir via a processing-dependent pathway that presumably involved adduct formation in antigen-presenting cells (Chessman et al. 2008).

Other examples of HLA restriction and instances of drug-induced hypersensitivity reactions include the association of the HLA-B*57:01 with drug-induced liver injury following flucloxacillin therapy as revealed by Daly et al. when they performed a genome-wide study (Daly et al. 2009); the HLA-B*15:02 allele is associated with carbamazepine-induced SJS and toxic epidermal necrolysis (TEN) in the Han Chinese population (Ferrell and McLeod 2008). Studies investigating the blister fluid of patients with carbamazepine reactions have revealed that the T cells are comprised mainly of CD8+ T cells (Nassif et al. 2002, 2004). It is thought that these cytotoxic T cells cause apoptosis of keratinocytes to occur (Paul et al. 1996).

10.4 In Vitro Assays to Investigate the Pathomechanisms of T Cell-Mediated Drug-Hypersensitivity Reactions

In vivo and/or in vitro testing can be used to establish whether a patient is allergic to a given drug. The patch, prick and intracutaneous tests represent such in vivo testing, although they are not very reliable and lack efficacy (Torres et al. 2003; Romano et al. 2004) which likely relates to the application of an inappropriate antigen to the skin during testing. To date, the nature of the antigenic determinant (parent drug, metabolite, protein adduct) that is taken up by cutaneous dendritic cells has not been defined. Drug provocation tests, although considered much more effective in the diagnosis of drug-hypersensitivity reactions (Messaad et al. 2004), particularly immediate type 1, IgE-mediated reactions, are not commonly used in clinical practice due to the significant risk to the patients safety and wellbeing (Aberer et al. 2003).

Table 10.1 Examples of HLA restriction and drug-induced hypersensitivity reactions

Drug	HLA association	ADR
Abacavir	B*5701	Immunologically mediated reaction with clinical phenotypes ranging from fever, rash to gastrointestinal and respiratory symptoms
Carbamazepine	B*1502 in Han Chinese	Stevens–Johnson syndrome and toxic epidermal necrolysis
Flucloxacillin	B*5701	Drug-induced liver injury

In vitro tests are routinely used for the investigation of drug-hypersensitivity reactions in specialist laboratories, as they pose no significant threat to patient safety in the clinic. However, as drug hypersensitivity relies on very complex and different pathomechanisms, it is not surprising that a single diagnostic test cannot cover all possible reactions. At present, no one assay can detect and measure all aspects of the underlying mechanisms of an ADR; thus, many immunological and analytical platforms have to be used in combination with one another to gain a deeper insight into the complex pathomechanisms involved in eliciting the immunological response. Well-known proliferation-based assays (standard LTT) have been recently supplemented by a panel of novel in vitro tests measuring upregulation of activation markers (CD69) (Beeler et al. 2008), evaluation of cytokine secretion (IL-2, IL-5, IFN-γ) (Lochmatter et al. 2009a) and analysis of cytotoxic effector cells (measurement of granzyme B, granulysin and CD107a) (Lochmatter et al. 2009a, b; Zawodniak et al. 2010; Porebski et al. 2011). All in vitro tests depend on the presence of drug reactive T cells in the peripheral blood of drug-allergic patients. The persistence and frequency of these cells has a crucial impact on the outcome of the in vitro test. In some allergic patients, these effector T cells persist within a memory pool in the peripheral blood up to 12 years after disease, but in some other individuals, they lose their reactivity 1–3 years after consumption of drugs causing hypersensitivity (Beeler et al. 2006). As it is not possible to predict how long the reactivity of drug-specific T cells will persist, it is advised to perform in vitro tests within a minimal interval of 3 weeks up to 1 year after a delayed hypersensitivity reaction (DHR).

In vitro assays offer the following benefits:

- Present no risk to the patient.
- Permit the pathophysiology to be investigated.
- Provide a platform in which a panel of drugs can be tested.

As the in vitro tests described herein measure the responses of drug-specific memory T cells, they cannot be used for the prediction of drug-hypersensitivity reactions.

It is important to note that, as with any test, there are limitations to their use, and these are discussed below and summarised in Table 10.2.

Table 10.2 Comparison of in vitro tests to investigate delayed drug hypersensitivity reactions

Type of test	Read-out system	Duration (days)	Advantages	Disadvantages	Comments
LTT	^{3}H-thymidine incorporation	6–7	Suitable for many drugs High specificity Well-documented sensitivity and experience	Long-lasting Radioactive Technical experience needed Proliferation only Severe cases often negative (SJS/TEN)	Most commonly used test Detect activated T cells, not useful in predicting DHRs in non-exposed individuals
Surface CD69 upregulation	FACS	2–3	Rapid Identification of reactive cell subset	Method is difficult to standardize	Result often in concordance to LTT
Cytokine secretion (IL-2, IL-5, IL-13, IFNg)	ELISA, ELISpot, FACS	3	Rapid Sensitive Informative regarding pathomechanism	Expensive Low specificity	Could be the most sensitive, but has reduced specificity
Cytotoxicity (Perforin, Granzyme B, Granulysin, CD107a)	ELISA, ELISpot, FACS	3	Rapid Informative regarding pathomechanism Possibly suitable for severe reactions	Sensitivity and specificity need more evaluation	Focus mainly on $CD8^{+}$ and NK cells

10.4.1 Lymphocyte Transformation Test

The lymphocyte transformation test (LTT) is an in vitro lymphocyte proliferation assay that is used for the detection of sensitised T cells in order to diagnose T cell-mediated drug-hypersensitivity reactions. The most common application of the LTT is in delayed-type hypersensitivity; however, it has also been used to investigate the role of T cells in immediate, IgE-mediated reactions (Luque et al. 2001). The LTT assay can be used to detect T cells that are specific to a drug and/or drug metabolite. In brief, the proliferation of PBMCs isolated from patient blood samples in response to drug exposure can be quantified by measuring the uptake of tritium-labelled thymidine (^{3}H-thymidine) into the DNA of drug-activated lymphocytes. In this assay, cells isolated from patient blood samples are incubated with the parent drug, drug metabolites and/or drug-protein conjugate if available; the stimulation index (SI) is calculated based on the uptake of thymidine, which is directly proportional to the degree of drug-specific T cell proliferation. This assay has been demonstrated as a useful in vitro technique to investigate a range of drug classes including β-lactams (Schnyder and Pichler 2000; Luque et al. 2001; Whitaker et al. 2011), anticonvulsants (Naisbitt et al. 2003) and sulphonamides (Castrejon et al. 2010). A positive LTT, typically with an SI > 2, indicates T cell sensitisation. It can be used to identify the drug that caused the immune reaction, although it is important to note that a negative LTT does not necessarily rule out hypersensitivity. There are limitations with the LTT both in terms of sensitivity and practicality. The reported sensitivity of the LTT assays is 60–70 %; this can vary depending on the drug being tested. In terms of practicality, LTT assays take a long time, require sterile conditions and involve the use of tritiated thymidine. Furthermore, the LTT does not provide data on the nature of the induced response; thus, secreted cytokines are often measured in parallel to the proliferative response. In vitro tests are usually not performed during the acute phase of the hypersensitivity reaction as during this phase, the assays generally yield negative results; therefore failure of the test may relate to the immune system being strongly activated which would give a high background in the controls and so could mask any proliferation observed caused by the drug itself. Alternatively, antigen-specific T cells may reside in tissues in acute-phase reactions, and thus proliferative responses are not always detectable with blood-derived cells. Despite this, it has been reported that in cases of severe SJS/TEN reactions, the LTT responses are more positive during the acute-phase reaction. This still remains a matter of debate as it has also been reported that low rates of positive LTTs were obtained after recovery from SJS/TEN (Tang et al. 2012). In contrast to the findings of Kano et al. (2007) that were attributed to defective regulatory T cell functions during acute-phase reactions (Takahashi et al. 2009), they did not find any more drug-specific T cells in the acute phase than in the recovery phase.

