

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 μ M) 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 V α and J α segments of the α -chain and the variable (V β), diversity (D β), and joining (J β) gene segments of the β -chain locus are re-organized (Hughes et al. 2003). The underlying structural and biophysical determinants of $\alpha\beta$ 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 V β -D β , D β -J β , and V α -J α junctions, thereby enabling junctional diversity. Thus, 10^{15} – 10^{20} unique $\alpha\beta$ 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 $\alpha\beta$ 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 microenvironment *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

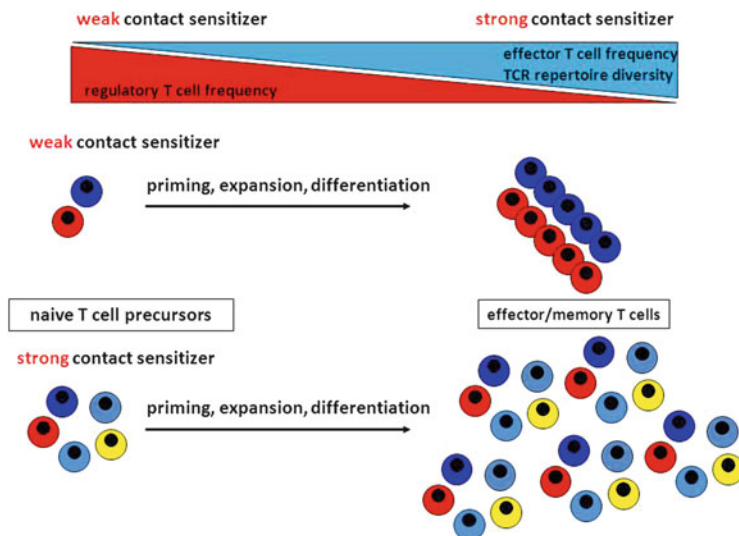


Fig. 8.1 Correlation of contact allergen potency with effector T cell and Treg pool size and TCR repertoire diversity. Differences in the size of the naïve T cell precursor pool may be reflected in the size of the effector/memory T cell pool and the diversity of the corresponding TCR repertoire. The 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 naïve 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 Carrier 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

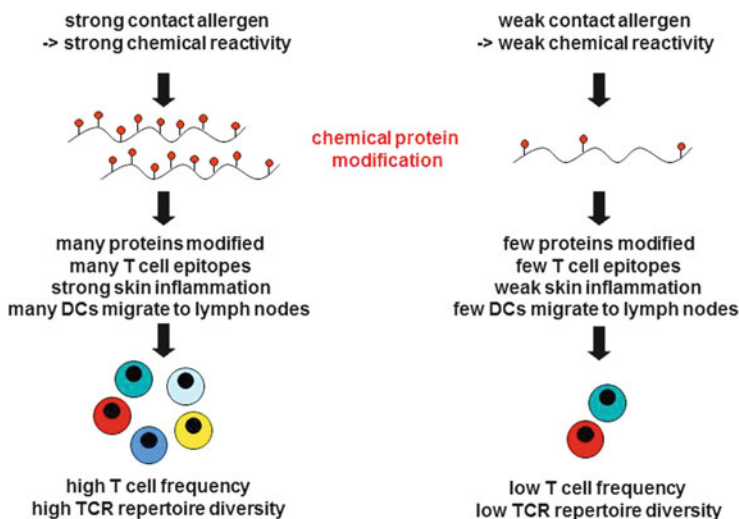


Fig. 8.2 Determinants of contact sensitizer potency. Chemical reactivity may be a crucial determinant of contact sensitizer potency. The extent of chemical protein modification should be translated into the strength of the innate inflammatory response that determines the number of contact allergen modified 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 V β families after 7 days. The stimulation with superantigen resulted in unique patterns of diversity that included polyclonal expansion of specific TCR V β families, as well as oligoclonal expansion of most other TCR V β 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^bPA₂₂₄-specific TCR in complex with cognate pMHC complex. They observed that while the CDR3 α loop made peptide contact via the amino acid side chains, the CDR3 β 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^bPA₂₂₄-specific TCR α -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 V α and V β 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 V β - and C β - or J β -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 V β 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 V β 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 V β 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 V β 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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