

Immune dysregulation increases the incidence of delayed-type drug hypersensitivity reactions

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3 1 **Immune dysregulation increases the incidence of delayed-type drug hypersensitivity reactions**

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8 3 **Short title:** Regulatory pathways and drug hypersensitivity

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34
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36
37 17 aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the
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39 18 work are appropriately investigated and resolved.

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3
4 19 **Abstract:** Delayed-type, T-cell mediated, drug hypersensitivity reactions are a serious unwanted
5 20 manifestation of drug exposure that develops in a small percentage of the human population. Drugs
6 21 and drug metabolites are known to interact directly and indirectly (through irreversible protein
7 22 binding and processing to the derived adducts) with HLA proteins that present the drug-peptide
8 23 complex to T-cells. Multiple forms of drug hypersensitivity are strongly linked to expression of a single
9 24 HLA allele and there is increasing evidence that drugs and peptides interact selectively with the protein
10 25 encoded by the HLA allele. Despite this, many individuals expressing HLA risk alleles do not develop
11 26 hypersensitivity when exposed to culprit drugs suggesting a non-linear, multifactorial relationship in
12 27 which HLA risk alleles are one factor. This has prompted a search for additional susceptibility factors.
13 28 Herein, we argue that immune regulatory pathways are one key determinant of susceptibility. As
14 29 expression and activity of these pathways is influenced by disease, environmental and patient factors,
15 30 it is currently impossible to predict whether drug exposure will result in a health benefit,
16 31 hypersensitivity or both. Thus, a concerted effort is required to investigate how immune dysregulation
17 32 influences susceptibility towards drug hypersensitivity.

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35 Introduction

36 Drug hypersensitivity refers to objectively reproducible symptoms or signs initiated by exposure to a
37 drug at a dose normally tolerated by non-hypersensitive persons (1). Hypersensitivity is also
38 commonly referred to as a form of off-target toxicity, which means that the development of tissue
39 injury is not predictable from known pharmacology of the drug and there is no simple association
40 between the dose of the drug administered and the development of clinical signs and symptoms.
41 Delayed-type reactions vary in severity and can target individual organs such as liver and skin in
42 isolation or as part of a generalized hypersensitivity syndrome. Common to the cellular
43 pathophysiology of drug hypersensitivity is the presence of drug-specific T-lymphocytes in blood and
44 inflamed tissue (2-4). In fact, cutaneous hypersensitivity reactions (maculopapular, pustular, and
45 bullous) are classified according to the effector molecules secreted by T-cells when activated with drugs
46 (5, 6).

47 In 2002, Mallal et al. reported a strong association between the presence of HLA-B*57:01, HLA-DR7,
48 and HLA-DQ3 and hypersensitivity to the HIV-1 reverse-transcriptase inhibitor abacavir (7).
49 Subsequent studies demonstrated that (i) all skin test confirmed cases of abacavir hypersensitivity
50 carry HLA-B*57:01 (8), (ii) abacavir interacts selectively with high affinity within the HLA-B*57:01
51 peptide binding cleft through non-covalent interactions (9-11), and (iii) abacavir only activates CD8+
52 T-cells (12-14). It is important to note that the abacavir association differs from all other forms of HLA-
53 linked hypersensitivity reaction. For example, drug-responsive CD4+ and CD8+ T-cells are observed in
54 patients hypersensitive to drugs such as carbamazepine, dapsone, flucloxacillin who express the
55 relevant HLA class I risk alleles, B*15:02, B*13:01 and B*57:01, respectively (15-17). These data
56 indicate that although there is a preference for drug (parent drug, metabolite) peptide complex HLA
57 T-cell receptor binding in patients, binding interactions are generally heterogeneous and this
58 contributes to the complete adaptive drug-specific T-cell response. Throughout this manuscript we
59 discuss the different forms of drug HLA interaction in detail highlighting similarities and differences in
60 pathways that lead to T-cell activation. However, we subsequently use the general term "drug peptide
61 complex" where appropriate to refer to any drug-derived structure that interacts with HLA proteins
62 and T-cell receptors to trigger T-cell activation. This is because the formation of an HLA, drug, peptide
63 and T-cell receptor complex is necessary for all pathways of T-cell activation. It is simply the nature of
64 the complex and form of binding interaction that differs. As the number of associations between drug
65 hypersensitivity and HLA allele expression increases (18-20), it is important to consider the additional
66 patient factors that confer susceptibility. This is of particular importance because not all patients
67 expressing a risk HLA are susceptible, while many patients lacking known risk alleles go on to develop
68 hypersensitivity when exposed to culprit drugs.

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3 69 Three factors are critical for the activation of T-cells with drugs; exposure to a drug peptide complex,
4 70 the availability of a T-cell repertoire for a drug peptide complex and a protein encoded by HLA alleles
5 71 for drug peptide complex binding. The argument is presented that although each factor detailed above
6 72 is critical for drug immunogenicity; separately or together, they cannot be used to predict patient
7 73 outcome following drug exposure. We hypothesize that when each factor is present, active immune
8 74 regulatory pathways (co-inhibitory receptors, Tregs, cytokines) are key determinants of whether drug
9 75 exposure will result in hypersensitivity. Since expression and activity of these regulatory pathways are
10 76 altered by disease, the genetic make-up of the host and environmental factors, it is currently
11 77 impossible to predict whether drug exposure will result in a health benefit, hypersensitivity or both
12 78 (Figure 1).
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23 80 **Different manifestations of drug hypersensitivity**