10.4.2 ELISpot

Enzyme-linked immunosorbent spot (ELISpot) is used for the detection of cytokine (IL-2, IFN-γ, IL-4, IL-5 and IL-13) secreting cells or cytotoxic protease release from T cells that have been isolated from drug-hypersensitive patients (Rozieres et al. 2009a). It is a very sensitive technique with a reported lower detection threshold of <25 secreting cells per million PBMCs (Schmittel et al. 2001). To date, ELISpot has been restricted to cases of delayed hypersensitivity reactions, and the sensitivity and specificity for diagnosis has not been defined. However, since ELISpot provides important information relating to the nature of the induced drug-specific response, panels of markers are currently been applied to the diagnosis of different forms of drug allergy. In brief, PBMCs are isolated from a drug-hypersensitive patient and cultured in the presence of the suspected culprit drug. Cytokines are secreted from the T cells that are sensitised to the culprit drug, and the result is displayed as spots with each spot corresponding to the number of cytokine-secreting T cells. The results from such experiments can then be compared with control T cells that have not been incubated with drug. Elevated IFN-γ is often observed in delayed hypersensitivity reactions and, therefore, may serve as a cytokine marker to detect delayed hypersensitivity reactions. The increased concentration of IFN-γ causes MHC class II on keratinocytes to be upregulated: these activated keratinocytes are then able to present the drug in question to CD4+ T cells (Schnyder et al. 1998; Yawalkar et al. 2000). IFN-γ ELISpot has been used for the detection of drug-specific T cells in patients with a history of delayed maculopapular exanthemas following treatment with penicillin (Rozieres et al. 2009a). This study also demonstrated the use of ELISpot to determine potential cross-reactivity with structurally related compounds as it was observed in one patient that was hypersensitive to ticarcillin that IFN-γ-producing T cells were reactive to several other β-lactam containing compounds (Rozieres et al. 2009a). More recently, ELISpot has been investigated for the detection of abacavir-induced hypersensitivity reactions in HLA-B*57:01-negative patients: this approach shows promise as a diagnostic tool in such patients (Esser et al. 2012).

10.4.3 Flow Cytometry

Flow cytometry is frequently used by immunologists to study cell surface receptors and markers and in cell sorting applications. Characterisation of T cell subsets using flow cytometry in the context of drug hypersensitivity has been reported with drug-specific cytokine production being observed in 75 % of the patients studied (Martin et al. 2010a). Furthermore, the proliferation of drug-specific T cells can be investigated using flow cytometry by using fluorescent dyes such as carboxyfluorescein succinimidyl ester (CFSE) to stain the cell sample that contains within it drug-specific T cells. After stimulation, cells divide and at each division become two

times less fluorescent: the decreasing amount of fluorescence is indicative of the number of cycles of cell division. This has been demonstrated by Beeler et al. whereby the fluorescence intensity was shown to decrease in sulfamethoxazole-stimulated cultures and not in control cultures in the absence of the drug (Beeler et al. 2006). Importantly, flow cytometry-based approaches to detect proliferation can be coupled to functional markers of activation, thus providing important mechanistic data on the nature of the induced immune response (Caruso et al. 1997).

10.4.4 T Cell Cloning Experiments

T cell cloning experiments provide a platform in which the pathophysiology of the adverse reaction can be studied with respect to the phenotype, specificity and functionality of T cells that have been isolated from hypersensitive patients (Pichler et al. 2002; Naisbitt 2004). More recently, T cells have been cloned from inflamed tissues: phenotypic and functional characterisation of these clones provides essential information on true effector cells in hypersensitive patients. Moreover, as clones express a single T cell receptor, they can be used to define the restriction of the fit of the drug antigen into immunological receptors and the potential for closely related drug moieties to stimulate specific T cells. These studies are of particular importance in anticonvulsant-hypersensitive patients where alternative medication often needs to be prescribed.

10.4.5 Development of a T Cell Priming Assay to Predict the Potential of a Drug to Cause Hypersensitivity Reactions in Humans

In vitro T cell priming assays utilising human cells have been established for directly reactive chemical sensitisers such as the dinitrohalobenzenes (Martin et al. 2010b; Dietz et al. 2010). The success of such assays is dependent on the availability of dendritic cells and their ability to recognise reactive chemicals when conjugated to protein. Dendritic cells function as effective antigen-presenting cells and present peptides derived from the conjugated protein to naive T cells. A short 1–2-week incubation of antigen-exposed dendritic cells with naive T cells primes the response, and following reexposure to the same antigen, proliferation and cytokine secretion are readily detectable. For effective antigen presentation, dendritic cells must be exposed to danger signals that upregulate cell surface co-stimulatory molecule expression and stimulate cytokine secretion. In terms of in vitro T cell priming, it is widely believed that the reactive chemical stimulates dendritic cells directly via REDOX signalling and/or causing low levels of cell

death. However, it must be noted that the mechanisms of compound-specific dendritic cell signalling in in vitro T cell priming assays and indeed the requirement for dendritic cell co-stimulatory signalling have not been fully defined. Development of T cell priming assays to predict the potential of a drug to cause hypersensitivity is much more challenging (Fig. 10.2). As previously discussed, many drug allergens are not directly protein reactive and gain protein reactivity through metabolic activation. Strategies are in development to incorporate appropriate metabolising systems into in vitro assays. Although some degree of success has been noted, the level of metabolite formed might not always be sufficient to generate antigenic determinants for dendritic cell co-stimulatory signalling and/or T cells. When drugs do not stimulate dendritic cells directly, it might be possible to artificially activate dendritic cells using soluble activators and several approaches are in development. Furthermore, the critical protein targets for *all* drugs in development might not be available in a simple dendritic cell/T cell system.