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25 81 Drug-induced cutaneous reactions: Although skin rashes are common forms of drug hypersensitivity,
26 82 serious and life-threatening reactions develop much less frequently. Examples of serious cutaneous
27 83 hypersensitivity reactions include Stevens-Johnson syndrome, toxic epidermal necrolysis and drug
28 84 reaction with eosinophilia and systemic symptoms (DRESS). Although less serious than the conditions
29 85 listed above, acute generalised exanthematous pustulosis and maculopapular exanthema are also
30 86 important adverse drug reactions. A broad spectrum of different drugs may cause cutaneous reactions
31 87 including the sulfonamides, allopurinol, carbamazepine, dapsone and many of the penicillins (21-24) .
32 88 Although there is some degree of pathophysiological overlap, there are some clinically defining
33 89 features for each type of severe cutaneous adverse drug reaction and these are briefly discussed
34 90 below.
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43 91 The most common skin manifestation is maculopapular exanthema which accounts for approximately
44 92 95% of all cutaneous reactions (25). These are reported as eruptions starting on the trunk and upper
45 93 extremities and progressively become more prevalent. These reactions are not life-threatening and
46 94 almost always subside when the culprit drug has been withdrawn (26). Antibiotics and a number of
47 95 tuberculosis medications such as rifampicin, isoniazid, pyrazinamide and ethambutol are common
48 96 causes of maculopapular exanthema (27).
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53 97 Acute generalised exanthematous pustulosis represents a more severe, usually drug-related skin
54 98 reaction characterised by the presence of sterile pustules on an erythematous surface along with fever
55 99 and neutrophilia in a patient. Furthermore, the involvement of activated neutrophils along with
56 100 excessive production of cytokines IL-8 and IL-17 is characteristic of acute generalised exanthematous
57 101 pustulosis, stimulating the recruitment to tissues and the induction of innate immune responses (28).
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3 102 DRESS is a severe skin reaction with an incidence of between 1:1000 and 1:10000 in patients exposed
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5 103 to culprit drugs such as anticonvulsants, antimicrobials and antivirals (29). The reaction is
6
7 104 characterised by skin eruptions, fever as well as symptoms in other organs, such as hepatitis, nephritis
8
9 105 and thyroiditis (30). DRESS has been shown to be regulated by the cellular actions of eosinophils
10
11 106 mediated via the secretion of IL-5 from drug-specific T-cells (31). Furthermore DRESS is often
12
13 107 associated with reactivation of several viruses, including HHV-6, CMV and EBV (32) (33).

14 108 Stevens-Johnson syndrome and toxic epidermal necrolysis define increasing degrees of severity of the
15
16 109 same skin disease and are often grouped together. The disease involves the mucosal membranes
17
18 110 including the eyes, mouth and genitals (30). The level of skin detachment can be used to categorise
19
20 111 the severity of the reaction. The clinical definition of Stevens-Johnson syndrome is when the
21
22 112 detachment of epidermal sheets remains on small areas and occurs on less than 10% of the body
23
24 113 surface area. Stevens-Johnson syndrome/toxic epidermal necrolysis overlap is when this value is
25
26 114 between 10-30% and toxic epidermal necrolysis patients experience large sheets of skin detachment
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28 115 exceeding 30% of the body surface area (34).

28 116 Drug-induced liver injury: The liver is the largest organ in humans; it is the major organ responsible for
29
30 117 the metabolism and detoxification of drugs. Hepatocytes (parenchymal cells) make up about 85% of
31
32 118 the liver while non-parenchymal cells, including liver sinusoidal endothelial cells, hepatic stellate cells,
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34 119 Kupffer cells and biliary epithelial cells make up the remaining 15% and play important roles in
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36 120 maintaining the homeostasis of the liver. Drug-induced liver injury is a major reason for drug attrition
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38 121 and withdrawal of drugs in clinical trials or drugs already licenced for clinical use (35). Worldwide, the
39
40 122 estimated annual incidence rate of drug-induced liver injury is 0.02% (36, 37). Hoofnagle and
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42 123 Björnsson have recently classified drug-induced liver injury into three categories (direct,
43
44 124 indirect and idiosyncratic) according to frequency, predictability and reaction mechanisms
45
46 125 (38). Direct liver injury is common and occurs rapidly when drugs are given at high doses (e.g.,
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48 126 paracetamol). Indirect liver injury has an intermediate frequency, is partially predictable and
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50 127 occurs as an indirect action of the drug on liver or the immune system (e.g., monoclonal
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52 128 antibodies). Finally, idiosyncratic liver injury occurs in only a small number of individuals, is
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54 129 not predictable and involves activation of the patients adaptive immune system. The mean
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56 130 onset of idiosyncratic liver injury with certain drugs exceeds 100 days (39). Amoxicillin, clavulanic acid,
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58 131 NSAIDs, flucloxacillin, lapatinib, lumiracoxib, ximelagatran among other drugs have been implicated
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60 132 with various degrees of unpredictable/idiosyncratic liver injury. Several forms of drug-induced liver
injury are strongly associated with expression of specific HLA alleles (40). This, alongside the delayed
onset of clinical symptoms, is indicative of the pathogenesis involving drug-specific T-cells. Recent

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3 135 studies have identified and characterized drug-responsive CD4+ and CD8+ T-cells from the peripheral
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5 136 blood of patients with tuberculosis medicine-, co-amoxiclav- and flucloxacillin-induced liver injury (41-
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7 137 43). Furthermore, T-cells have been shown to infiltrate liver and kill hepatocytes through the release
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9 138 of cytolytic molecules (44, 45).

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12 13 140 **Does drug exposure impact on susceptibility to hypersensitivity?**

14
15 141 For this discussion, we assume that the initiating event for T-cell activation is either a drug or drug
16
17 142 metabolite binding directly to the HLA T-cell receptor complex (through either covalent or non-
18
19 143 covalent binding) or a drug or drug metabolite binding indirectly to non-HLA proteins (through
20
21 144 covalent binding; the HLA binding epitope being a peptide derived from the modified protein, which
22
23 145 may or may not contain the drug moiety).