One of the most important areas of progress in drug-hypersensitivity research is the discovery of surprisingly strong associations between hypersensitivity and expression of particular HLA alleles (Phillips et al. 2011). Indeed, for abacavir and carbamazepine has been possible to relate the genetic association to the pathogenic immune response. T cell responses in hypersensitive patients were shown to be exclusively restricted by the HLA risk allele (B*57:01 for abacavir, B*15:02 for carbamazepine), whilst it was also possible to prime T cell responses from drug-naive volunteers carrying the relevant HLA (Chessman et al. 2008; Ko et al. 2011). Thus, researchers attempting to develop predictive T cell priming assays must be conscious of this issue. The approach we have adopted in Liverpool is to establish an HLA-typed bank of viable lymphocytes. Four hundred volunteers were characterised using sequence-based HLA typing. Each volunteer donated 100 mL of peripheral blood; mononuclear cells were isolated and stored for functional studies. Three potential approaches summarised below are currently under investigation to study in vitro T cell priming:

1. *Analysis of HLA-restricted T cell activation following the direct stimulation of PBMCs*: Analysis of drug antigen-specific proliferation and cytokine secretion from PBMCs has yielded largely negative results, which likely relates to the low precursor frequency. Thus, we are now investigating an alternative approach to determine if it might be possible to characterise a transcriptional response in patients carrying HLA risk alleles.
2. *Drug stimulation of pre-activated memory T cells* (Engler et al. 2004): In these experiments, peripheral blood lymphocytes undergo several rounds of antigen-driven stimulation using frozen peripheral blood mononuclear cells as antigen-presenting cells. The aim of this protocol is to expand the number of antigen-responsive T cells prior to analysis of antigen specificity using readouts for proliferation, cytokine secretion and cytotoxicity. Using this approach, it has been possible to detect T cell responses to a limited number of drug antigens. The low sensitivity of the assay likely relates to the provision of inappropriate

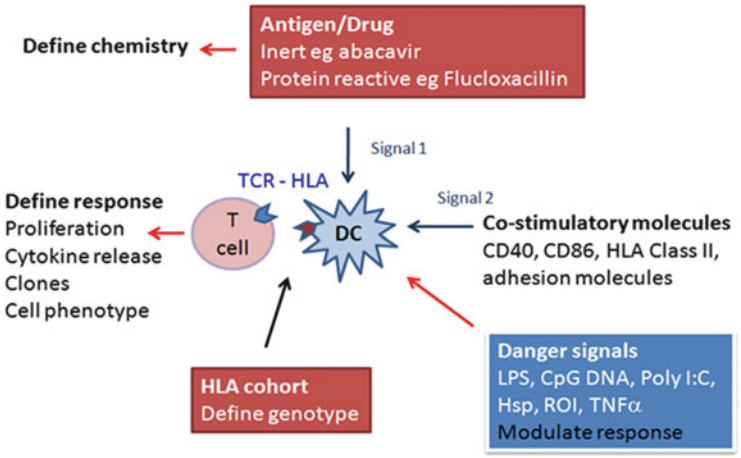


Fig. 10.2 Schematic showing the chemical and immunological factors to consider when developing an in vitro drug antigen-specific T cell priming assay

antigenic determinants and the need for repeated rounds or antigen-driven stimulation, which introduces a degree of variability into the assay.

3. *Drug stimulation of naive T cells*: This assay is based on the work of Dietz et al. and relies on the isolation and culture of highly pure T cell and antigen-presenting cell populations (Dietz et al. 2010). Immature monocyte-derived dendritic cells and naive T cells are used as antigen-presenting cells and responder cells, respectively. After a 10-day culture period, primed T cells are reexposed to the drug antigen and dendritic cells and antigen specificity is measured shortly after. In addition to classical readouts for proliferation, cytokine secretion and cytotoxicity, a change in phenotype from naive to memory can be quantified by flow cytometry. This approach, once established, yields reproducible results when CD4+CD25+Foxp3+ regulatory T cells have been depleted prior to antigen exposure and the drug antigen is presented by dendritic cells. Moreover, the assay is flexible in that one can control (1) drug delivery in an appropriate antigenic form, (2) the requirement for and maturation status of professional antigen-presenting cells and (3) the genetic background of the volunteer.

10.5 Conclusions

Based on this discussion, it is clear that several important inroads have been made with respect to the development of in vitro assays to (1) diagnose drug hypersensitivity and (2) test for in vitro T cell priming. However, it must be noted that most studies to date have used drugs with well-defined antigenic determinants and, when

relevant, known HLA associations. Translation of these mechanistic studies into sensitive and specific test systems to predict the potential of novel drug candidates to cause hypersensitivity reactions in humans is a long way from fruition. Researchers in the field must face up to and overcome issues relating to the provision of appropriate antigenic determinants and genetic variation in the human population. The application of new and emerging bioanalytical platforms to directly relate the chemistry of drug antigen formation to immune function should greatly assist the challenges that we face.

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Chapter 11
Hypersensitivity Reactions to Beta-lactams

Maria J. Torres, Cristobalina Mayorga, Natalia Blanca-López, and Miguel Blanca

Abstract Beta-lactam antibiotics (BLs) are the most frequent cause of hypersensitivity reactions mediated by specific immunological mechanisms, with two main types, IgE reactions or T-cell-dependent responses. From a practical point of view, these reactions can be classified into immediate, for those appearing within 1 h after drug intake, and non-immediate, for those appearing at least 1 h after and usually within 24 h of BL administration. The clinical symptoms differ according to this classification. Urticaria and anaphylaxis are the most frequently recorded symptoms in immediate reactions and maculopapular exanthema and delayed urticaria in non-immediate reactions. Although the exact diagnostic approach differs depending on the underlying mechanism, it is based on the performance of skin testing, laboratory tests, and drug provocation tests.

T cells are a key factor in all types of hypersensitivity reactions to BLs, regulating both IgE production or acting as effector cells, with a different profile of cytokine production. A Th1 pattern is observed in both $CD4^+$ and $CD8^+$ peripheral T cells in non-immediate reactions, whereas a Th2 pattern is expressed in $CD4^+$ T cells in immediate reactions.

Keywords Beta-lactams • Hypersensitivity • Hapten • IgE • T cells

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Abbreviations

AGEP	Acute generalized exanthematous pustulosis
APC	Antigen-presenting cells
AX	Amoxicillin
BAT	Basophil activation test
BLs	Beta-lactam antibiotics
BP	Benzylpenicillin
BPO	Benzylpenicilloyl
CLA	Cutaneous lymphocyte antigen
CPO	Cephalosporoyl
DC	Dendritic cells
DIHS	Drug-induced hypersensitivity syndrome
DPT	Drug provocation test
DRESS	Drug hypersensitivity syndrome with eosinophilia and systemic symptoms
FDE	Fixed drug eruption
LTT	Lymphocyte transformation test
MDM	Minor determinants mixture
MPE	Maculopapular exanthema
PLL	Poly-L-lysine
PPL	Benzylpenicilloyl-poly-L-lysine
RAST	Radioallergosorbent test
SJS	Stevens–Johnson syndrome
TEN	Toxic epidermal necrolysis

11.1 Introduction

Drug hypersensitivity includes reactions mediated by immunological mechanisms, the most frequent of which are those induced by specific IgE antibodies or by T-cell-dependent mechanisms (Blanca et al. 2009; Torres et al. 2003). The drugs most often involved in these immunological reactions are the beta-lactam antibiotics (BLs), which have therefore become the best studied model. Other hypersensitivity reactions are non-immunologically mediated—the most frequent of these being cross intolerance to nonsteroidal anti-inflammatory drugs. This type of reaction is not induced by BLs, and it does not therefore come within the scope of this review.

According to the time interval between the drug administration and the development of the symptoms, hypersensitivity reactions to BLs can be classified as immediate (appearing within 1 h of drug intake) or non-immediate reactions (appearing more than 1 h after drug intake) (Blanca et al. 2009). The former are mediated by specific IgE antibodies and the latter mainly by a T-cell-dependent mechanism.