24 146 In consideration of the latter first, most research has been conducted on biological samples from
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26 147 patients with β -lactam hypersensitivity. For adduct formation, the β -lactam ring is targeted by lysine
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28 148 residues. Nucleophilic attack leads to ring opening and binding of the penicilloyl group to the lysine
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30 149 residue (46). β -lactam antibiotics modify serum proteins such as serum albumin and multiple
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32 150 intracellular proteins (47-51). Protein adducts are transported to antigen presenting cells via exosomal
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34 151 transport (50, 52) and β -lactam-modified protein and peptide adducts have been shown to activate
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36 152 patient T-cells (15, 53-57). Importantly, these adducts are formed in *all* drug exposed patients (48, 58-
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38 153 60), those who develop skin and liver reactions as well as those that safely tolerate the drug.
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40 154 Moreover, through the synthesis of β -lactam-modified peptides as standards for mass spectrometric
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42 155 analysis, Meng et al (58) were able to quantify and compare the level of drug albumin binding in
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44 156 hypersensitive and tolerant patients. No clear differences in the level of β -lactam antibiotic lysine
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46 157 modification was detected between the two patient groups, and importantly, the level of modification
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48 158 in all patients exceeded the threshold required for activation of β -lactam antibiotic-responsive T-cells.
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50 159 Obviously, additional studies are required to explore whether hapten thresholds are exceeded in
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52 160 patients receiving others β -lactam antibiotics and hapteneic drug metabolites. However, currently
53
54 161 available data suggests that although the formation of drug protein adducts may be an important, if
55
56 162 not critical factor for drug immunogenicity, the level of therapeutic drug exposure does not seem to
57
58 163 be a key determinant of patient outcome. One way to confirm this would be a detailed comparison of
59
60 164 the incidence of hypersensitivity reactions in patients receiving higher and lower β -lactam doses or
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62 165 longer and shorter treatment courses, as long as this doesn't impact on clinical care.

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64 166 An assortment of drug structures activate T-cells through a direct non-covalent interaction with HLA
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66 167 and/or specific T-cell receptors. The p-I concept has been coined to explain this phenomenon and

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3 168 differentiate this pathway of T-cell activation from the hapten concept. A number of pieces of
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5 169 experimental evidence support this direct binding concept: first, the addition of parent drug to human
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7 170 immune cell culture systems that express low levels of drug metabolizing enzymes leads to a T-cell
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9 171 response characterized by proliferation and cytokine and cytolytic molecule release (61-63); second,
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11 172 inhibition of protein processing within antigen presenting cells, which blocks T-cell responses to
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13 173 protein antigens has no effect on the activation of T-cells with drugs (64, 65); and third, the kinetics of
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15 174 T-cell activation with drugs is rapid, within minutes (14, 66), which is in stark contrast to classical
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17 175 antigen presentation pathways that require several hours. Many drugs have been shown to activate
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19 176 T-cells from hypersensitive patients via this pathway, including sulfamethoxazole (65), carbamazepine
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21 177 (67, 68) and allopurinol (66). However, with the exception of abacavir, the nature of the drug peptide
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23 178 HLA T-cell receptor interaction is yet to be defined. The selective interaction of abacavir with HLA-
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25 179 B*57:01 alters the spatial arrangement of molecules within the peptide binding groove. This results in
26
27 180 the display of novel "altered" HLA-B*57:01 peptide sequences that seemingly go on to stimulate T-
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29 181 cells that bring about abacavir hypersensitivity (9-11, 69). Adam et al. (69) demonstrated that
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31 182 abacavir-responsive T-cells stemming from naïve and memory compartments are detectable in 100%
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33 183 of donors expressing HLA-B*57:01. This led the authors to suggest that abacavir T-cell reactivity by-
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35 184 passes normal co-stimulatory/regulatory requirements. However, we draw readers attention to the
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37 185 fact that it has not been possible to explain why only half of HLA-B*57:01+ donors (who all possess
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39 186 abacavir-responsive T-cells) exposed to abacavir develop hypersensitivity. It should also be noted that
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41 187 p-I- and hapten-responsive T-cells are not always detected in isolation. For the β -lactam antibiotics
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43 188 (55, 70) and sulfonamides/sulfones (17, 71, 72), the only drug exemplars studied to date, drug p-i- and
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45 189 hapten-responsive T-cells are found together.

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47 190 Drugs administered at a high mass dose more frequently cause hypersensitivity reactions, when
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49 191 compared with drugs administered at lower doses (73). However, in humans, individual drugs tend to
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51 192 be administered at similar doses using dosing regimens directed to achieve drug concentrations within
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53 193 a therapeutic window for a sustained duration of time. Humans are therefore exposed to similar
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55 194 plasma concentrations of the parent drug. A handful of studies describe associations between
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57 195 metabolism (increased production of metabolite or increased exposure to parent drug) and the
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59 196 incidence of drug hypersensitivity reactions (74). For example, CYP2C9*3, which decreases phenytoin
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197 clearance is associated with an increased occurrence of anticonvulsant hypersensitivity (75, 76).
198 Similarly, impaired renal function and increased plasma levels of oxypurinol (the metabolite that
199 drives T-cell responses in hypersensitive patients (77)) correlate with the poor prognosis of
200 allopurinol-induced severe cutaneous hypersensitivity reactions (78). However, these findings seem

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3 201 to be an exception, rather than a rule, as few other studies have reported associations between drug
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5 202 disposition and hypersensitivity.

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7 203 It is clear that a threshold level of drug exposure must be surpassed for the activation of T-cells. In
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9 204 agreement with this, most drugs that have been withdrawn from the market or have received black
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11 205 box warnings due to liver injury are administered at daily doses greater than 50 mg per day (79, 80).
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13 206 However, it is difficult to argue susceptibility to drug hypersensitivity is solely dependent upon plasma
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15 207 drug concentrations or the drug concentration at the site of T-cell activation. The vast majority of
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17 208 patients tolerate therapeutics drug concentrations with little or no adverse effects. Thus, for the
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19 209 purpose of this review we argue that everyone taking medicinal drugs may be exposed to therapeutic
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21 210 concentrations that are capable of forming HLA drug peptide complexes and delivering them to T-
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23 211 cells.

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26 213 **Does the display of drug peptide complexes by human leukocyte antigen proteins impact on**
27 214 **susceptibility to hypersensitivity?**

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29 215 A plethora of studies, starting with abacavir discussed above, have identified astonishingly strong
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31 216 associations between HLA class I alleles and susceptibility to drug hypersensitivity reactions, which
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33 217 implies a direct effect of the gene product on the disease (81, 82) (Table 1 shows several HLA class I
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35 218 allele-associated drug hypersensitivity reactions with known drug peptide complex HLA binding
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37 219 interactions for T-cell activation). This suggests that mechanistically, restriction of the fit of the drug
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39 220 and peptide into HLA proteins is important for T-cell activation. HLA-B*57:01, which is associated with
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41 221 abacavir hypersensitivity, has a positive predictive value of 55 % and a negative predictive value of
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43 222 100 % (8). This means that only individuals carrying the allele are at risk and 1 out of 2 carriers develop
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45 223 hypersensitivity following abacavir exposure. Genetic screening prior to abacavir use is routine
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47 224 practice and eradicates the appearance of hypersensitivity. Other forms of HLA class I associated
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49 225 hypersensitivity (e.g., flucloxacillin [HLA-B*57:01] (83), allopurinol [HLA-B*58:01] (21), carbamazepine
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51 226 [HLA-B*15:02] (84) and dapsone [HLA-B*13:01] (85)) display similar negative predictive values (99-
52
53 227 100%) in specific patient groups; however, the positive predictive value is much lower. This suggests
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55 228 that the HLA allele is essential for drug peptide complex display, but other factors determine whether
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57 229 drug exposure results in a T-cell response and hypersensitivity. In a final group of HLA class I associated
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59 230 reactions (e.g., carbamazepine [HLA-A*31:01] (86), co-amoxiclav [HLA-A*02:01] (87),
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231 sulfamethoxazole [HLA-B*38:02] (88), minocycline [HLA-B*35:02] (89) and terbinafine [HLA-A*33:01]
232 (90)), the carrier frequency in hypersensitive patients is 50% or lower. Thus, in these reactions, the
233 drug-peptide complex is displayed by a number of different HLA proteins to activate T-cells. Additional

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3 234 forms of drug hypersensitivity are (i) linked to expression of HLA class II allele(s) or (ii) not known to
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5 235 be associated with expression of a specific HLA allele despite the fact that drug-specific CD4+ and CD8+
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7 236 T-cells are detectable. Importantly, it has not been possible to show that selective drug peptide
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9 237 complex binding to HLA class II proteins, identified as risk factors, leads to the activation of CD4+ T-
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11 238 cells (authors unpublished data).

12 239 Drug-peptide complex HLA protein binding is without doubt critical for the development of drug
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14 240 immunogenicity; however, from the above discussion it is clear that for most HLA allele associated
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16 241 reactions, expression of the HLA protein alone does not determine whether drug exposure will result
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18 242 in hypersensitivity.

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21 244 **Does expression of specific T-cell receptors impact on susceptibility to hypersensitivity?**

23 245 Advances in high-throughput sequencing technologies has enabled the detailed analysis of global T-
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25 246 cell repertoires in patients with and without immunological diseases. Glanville et al. (91) recently
26
27 247 defined the minimal requirements for T-cell receptor specificity through an analysis of T-cell receptor
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29 248 sequences using a panel of HLA binding peptides. Focussing on 5711 T-cell receptor V β chain
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31 249 sequences from CD4+ T-cells derived from 22 donors with mycobacterium tuberculosis, they identified
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33 250 141 T-cell receptor specificity groups including 16 groups containing T-cell receptors from at least 3-4
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35 251 individuals with shared alleles. The T-cell receptors shared HLA alleles from different donors for shared
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37 252 peptide presentation. These data indicate that a diverse array T-cell receptor sequences are available
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39 253 in any individual that interact with peptide ligands from a single protein antigen. Similar technologies
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41 254 should be applied to the study of drug hypersensitivity to explore whether shared drug peptide
42
43 255 complex specificity clusters are present across different donors and whether this correlates with
44
45 256 disease.

46 257 Our knowledge of how T-cell receptor sequences impact on drug hypersensitivity is in its infancy.
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48 258 Through global expression level analysis and assessment of the third complementary-determining
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50 259 region length distribution of the T-cell receptor profile in patients with carbamazepine-induced
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52 260 Stevens-Johnson syndrome, Ko et al. (92) identified VB-11-ISGSY as a dominant clonotype shared
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54 261 amongst different hypersensitive, but not drug-tolerant, donors. Furthermore, carbamazepine-
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56 262 specific cytotoxic T-cells could be primed from PBMC of healthy human donors that were carriers of
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58 263 both HLA-B*15:02 and VB-11-IsGSY. More recently, the same group working on the same patient
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60 264 cohort reported the detection of a public T-cell receptor composed of paired TCR α CDR3 "VFDNTDKLI"
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62 265 and TCR β CDR3 "ASSLAGELF" clonotypes and that similar receptor clusters are found in the blister
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64 266 fluid cells and peripheral blood (93). These data suggest that the correct combination of HLA, drug

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3 267 peptide complex and T-cell receptor may be important drivers for carbamazepine-induced Stevens-
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5 268 Johnson syndrome. Unpublished data analysing blister fluid from a different cohort of patients with
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7 269 Stevens Johnson syndrome after administration of multiple drugs also show an enrichment of T-cells
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9 270 that display a selective repertoire of T-cell receptor sequences at the most early phase of the adverse
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11 271 event (Vocanson, personal communication). However, the T-cell receptor identified differs across
12
13 272 patients, even those exposed to the same culprit drug. Moreover, a dominant clonotype was not
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15 273 detected in all patients.