Although all BLs, including those more recently introduced on the market, can induce hypersensitivity reactions, the particular BL involved depends on the patterns of prescription and consumption in the population evaluated (Blanca 1995). Benzylpenicillin (BP) was the first BL identified as responsible for allergic reactions, but it has progressively been replaced by amoxicillin (AX) (Blanca 1995). Other BLs, such as cephalosporins (Blanca 1995) and more recently clavulanic acid, also contribute to inducing hypersensitivity reactions, most of them immediate (Torres et al. 2010a). This tendency will probably change over the next few decades as patterns of consumption are modified (Blanca 1995; Torres et al. 2010a).

The prevalence and incidence of hypersensitivity to BLs is unknown, with data differing depending on the study (Rebelo-Gomes and Demoly 2005). Surveys carried out in large series of patients with cutaneous symptoms showed that 19 % of all the patients evaluated with a history of hypersensitivity reactions to BLs were finally allergic (Rebelo-Gomes and Demoly 2005), with lower values when children were evaluated (Caubet et al. 2011).

The immunological mechanisms involved in hypersensitivity reactions to BLs follow the classification of hypersensitivity reactions described by Gell and Coombs (1968), although further complexity has been added (Pichler 2003):

- Type I or immediate reactions, mediated by drug-specific IgE antibodies.
- Type II or cytotoxic reactions, responsible for immune hemolytic anemia and thrombocytopenia as classical representatives.
- Type III reactions, also known as cytotoxic and immune complex reactions. These are now considered rather rare and are mediated by drug-specific, complement-fixing IgG or IgM antibodies. The classical entity seen in this group is serum sickness.
- Type IV or delayed-type hypersensitivity reactions, where different T lymphocyte subpopulations participate as well as other immune system cells.

In this review we will analyze in detail the general characteristics of hypersensitivity reactions to BLs, focusing on the mechanisms in both IgE-mediated (Type 1) and T-cell-dependent (Type IV) reactions.

11.2 Skin as a Target of Hypersensitivity Reactions

The skin is generally the target organ in hypersensitivity reactions to BLs (Blanca et al. 2009; Torres et al. 2003). In the case of IgE-mediated reactions, symptoms can be limited to the skin, as happens in urticaria with transient pruriginous wheals occurring simultaneously at different sites of the body. This may or may not be accompanied by angioedema, consisting of inflammation of the subcutaneous tissue. IgE-mediated reactions also include anaphylaxis, which involves generalized pruritus, erythema, and angioedema: difficulty breathing; upper/lower airway obstruction; and, in more severe cases, cardiovascular collapse leading to

anaphylactic shock. The reasons why some persons develop urticaria while others develop anaphylaxis are currently not well understood.

The symptoms in T-cell-mediated reactions usually appear after 24–48 h, although they can develop as soon as 1 h after drug administration leading to a full expression in a few hours (Padial et al. 2008; Blanca-Lopez et al. 2009; Warrington et al. 1993). The most frequent entities are usually mild, such as MPE and delayed urticaria (Blanca et al. 2009; Romano et al. 1995; Terrados et al. 1995; Garcia et al. 1997), but other more severe manifestations can also appear. These latter include acute generalized exanthematous pustulosis; drug hypersensitivity syndrome with eosinophilia and systemic symptoms/drug-induced hypersensitivity syndrome (DRESS/DIHS); bullous eruptions such as erythema multiform, Stevens–Johnson syndrome (SJS), and toxic epidermal necrolysis (TEN) (Doña et al. 2009); localized or generalized fixed drug eruption (FDE) and contact dermatitis (de San et al. 1999); and serum sickness-like syndrome (Clark et al. 2006). It is important to note that it is difficult to differentiate these symptoms from those induced by viral or autoimmune diseases, especially in children where viral infections are frequent (Mayorga et al. 2009).

The reasons why BLs, which are usually administered by oral or parenteral routes, induce symptoms mainly affecting the skin are not completely understood. In immediate reactions the release of histamine and other inflammatory mediators produces the typical skin effects of wheals and erythema as well as pruritus. In non-immediate reactions induced by drugs that require metabolism, there is presence in the skin of an incomplete metabolic system that does not totally detoxify drugs. This is more difficult to explain in non-immediate reactions induced by BLs where no metabolic pathway is needed.

Although classically the skin has been considered just a physical and biochemical barrier of the organism, its importance as an immunological organ has been stressed in recent years. The skin contains different cells related with the immunological response, including mast cells, macrophages, dermal dendritic cells, keratinocytes, and Langerhans cells; these have been denominated the static skin components that produce proinflammatory cytokines (Metz and Maurer 2009; Fernandez et al. 2009; Ramirez-Gonzalez et al. 2009). These cytokines induce the recruitment of cells from peripheral blood as antigen-presenting cells (APC), such as Langerhans cells, dendritic cells (DCs), monocytes and macrophages, as well as T lymphocytes expressing skin-homing receptors like the cutaneous lymphocyte antigen (CLA) and different chemokine receptors (CCR10, CCR4, CCR6,) representing the cellular basis of the immunological memory in the skin (Fernandez et al. 2009; Blanca et al. 2000; Bos and Kapsenberg 1993). These cells form the dynamic component of the cutaneous immunological system.

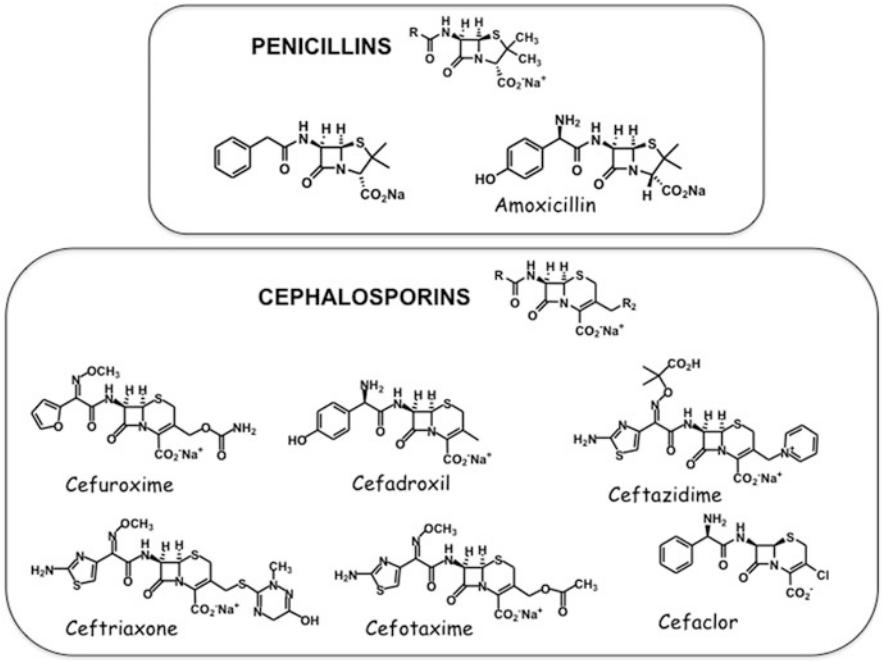

Fig. 11.1 Chemical structure of different beta-lactam antibiotics: penicillins and cephalosporins

11.3 Beta-Lactams as Haptens

The chemical structure of BLs is formed by a four-member ring, the so-called BL ring. In penicillins it is bound to a five-member thiazolidine ring and in cephalosporins a six-member dihydrothiazine ring (Fig. 11.1). Penicillins have only one side chain at the R1 position and cephalosporins two, one at the R1 and the other at the R2 position. The chemical substitutions at the different side chains produce a wide range of BLs with different antibacterial activity and spectra that are also differentially recognized by the immunological system (Blanca et al. 1994).