16 274 The proposal that susceptibility to drug hypersensitivity relates to expression of a single T-cell
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18 275 clonotype contrasts with published literature showing the polyclonal expansion of T-cells by certain
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20 276 drugs. Abacavir, which interacts non-covalently with HLA-B*57:01, activates T-cells in 100% of human
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22 277 donors that carry the risk allele (even though only half develop hypersensitivity when exposed to
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24 278 abacavir) (94). Analysis of T-cell receptors expressed on abacavir-responsive T-cells did not reveal
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26 279 skewed patterns (9). This is consistent with abacavir activating an array of different T-cell receptors.
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28 280 Similarly, nitroso sulfamethoxazole, a cysteine-reactive metabolite of sulfamethoxazole has been
29
30 281 shown to prime naïve CD4+ and CD8+ T-cells from 59/60 healthy human donors (95, 96). Spectratyping
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32 282 revealed that nitroso-sulfamethoxazole-specific T-cell responses were controlled by public T-cell
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34 283 receptors present in all individuals alongside private T-cell repertoires specific to each individual (97).
35
36 284 Finally, elegant studies by Azoury et al. (98, 99) utilized immunodominant β -lactam-modified peptides
37
38 285 derived from albumin to calculate the frequency of naïve CD4+ T-cells that recognize the drug peptide
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40 286 complex. The haptened peptides were recognized by naïve T-cells from 13/14 human donors.

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42 287 These data, although utilizing a limited number of drugs, cover three forms of drug HLA binding
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44 288 derivative (parent drug, drug metabolite and haptened peptide) and show that PBMC from each
45
46 289 and every one of us contain naïve T-cells capable of recognizing and responding to drugs. Although
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48 290 certain HLA drug peptide complexes may associate preferentially with specific T-cell receptors and this
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50 291 may impact on the development of hypersensitivity: as has been described with HLA-B*15:02 and
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52 292 patients with carbamazepine-induced Stevens Johnson syndrome. It needs to be emphasized that the
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54 293 Caucasian population very rarely express HLA-B*15:02; they do however still develop carbamazepine
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56 294 hypersensitivity. The only explanation for this is that carbamazepine interacts with multiple HLA
57
58 295 proteins and T-cell receptors to bring about hypersensitivity reactions.

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60 297 To summarize the discussion thus far, most, if not all, drug-treated patients have a T-cell repertoire
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62 298 for drug peptide complexes and are exposed to drugs in sufficient quantities to activate the T-cells.
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64 299 Although expression of a specific HLA protein is important, for many forms of hypersensitivity, HLA

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3 300 risk allele expression *per se* does not predict the outcome of drug exposure. Therefore, for the
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5 301 remainder of this article we focus on the hypothesis that immune regulatory pathways are key
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7 302 determinants of whether drug exposure in genetically predisposed individuals will result in
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9 303 hypersensitivity. Figure 2 illustrates that drug exposure, expression of HLA alleles and T-cell receptors
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11 304 are all important determinants of immunogenicity, whereas regulatory pathways are determinants of
12
13 305 hypersensitivity. The pathways of drug-specific T-cell activation are also depicted with reference to
14
15 306 the possible different requirements for immune regulation.

16 307 While immune cells survey the tissue microenvironment for drug-derived signals, a key task is to
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18 308 maintain tissue homeostasis. The outcome of immune surveillance may be unresponsiveness (the
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20 309 immune system does not detect the drug-derived signal), a conventional effector response (leading
21
22 310 to hypersensitivity with a drug-derived signal) or tolerance (a state of immunological
23
24 311 unresponsiveness to the drug-derived signal). Tolerance can be natural or induced and these terms
25
26 312 are discussed in more detail below with reference to regulatory T-cells. In the context of drug
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28 313 hypersensitivity it is important to consider variation in natural tolerance and whether drug treatment
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30 314 actively induces or alters toleragenic pathways and indeed the potential for certain drug peptide
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32 315 complexes to bypass natural tolerance. The way the immune system regulates immune responses,
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34 316 and is able to adapt to change, is through the expression of an array of cell surface co-stimulatory and
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36 317 co-inhibitory signalling receptors (Figure 3). Co-stimulatory receptors collect information from
37
38 318 stressed or damaged cells and tissue and determine whether an effector response should be directed
39
40 319 towards an antigen. The co-inhibitory receptors act alongside regulatory T-cells (Tregs) and
41
42 320 stimulatory and inhibitory cytokines (e.g., IL-10, TGF- β) to preserve the regulatory environment to
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44 321 prevent unwanted immune responses against self and non-damaging agents and to prevent excessive
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46 322 responses to antigens when a T-cell response has been initiated. Factors that influence the balance
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48 323 between co-stimulatory and co-inhibitory signalling include the genetics of the host, disease and
49
50 324 environmental factors.

51 325 It is possible that each and every one of us may develop a hypersensitivity reaction following drug
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53 326 treatment if the balance between co-stimulation and co-inhibition is skewed at the time of exposure.
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55 327 This represents a frightening concept for Pharma and healthcare professionals, since the factors that
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57 328 control this balance are difficult to predict and will vary across individuals and within an individual
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59 329 when they are exposed to different immunomodulatory environments (e.g., infections or damaging
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330 agents). For this reason, although it might be possible to work towards a framework to predict the
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332 intrinsic immunogenicity of a drug, prediction of the number of individuals that will ultimately develop
a clinical drug hypersensitivity reaction is very difficult.

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5 334 **Clinical evidence to exclude drug exposure, the availability of a T-cell repertoire or a single genetic**
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7 335 **factor as key determinants that impact on susceptibility to drug hypersensitivity**
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9 336 We have worked together with respiratory physicians to understand the chemical and cellular basis
10 337 of β -lactam hypersensitivity in patients with cystic fibrosis. This patient population is an important
11 338 study group as they have been monitored closely throughout childhood and adult life and as such they
12 339 have almost complete drug histories as well as detailed records of the nature and timeframe of
13 340 hypersensitivity reactions that occur more frequently when compared to the general population (100-
14 341 102). Piperacillin is a commonly used β -lactam antibiotic for the treatment of recurrent respiratory
15 342 infections. Patients receive repeated courses of the drug at the same dose (12g/day; iv injection) and
16 343 duration (14 days). If one assumes that a patient receives 3 treatment courses a year, the overall mass
17 344 of piperacillin a patient will be exposed to over a 20 year period would exceed 10kg. Thirty five percent
18 345 of patients with cystic fibrosis develop delayed-type piperacillin hypersensitivity reactions
19 346 characterized clinically with maculopapular or urticarial rashes, fever and arthralgia (100). Drug-
20 347 responsive T-cells are detected in approximately 75% of hypersensitive patients, but not tolerant
21 348 controls using the lymphocyte transformation test (60). Moreover, CD4+ and CD8+ T-cells that secrete
22 349 proinflammatory cytokines, including IL-22 and cytolytic molecules, when exposed to piperacillin are
23 350 present in inflamed skin (2). Drug-responsive T-cells are also detectable in drug tolerant patients
24 351 (unpublished data) and drug-naïve donors (2, 96), but only when immune regulation has been
25 352 perturbed *ex vivo* and the drug peptide adduct is presented by dendritic cells pre-treated with LPS to
26 353 provide co-stimulation.

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40 354 The mean time to onset of piperacillin hypersensitivity is the ninth day of the ninth treatment course
41 355 (i.e., the average patient will tolerate eight separate courses of piperacillin), which might lead one to
42 356 assume that susceptibility is linked to accumulation of, or repeated exposure to, the drug peptide
43 357 complex. However, over a 20 year assessment period at the St. James Cystic Fibrosis Unit (Leeds, UK)
44 358 patients have been diagnosed with hypersensitivity after every treatment course (1-15; personnel
45 359 communication, Dr Paul Whitaker). These clinical data are impossible to rationalize in terms of drug
46 360 exposure/accumulation, the availability of a T-cell repertoire for the drug peptide complex or indeed
47 361 a single genetic factor such as HLA.

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54 362 As depicted in figure 2, the pathway of T-cell activation for drugs such as allopurinol and
55 363 carbamazepine are very different to that of β -lactam antibiotics. It is possible that reactions with these
56 364 drugs occur after T-cell responses develop in the presence of other classical peptide antigens (i.e., the
57 365 drug peptide complex cross-reacts with the peptide antigen). In this case, the drug will not always

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3 366 activate a *de novo* response for hypersensitivity to develop and the regulatory requirements for
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5 367 activation will be lower. The caveat to this argument however is that both of these drugs have been
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7 368 shown to prime naïve T-cells using autologous dendritic cells to present the drug peptide complex in
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9 369 an appropriate immunological form (103).

10 370 **The immune regulatory network**

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13 371 Several mechanisms have evolved to regulate T-cell responses and prevent the development of
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15 372 autoimmune disease and other inflammatory conditions. The best known mechanisms of peripheral
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17 373 tolerance include thymic selection of T-cells, the suppressive activity of Tregs (104) and the increased
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19 374 expression of cell surface receptors, the so-called immune checkpoints (105, 106). The importance of
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21 375 immune regulation and power of the regulatory network has been demonstrated clinically through
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23 376 the application of immune checkpoint inhibitors for the treatment of cancer (107). Furthermore,
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25 377 mutations in FOXP3, the regulatory transcription factor for Tregs, results in dysfunctional Tregs and
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27 378 the development of autoimmune disease and allergy (108). IPEX syndrome – a loss of function
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29 379 mutation in FOXP3 (and other regulatory pathways such as CTLA4) - is the most extreme clinical
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31 380 scenario. IPEX syndrome is often fatal presenting clinically for a variety of autoimmune-like
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33 381 syndromes. It would be interesting to investigate whether patients with IPEX syndrome also develop
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35 382 more drug hypersensitivity reactions. Tregs are now easy to expand *ex vivo* and have been used in
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37 383 Phase I clinical trials for the treatment of autoimmune disease to prevent transplant rejection (109).
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39 384 In the following sections we briefly discuss the major immune regulatory pathways and how
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41 385 dysregulation of these pathways may impact on drug hypersensitivity.

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41 387 **Immune checkpoints**

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43 388 Immune checkpoints are a series of receptor ligand interactions between T-cells and antigen
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45 389 presenting/tissue cells which specifically co-ordinate the secondary co-stimulatory signal required for
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47 390 immune activity following TCR binding. Checkpoint proteins negatively regulate the activation of naïve
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49 391 T-cells. Furthermore, checkpoint receptor expression is upregulated on T-cells when they are
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51 392 activated, providing a negative feedback loop to restrict the effector response. PD-1 and CTLA-4, which
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53 393 are expressed on T-cells, are the most studied immune checkpoints. PD-1 interacts with ligands PD-L1
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55 394 and PD-L2, which activates tyrosine phosphatases that inactivate tyrosine kinase-mediated activation
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57 395 signals (110). CTLA-4 binds to ligands CD80 and CD86 on antigen presenting cells displaying antigen.
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59 396 T-cell inhibition is achieved through competitive antagonism of CD28 signalling and direct delivery of
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397 an intracellular signal (111). Other less well characterized immune checkpoints include TIM-3

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3 398 (suppresses Th1/Th17 CD4+ responses (112)) and LAG-3 (contributes towards Treg activity and directly
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5 399 suppresses CD8+ T-cells (113)). The complex interaction between immune checkpoints and naïve and
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7 400 memory T-cell subsets and how intra- and inter-individual variation impacts on susceptibility to
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9 401 adverse immunological reactions is ill-defined.