BLs are haptens that cannot be recognized by the immunological system and bind spontaneously to exogenous or endogenous proteins that can later be processed and recognized by the immunological system (Burke et al. 1991; Levine and Ovary 1961). This binding with the lysine residues produces the opening of the BL ring inducing, in the case of BP, the benzylpenicilloyl (BPO) structure, the first antigenic determinant identified (Burke et al. 1991; Levine and Ovary 1961) (Fig. 11.2). This has been used for skin test diagnosis by conjugating with PLL as a carrier in what has been called the major determinant, analogous to what occurs with protein allergens, because it was the most frequent structure recognized (Adkinson et al. 1971). Human serum albumin is the candidate carrier protein and BP and benzylpenicillenic acid selectively target different residues, Lys199 and

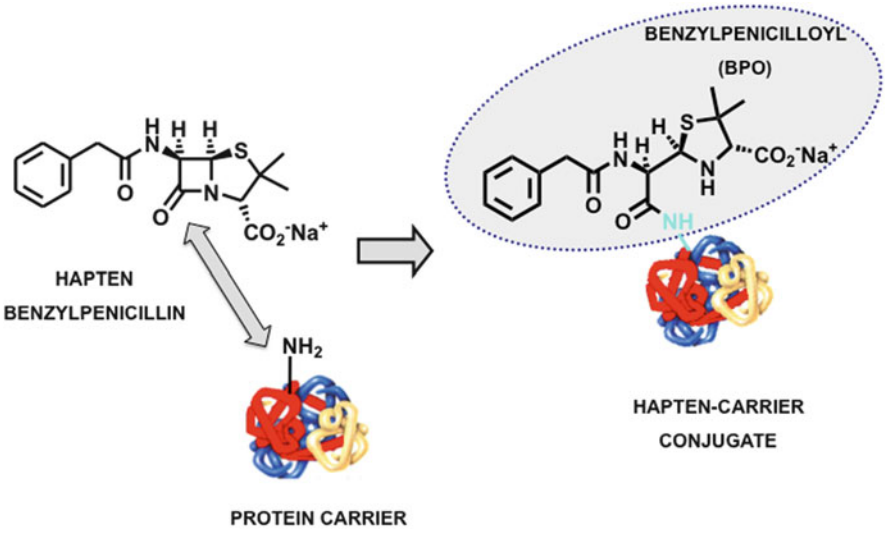

Fig. 11.2 Formation of the major determinant of benzylpenicillin

Lys525, respectively (Meng et al. 2011). In the degradation pathway of BP, other metabolites can appear, such as benzylpenicilloic or benzylpenilloic, that can also induce positive responses in the skin; these are commercialized as a minor determinants mixture (MDM) (Parker et al. 1962; Levine and Redmond 1969). Similar determinants are also generated from AX, such as amoxicilloic acid and diketopiperazine (Torres et al. 2010b).

The contribution to the antigenic determinant and the way the BL molecule is recognized by IgE antibodies depend on the chemical structure. Using murine monoclonal antibodies to BP three epitopes have been identified: the side chain, the structure formed by the binding of the carbonyl of the BL to the amino group of proteins, and the thiazolidine ring (De Haan et al. 1985). Another study generating a complete panel of antibodies of different isotypes to AX (Mayorga et al. 1995) showed that the side chain was the most important part of the molecule contributing to the epitope, with the whole structure necessary for optimal recognition. Studies carried out using human IgE antibodies showed that although differences in the side chain structure were important for IgE recognition, the whole structure including the protein carrier plus the BL was also necessary for the constitution of the complete antigenic determinant (Perez-Inestrosa et al. 2005; Sánchez-Sancho et al. 2003; Moreno et al. 1995).

These in vitro studies agree with clinical evidence that selective responses to AX occur in a considerable number of cases as well as with varying degrees of cross-reactivity between the different BLs (Moreno et al. 1995; Blanca et al. 1988, 1990). Up to 30 % of allergic patients react with AX but have good tolerance to BP, though this percentage is tending to increase over the years (Blanca et al. 1988, 1990). IgE antibodies from selective responders to AX mainly recognize the AX side chain

structure, whereas those patients with cross-reactive responses to BP do not differentiate between the AX and BP side chain structures and also recognize the nuclear part of the BL structure (Moreno et al. 1995).

The situation with cephalosporins is more complex, as there are four generations of chemical structures and many more molecules with differences in the degradation pathway and generation of metabolites (Perez-Inestrosa et al. 2005). With these drugs the opening of the BL ring by nucleophilic attack of proteins and other reagents generates an intermediate cephalosporoyl (CPO) determinant which is chemically unstable, suffering multiple fragmentation reactions (Fig. 11.3). Those cephalosporins that have a good R2 leaving group undergo the process of expulsion when they conjugate to carrier proteins by the opening of the BL ring. For these cephalosporins the unstable dihydrothiazine moiety is enough to undergo further degradation processes. As a result, conjugation of cephalosporins by the BL ring leads to loss of the R2 side chain and to fractionation of the dihydrothiazine ring, and this does not therefore form part of the epitope presented in the hapten–carrier conjugate. Only the R1 side chain and a fragment of the BL ring remain bound to the carrier protein, constituting the epitope resulting from these conjugates. The presence of an R2 side chain that may act as a good leaving group is closely related to enhanced reactivity of the BL ring for nucleophilic attack (Perez-Inestrosa et al. 2005).

In depth studies of IgE, recognition to these resultant structures has involved the synthesis of well-defined structures comprising the entire acyl side chain of different cephalosporins and the aminoacidic residue included in the BL moiety of the cephalosporins studied, linked as amide functions to an aliphatic (*n*-butyric) chain (Sánchez-Sancho et al. 2003; Montañez et al. 2011). The results showed that the proposed skeleton epitopes involving the appropriate functionality and R1 side chain were selectively recognized by IgE from patients allergic to cephalosporins with the same or similar side chain structures.

11.4 Immunological Mechanisms Involved

Although Gell and Coombs described four main mechanisms of hypersensitivity, the two mechanisms mainly involved in BLs are specific IgE antibodies and T-cell mediation. However, T cells have an essential role in all types of hypersensitivity reactions to drugs, regulating both IgE production or effector cells, depending on the profile of cytokine production (Mosmann et al. 1986). Differences in the cytokines and transcription factors have been detected in immediate and non-immediate reactions, with a Th1 pattern with T-bet expression in both $CD4^+$ and $CD8^+$ T cells in non-immediate reactions, and a Th2 pattern with c-Maf expression in $CD4^+$ T cells in immediate reactions (Cornejo-Garcia et al. 2007).

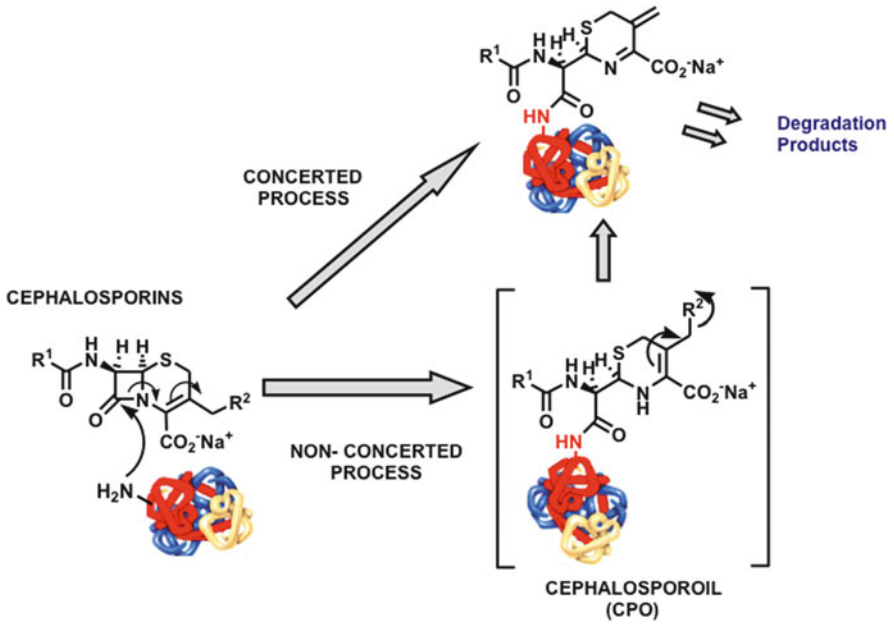

Fig. 11.3 Formation of cephalosporin determinants

11.4.1 IgE-Mediated Reactions

In the sensitization phase BLs are taken up by the DCs, processed, and presented to T cells in a Th2 microenvironment. These will proliferate and interact by two signals (CD40–CD40L and the Th2 cytokines) with B cells, inducing the corresponding switching to produce specific IgE antibodies to the hapten–carrier conjugate which binds to high affinity Fcε-RI on the surface of mast cells and basophils. After a drug reexposure, this conjugate is recognized by specific IgE antibodies at the cell surface, and after cross-linking the activation of a calcium-dependent protein kinase cascade occurs with a subsequent release of inflammatory mediators such as histamine, prostaglandin D2, sulphidoleukotrienes, tryptase, and many cytokines. These mediators induce the symptoms of IgE-mediated reactions.

11.4.2 T-Cell-Dependent Responses

The skin is the target organ in the majority of hypersensitivity reactions to BLs. This type of reaction can be monitored during the acute episode and the resolution period in the affected skin and the peripheral blood in order to provide clues concerning the pathophysiological mechanisms involved. This process parallels the skin lesion involvement, and it is thought that after an antigenic stimulus

originated in the skin, the specific immunological process is triggered, with the arrival of lymphocytes via the peripheral circulation with the interplay of different ligands and receptors, including adhesion molecules and chemokine receptors (Mayorga et al. 2009).

The involvement of T cells in allergic drug reactions has mostly been studied in non-immediate reactions, where the presence has been shown of activated T cells expressing the CLA during the acute response that normalize when the clinical reaction subsides (Picker et al. 1993; Leyva et al. 2000) as well as increased production of the IFN-γ, TNF-α, IL-2 and transcription factor, T-bet, and cytotoxic markers perforin and granzyme B (Mayorga et al. 2009).

Considering the specific subpopulations involved, although in general there is a predominant $HLADR^+$ activation in the $CD8^+$ cells in circulating cells in patients with severe skin symptoms with $CD4^+$ cells in weak maculopapular eruptions (Hari et al. 2001), specific differences nevertheless exist depending on the exact entity induced. This has been confirmed by immunohistochemical studies performed in the skin, showing a mononuclear cell infiltrate composed mainly of T cells, expressing activation markers (CD25, CD69, and HLADR) and the skin-homing receptor CLA in both $CD4^+$ and $CD8^+$ subsets, with $CD4^+$ cells generally predominating over $CD8^+$ cells (Pichler 2003; Rozieres et al. 2009; Torres et al. 2004). Other cells such as neutrophils, eosinophils, macrophages, or keratinocytes can take part, as in MPE where increased numbers of eosinophils have been found in the papillary dermis (Mayorga et al. 2009).

Data from different studies show a parallelism between the results found in the skin and those in the peripheral blood, with a higher participation of $CD4^+$ cells in the more severe reactions (Torres et al. 2006). The trafficking of T cells is regulated by differential cell-surface expression of chemokine and tissue homing receptors to the skin, varying between entities (Foster 2001). In addition to high CLA expression, other receptors are involved, with chemokines playing a fundamental role (Kunkel and Butcher 2002). Some reports have shown that most CLA^+ T cells express other skin-homing chemokine receptors such as CCR4 and CCR10 (Tapia et al. 2004; Homey et al. 2002; Soler et al. 2003) and a parallel increase in chemokine CCL27 (CCR10 ligand) production in keratinocytes (Mizukawa et al. 2002). In MPE, during the acute phase of the reaction, the presence of Th1 (CXCR9 and CXCR10) and skin-homing (CCL20 and CCL27) chemokines has been identified in the skin, and their corresponding receptors (CXCR3, CCR6, and CCR10, respectively) in peripheral blood, which demonstrates the complexity of lymphocyte recruitment (Fernandez et al. 2008). With respect to the cytokines and cytotoxic marker expression in the skin, we found significant increases in TNF-α (Fernandez et al. 2008), which can be related with the expression of CCL27 by keratinocytes (Homey et al. 2002), and in IFN-γ, which is the stimulus for keratinocytes to produce CXCL9 and CXCL10 (Flier et al. 2001) (Fig. 11.4).

It is clear that immunological cellular mechanisms are responsible for the non-immediate reactions to BLs (Pichler et al. 1998). This group includes a variety of clinical entities formerly classified as Type IV reactions according to Gell and Coombs and that have now been divided into four separate subgroups (Type IV

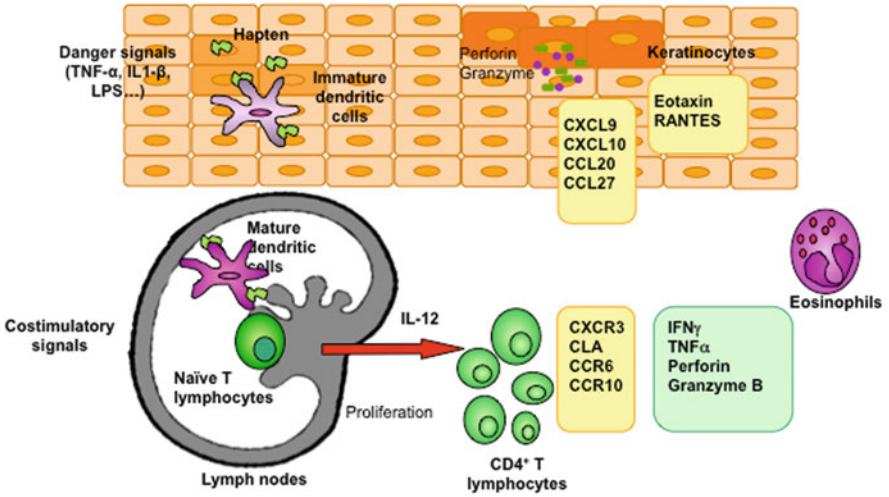

Fig. 11.4 Immunological mechanism involved in non-immediate reactions to drugs, maculopapular exanthema reaction

a–d) (Pichler 2003). These are elicited by different subsets of T cells, with distinctive functions leading to tissue damage. BLs are involved in all four Type IV reactions: Type IVa (Th1) reactions with macrophage activation, eosinophil-rich exanthemas (Type IVb); bullous skin diseases (Type IVc); and neutrophil-rich inflammations like AGEP (Type IVd).