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11 402 In recent years, we have investigated whether receptor blockade with immune checkpoint inhibitors
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13 403 remove the immune brakes and enhance the priming of naïve T-cells by drugs. Naïve T-cells were
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15 404 cultured *in vitro* with drug and autologous dendritic cells in the presence and absence of immune
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17 405 checkpoint inhibitors targeting PD-1, CTLA-4 and Tim-3 for 14 days to allow priming to occur. Drug
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19 406 exposure was associated with an increase in expression of all three immune checkpoints on dividing
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21 407 T-cells during the culture period, presumably a regulatory event to keep the drug-specific response in
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23 408 check (114). After the 14 day culture period, the primed T-cells were restimulated with drug and a
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25 409 second batch of autologous dendritic cells and the strength of the T-cell response was assessed. PD-1
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27 410 and CTLA-4 block enhanced the priming of naïve T-cells to drugs, whereas Tim-3 block had no effect
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29 411 (97, 114). A similar effect (enhanced priming of naïve T-cells to drugs) has been demonstrated *in vivo*
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31 412 with PBMC from patients receiving immune checkpoint inhibitor therapy (unpublished data).
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33 413 Furthermore, it is becoming apparent that patients receiving immune checkpoint inhibitor therapy
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35 414 develop more frequent drug hypersensitivity reactions. Ford et al. (115) recently described the
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37 415 development of sulfasalazine (a combination of salicylic acid and sulfapyridine)-induced cutaneous
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39 416 hypersensitivity in 4 patients with metastatic melanoma that had previous been treated with the anti
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41 417 PD-1 inhibitor pembrolizumab or the anti CTLA-4 inhibitor ipilimumab. Presumably the T-cell response
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43 418 and subsequent hypersensitivity reaction was induced by the sulfonamide component of sulfasalazine
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45 419 when natural immune checkpoints had been suppressed. Phillips et al. have recently reported on the
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47 420 treatment outcomes of 285 patients that developed cutaneous adverse events attributed to immune
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49 421 checkpoint inhibitor therapy (116). It would be interesting to consider the number of these patients
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51 422 receiving concomitant therapy with low molecular weight drugs.

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53 423 A report of the post-approval safety of the B-raf inhibitor vemurafenib described seven patients that
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55 424 developed serious cutaneous hypersensitivity reactions and importantly, six of these patients received
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57 425 anti-PD-1 antibody therapy prior to starting vemurafenib (117). Phase II studies of ipilimumab plus or
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59 426 minus dacarbazine therapy concluded that ipilimumab monotherapy had a manageable adverse
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427 events profile (118), while dual therapy provided no improvement in efficacy and was not tolerable
428 due to serious liver injury (119). Dacarbazine use alone is only associated with rare cases of liver injury
429 (120). The immune checkpoint inhibitor again seems to alter the co-stimulatory/co-inhibitory balance,
430 permitting the development of dacarbazine-induced liver injury in almost all treated patients. Finally,
431 it has been reported that polymorphisms in regulatory targets of immune responses such as CTLA-4

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3 432 and IL-10 could modulate susceptibility to nonsteroidal anti-inflammatory drug (121) and efavirenz
4 433 (122) hypersensitivity reactions. Collectively, these data indicate that immune checkpoints act to
5 434 regulate the strength of the drug-specific T-cell response and hence impact on the balance between
6 435 tolerance and hypersensitivity (Figure 4). These interactions will become increasingly relevant as the
7 436 focus on combination therapies for the treatment of various malignancies increases. Combination
8 437 therapies in oncology started by using two checkpoint inhibitors in combination (α CTLa-4/ α PD-1)
9 438 which illustrated increased efficacy but also an increased incidence of toxicity with a severe toxicity
10 439 incidence of 56% of patients (123). Latterly there have been an increasing number of trials combining
11 440 checkpoint inhibitors with additional systemic anticancer therapies including chemotherapy
12 441 (KEYNOTE189 [ClinicalTrials.gov number, NCT02578680], IMpassion150 [ClinicalTrials.gov
13 442 number, NCT03125902]) and tyrosine kinase inhibitors (KEYNOTE426 [ClinicalTrials.gov
14 443 number, NCT02853331]). This has culminated in the use of all three agents in some anticancer regimes
15 444 eg atezolizumab, bevacizumab, carboplatin and paclitaxel used in combination for the treatment of
16 445 non-small cell lung cancer (NSCLC) within IMPower150 (ClinicalTrials.gov number, NCT02366143).
17 446 Given the propensity for immune checkpoint inhibitors to interact and display phenotypically typical
18 447 hypersensitivity reactions the ability to predict individuals at risk of hypersensitivity or particular drug
19 448 combinations which carry an increased risk is increasingly important. It also remains to be seen if there
20 449 is a characterizable dose-toxicity relationship or whether there is a temporal relationship to
21 450 hypersensitivity. It is known that as monoclonal antibodies, immune checkpoint inhibitors have long
22 451 half-lives (6.1-25 days) (124) and receptor occupancy exists for weeks. However it is currently unclear
23 452 if there is a dynamic relationship with hypersensitivity and the duration of risk.