Although most information about non-immediate reactions to BLs concerns the specific effector T-cell response as described above, other cells, like DCs, also participate. These are professional APCs essential for initiating T-cell responses, the activities of which depend on the maturational status and which will modulate the tolerant or the effector immune responses mediated by different subtypes of T lymphocytes (Banchereau and Steinman 1998), with immature DCs (imDCs) and semimature DCs related to tolerant immunologic responses and mature DCs (matDC) to effector responses (Lutz and Schuler 2002). Several studies have supported the role of DCs in the response to haptens (Enk et al. 1993; Arrighi et al. 2001; Enk and Katz 1992), especially in contact dermatitis induced by low-molecular-weight compounds, such as nickel (Boisleve et al. 2004) in which the hapten by itself is able to produce DC maturation (Arrighi et al. 2001; Boisleve et al. 2004). Although there is little information regarding the involvement of DCs in non-immediate hypersensitivity reactions to BL, some studies indicate that BLs interact differently with DCs in drug-allergic and nonallergic patients by the modification of the maturational level of imDCs. AX increases the expression of DC maturation and activation markers and decreases their endocytosis (Rodriguez-Pena et al. 2006). Furthermore, AX-treated imDCs from patients with delayed hypersensitivity induced an increased proliferation of allogenic T cells (Rodriguez-Pena et al. 2006). However, AX was unlikely to induce full maturation

of DCs because after 72 h of treatment, compared with other agents that interact directly with receptors (LPS and TNF-a), it just induced a semi-maturation status (Lutz and Schuler 2002; Granucci et al. 2001).

The immune response involves a tight interaction between the innate and the adaptive immune systems, in which the NK–dendritic cell cross-talk has an important role (Walzer et al. 2005; Marcenaro et al. 2005). As has been shown, DCs can activate NK cells (Ferlazzo et al. 2004) and vice versa, and activated NK cells can induce DC maturation or the death of imDC through the production of either cytokines or cytotoxic factors (Vitale et al. 2004; Zhang et al. 2006), which can be a mechanism for DC homeostasis to maintain the balance between tolerant and immunologic responses (Walzer et al. 2005). In this sense, in non-immediate reactions to AX, this drug is able to activate NK-producing cytotoxic markers (perforin and granzyme B) mainly in the $CD56^{dim}$ subpopulation and a Th1 cytokine (IFNγ) in $CD56^{bright}$ cells (Chaves et al. 2010). The increase in the inflammatory NK subpopulation is mainly observed in the presence of AX-matured DCs. Moreover, AX only increases the NK cytotoxic effect in allergic patients, by an increase in annexin V binding to DC, preferentially imDC (Cahves et al. 2010).

Several studies have reported the bidirectional modulation induced by NK/DC cross-talk that includes maturation of DC and activation of NK cells (Marcenaro et al. 2005; Moretta 2002; Ferlazzo et al. 2002; Gerosa et al. 2002). Data in allergic patients show that the cytotoxic activity and cytokine release are well-differentiated processes in NK cells and depend on the status of the DC present in the culture, with imDC involved in NK cytotoxicity, whereas matDC are involved in cytokine production. All these data support the hypothesis that the cross-talk between both the innate and the adaptive immune systems, represented by NK cells and DCs, is critical in the immunopathology of adverse drug reactions, having as a consequence the amplification of the harmful effects observed in these reactions to drugs.

11.5 Clinical Approach to Hypersensitivity Reactions to BLs

The clinical symptoms and the diagnostic approach differ between patients with immediate reactions and those with non-immediate reactions. Table 11.1 lists the clinical entities of the patients with hypersensitivity reactions to BLs as well as the diagnostic methods currently used.

Table 11.1 Types of reactions, mechanisms involved, and clinical symptoms according to the Gell and Coombs classification

Type of reaction	Clinical
Type I	Urticaria
Immediate	Angioedema
IgE-mediated	Anaphylaxis
Type II	Immune hemolytic anemia
Cytotoxic	Thrombocytopenia
Antibody mediated	Blood cell dyscrasias
	Organ-specific reactions
Type III	Serum sickness-like syndrome
Immunocomplex	Vasculitis
Immunocomplex mediated	Organ-specific reactions
Type IV	Maculopapular exanthema
Delayed-type	Urticaria Stevens–Johnson syndrome
T-cell mediated	Toxic epidermal necrolysis
	Organ-specific reactions
	Acute generalized exanthematic pustulosis
	DRESS/DIHS
	Fixed drug eruption
	Contact eczema

11.5.1 Diagnostic Workup

The diagnostic approach to patients with hypersensitivity reactions to BLs is based on the performance of a clinical history followed by skin testing and a drug provocation test (DPT) when indicated. In some cases laboratory tests can also help with the diagnosis.

Skin testing is recommended to be done using benzylpenicilloyl-poly-L-lysine (PPL) and MDM, consisting of BP and benzylpenilloic acid (Blanca et al. 2009) and AX (Blanca et al. 2009; Torres et al. 2003). When the BLs involved in the reaction are different to BP and AX and skin tests to PPL, MDM, and AX are negative, skin testing with the culprit BL, such as cephalosporins or clavulanic acid, should be done (Blanca et al. 2009; Torres et al. 2003, 2010a).

Depending on the type of reactions to be explored, skin tests can be done by prick, intradermal, or patch testing. In IgE-mediated reactions the sensitivity of skin testing is lower than previously thought, with the number of patients reacting to penicillin decreasing and those reacting to AX increasing (Blanca et al. 2009; Torres et al. 2003). In T-cell-dependent reactions, sensitivity of the order of 50–60 % has been reported although recent studies have shown lower figures, with 10–20 % or even lower in children where most cases tolerate the BL implicated in the reaction (Blanca et al. 2009; Torres et al. 2003; Caubet et al. 2011; Padial et al. 2008; Blanca-Lopez et al. 2009; Terrados et al. 1995). In these reactions aminopenicillins are the drugs most frequently implicated, followed to a lesser extent by cephalosporins, with most patients tolerating BP

(Blanca et al. 2009; Torres et al. 2003; Caubet et al. 2011; Padial et al. 2008; Blanca-Lopez et al. 2009; Terrados et al. 1995).

In subjects with negative skin tests, a DPT can be done to confirm the diagnosis, especially in mild reactions. This approach consists of an increasing administration of the suspected drug to confirm the reaction or assess tolerance. This is contraindicated in severe IgE and T-cell reactions but is mainly done in urticarial reactions and MPE. This is particularly important in children with skin rashes attributed to BL administration, where penicillin allergy is overdiagnosed and symptoms are only reproducible in less than 7 % of the children studied by DPT (Caubet et al. 2011).

Laboratory methods widely used to detect BL-specific IgE antibody include immunoassays with different determinants such as BP, AX, and cephalosporins conjugated to carrier molecules (human serum albumin, aliphatic spacers or PLL) and then bound to solid phases (sepharose, cellulose discs) (Garcia et al. 1997; Blanca et al. 1992). A commercial assay for routine analysis is the CAP System FEIA method (Phadia, Uppsala, Sweden), which has a high surface capacity solid-phase assay using a secondary fluoro-labeled antibody. The specificity of this method ranges from 83.3 to 100 % with a sensitivity varying from 12.5 to 25 %, depending on the study (Blanca et al. 2001; Sanz et al. 2009).

Another procedure that is increasingly used is the basophil activation test (BAT). This is based on the capacity of basophils to upregulate activation markers like CD63 or CD203c after the interaction of the drug with specific IgE antibodies at their cell surface. It has a sensitivity of 48.6 % and a specificity of 93 % (Garcia et al. 1997; Blanca et al. 2001; Sanz et al. 2009). In general, in vitro tests, although less sensitive than skin testing, are complementary for the diagnosis of immediate reactions to BLs, with some cases being skin test negative but in vitro test positive (Torres et al. 2002).

The lymphocyte transformation test (LTT), although not routinely used, can be used for the evaluation of non-immediate reactions to BLs (Nyfeler and Pichler 1997; Luque et al. 2001). In our experience, 57 % of patients have a positive LTT to at least one of the penicillins tested, although when different BLs are used in the stimulation, a heterogeneous response is observed (Luque et al. 2001). Moreover, a study showed that the inclusion of autologous DCs as APC increases the sensitivity of the LTT to AX with no changes in the specificity (Rodriguez-Pena et al. 2006), properties that have been confirmed with other drugs (Lopez et al. 2009; Torres et al. 2008) (Table 11.2).

11.5.2 Cross-Reactivity

Patients with specific IgE antibodies to one BL can recognize a different BL due to similarities in their chemical structure and may therefore experience an allergic response. This has been described more often between penicillins and first-generation cephalosporins, although it can appear between any BL. Indeed,

Table 11.2 Diagnostic methods used in the diagnosis of hypersensitivity reactions to BLs

Type of reaction	Diagnostic methods
Immediate	*Skin tests*
	• Prick tests
	• Intradermal tests
	Laboratory tests
	• Immunoassays (ELISA, RAST, CAP)
	• Basophil activation test
	Drug provocation tests[a]
Non-immediate	*Skin tests*
	• Intradermal tests (delayed reading)
	• Patch testing
	Laboratory tests
	• Lymphocyte transformation tests
	Drug provocation tests[a]

[a]If no contraindications or risk factors exist

cross-reactivity has been described between penicillins and cloxacillin, ampicillin, methicillin, and AX (Blanca et al. 2009; Moreno et al. 1995; Torres et al. 1999, 2001; Co Minh et al. 2006).

The pattern of response varies between patients, and patients with a selective response to AX have a reaction after AX administration or even with AX skin testing while having good tolerance after BP administration. Similar patterns have been detected in patients allergic to cloxacillin or penicillin V (Padial et al. 2008; Blanca-Lopez et al. 2009; Romano et al. 1995; Terrados et al. 1995). Moreover, this recognition is maintained over time independently of the BL administered (Fernandez et al. 2005). This has recently been shown with clavulanic acid (Torres et al. 2010a) where patients developing an IgE response to AX–clavulanic acid administration responded to BP, AX, or clavulanic acid depending on their age. Cross-reactivity with carbapenems and monobactams in penicillin-allergic patients seems to be very low, less than 1 % with imipenem (Romano et al. 2006) and none with aztreonam (Vega et al. 1991).

Considering cephalosporins, cross-reactivity in patients who had an IgE response to penicillin was around 10 % (Romano et al. 2004). As mentioned above, the degradation pathway of cephalosporins is quite different from that of penicillins, and they are rapidly degraded and just the side chain at the R1 position remains, with this part of the molecule being critical for recognition and therefore for inducing cross-reactivity (Mosmann et al. 1986). This is the reason why first-generation cephalosporins have higher cross-reactivity with penicillins than those newly introduced into the market. Cross-reactivity is higher when the side chain is identical, increasing to 30 % in cases of selective responders to AX when cefadroxil is administered (Miranda et al. 1996). Similar results are found in patients with immediate allergic reactions to cephalosporins that may or may not cross-react with penicillin, and even tolerate other cephalosporins with different side chains. A high degree of cross-reactivity has been detected between cephalosporins with similar or identical side chains at the R1 position, as is the case of ceftriaxone, cefotaxime, or cefepime (Romano et al. 2000; Antúnez et al. 2006a).

Finally, cross-reactivity in non-immediate hypersensitivity reactions seems to be very low between penicillins and cephalosporins and even within the penicillins where cross-reactivity to other penicillins with different side chains is infrequent (Padial et al. 2008; Blanca-Lopez et al. 2009; Romano et al. 1995; Terrados et al. 1995).

11.5.3 Natural Evolution

Patients with IgE-mediated responses to BLs experience a decrease in the production of the antibodies over time that results in the negativization of skin and laboratory tests like the radioallergosorbent test (RAST) and BAT (Blanca et al. 1999; Fernández et al. 2009). This was first observed in patients with clear anaphylactic reactions but who were skin test negative when a long time had passed between the reaction and the allergological work-up (Blanca et al. 1999). The rate of negativization of the skin test depended on the BL inducing the reaction, with those cases involving IgE recognition of BP decreasing more slowly than those with selective responses to AX (Romano et al. 2004). Whether this decrease in sensitivity is accompanied by good tolerance or not is still not clearly known. Moreover, after new exposure a booster effect can appear and resensitization occurs, as has been detected in both skin tests and laboratory tests (Antúnez et al. 2006b). This is the reason why it is recommended to repeat the study in those cases with a clear reaction and a negative allergological work-up before confirming that patients are nonallergic (Blanca et al. 2009). Additionally, after a new contact a booster response occurs to the original sensitizer independently of the BL administered, such that IgE antibodies can increase to BP although the new contact was with AX, for example (Antúnez et al. 2006b).

In the case of T-cell responses, although the possibility of a decrease in the response also exists, sensitivity is maintained longer with the presence of drug-reactive T cells, even many years after the avoidance of contact with the culprit drug (Padial et al. 2008; Blanca-Lopez et al. 2009; Romano et al. 1995; Terrados et al. 1995; Beeler et al. 2006).

11.6 Concluding Remarks

T cells have an essential role in hypersensitivity reactions to BLs, in the case of immediate reactions by regulating IgE production and in the case of non-immediate reactions by acting as effector cells. The monitoring of the acute hypersensitivity response in non-immediate reactions showed that both $CD4^+$ and $CD8^+$ T cells are involved, with the expression of a number of cell-surface markers that enable them to migrate to the skin. Furthermore, the expression of the different markers is

related with the different entities induced and their severity. Exploration of the T-cell function can be used as a diagnostic tool.

New insights into the interaction of T cells with dendritic cells and NK cells show that the development of the reaction involves an interaction between the innate and the acquired immune systems.

Conflict of Interest None of the authors have any conflict of interest nor have they received any money for the present study. Research is part of their daily activities. All the authors had full access to all the data and can take responsibility for the integrity of the data and the accuracy of the data analysis. The study was funded by FIS-Thematic Networks and Co-operative Research Centres (RIRAAF/RD07/0064), Junta de Andalucia (CTS 06603, PI-0545-2010), and FIS (09/01768, PS09/00966).

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