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39 453 Tim-3 is an immune checkpoint receptor that interacts with its ligand galectin 9 to modulate Th1 CD4+
40 454 T-cell responses. The expression of Tim-3 has recently been shown to be significantly reduced on
41 455 peripheral blood CD4+ T-cells in the acute phase of drug-induced maculopapular exanthema (125),
42 456 a classical Th1-mediated iatrogenic disease. Furthermore, galectin 9 expression and release was
43 457 reduced on dendritic cells. These data indicate that the Tim-3 immune checkpoint also contributes to
44 458 the maintenance of drug tolerance and the prevention of hypersensitivity reactions.

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49 459 Contact allergy is a CD8+ T-cell mediated delayed-type hypersensitivity reaction brought about by low
50 460 molecular weight haptens. Unlike drug hypersensitivity, where for the most part murine models do
51 461 not exist, contact allergy can be reproduced easily in mice through direct application of the hapten to
52 462 skin. In recent years, contact allergy has been used to explore how Toll-like receptors, the
53 463 inflammasome and endogenous danger signals impact on the hapten specific CD8+ T-cell response
54 464 and skin inflammation (126-130). Most recently, Gamradt et al., (131) discovered that intrinsic control
55 465 mechanisms such as immune regulatory (PD-1 and TIM-3) signalling determine whether the cytotoxic

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3 466 CD8+ T-cells will be reactivated and hence prevent tissue injury. Blocking of immune checkpoints *in*
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5 467 *vivo* lead to severe contact hypersensitivity responses with low hapten doses.

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7 468 Immune checkpoint blockade has been used in mice to attempt to develop animal models of drug-
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9 469 induced liver injury with a delayed onset (132-135). Treatment of mice with therapeutic doses of
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11 470 human liver injury inducing compounds such as amodiaquine, isoniazid and nevirapine did not result
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13 471 in significant tissue damage. However, when the drugs were administered in the presence of PD-1 and
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15 472 CTLA-4 block, mild, but significant, delayed onset liver injury was observed. Liver injury was associated
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17 473 with hepatic recruitment of immune cells including CD8+ T-cells, suggesting that they participate in
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19 474 the pathogenesis. Although this work represents an important step forward – an *in vivo* model is now
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21 475 available to begin to study drug-induced delayed-typed liver injury - additional studies are required to
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23 476 determine why the liver injury does not progress to the serious forms of tissue damage seen in human
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25 477 patients.

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27 478 From the above discussion one can begin to visualize how immune checkpoint signalling impacts on
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29 479 the co-regulatory/co-stimulatory network that determines whether an effector response will ensue
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31 480 following antigen exposure as well as the strength and duration of the response. As one pathway is
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33 481 blocked other pathways exert an increased influence in an attempt to maintain tolerance. As we move
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35 482 forward combined immune checkpoint therapy will become more commonplace. This will result in an
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37 483 increase in serious autoimmune side effects. However, it is highly likely that drug hypersensitivity
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39 484 reactions will also become more prevalent.

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43 486 **Regulatory T-cells (Tregs)**

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45 487 Tregs regulate or suppress other cells in the immune system. They control the immune response to
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47 488 self and foreign antigens and help prevent autoimmune disease and allergy. Natural Tregs are
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49 489 identified by expression of the regulatory transcription factor FOXP3. Natural Tregs express CD4+ and
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51 490 CD25+ (136); however, CD25+ is also expressed on other forms of T-cell including activated T-cells.
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53 491 Thus, there was a search for additional classification markers. CD127+ has been identified as a marker
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55 492 that is only expressed at low levels on Tregs and can be used alongside CD4+, CD25+ and FOXP3 to
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57 493 identify natural Tregs (137, 138). Tregs can also be classified according to the expression of a naïve T-
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59 494 cell marker CD45RA (139). CD45RA+FOXP3^{low}CD4+ (CTLA-4^{low}, CD25^{high}, CD127^{low}) cells are referred to
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495 as naïve or inducible Tregs. These cells exhibit weak suppressive activity until they differentiate
496 following antigen-mediated T-cell receptor engagement. They differentiate into effector Tregs
497 (CD45RA-FOXP3^{high}CD4+) that display a range of additional markers including CTLA-4, CD25+, PD-1,

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3 498 TIM-3 and secretory molecules such as IL-10 and TGF- β . These cells display a strong inhibitory activity
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5 499 and increase in number in blood with age. Tregs exert their suppressive function through a range of
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7 500 pathways ((139-141) Figure 5). These include the inhibition of antigen presenting cells through
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9 501 expression of immune checkpoint receptors, the release of cytokines such as IL-10 and TGF- β that
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11 502 decrease dendritic cell function and the production of pro-inflammatory cytokines and restriction of
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13 503 IL-2 for effector T-cells through CD25+ ligation.

14 504 A plethora of studies have shown that FOXP3+ Tregs suppress hypersensitivity reactions to chemical
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16 505 contact allergens in mice by blocking effector CD8+ T-cell responses (142-144). Gomez de Agüero et
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18 506 al (145) reported that Langerhans cells (cutaneous dendritic cells) are critical in the regulatory process
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20 507 through inducing the depletion of antigen-responsive T-cells and by activating FOXP3+ Tregs.
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22 508 Furthermore, *in vivo* expansion of Treg populations has been shown to induce long-term suppression
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24 509 of contact hypersensitivity (146). In humans, Cavani et al (147) have reported that CD25+ regulatory
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26 510 T-cells maintain tolerance to the contact metal allergen nickel in non-hypersensitive individuals. T-
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28 511 cells showed a limited capacity to proliferate in the presence of nickel *ex vivo*. However, T-cell
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30 512 activation was strongly increased when Tregs were depleted from the PBMC population. Collectively,
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32 513 the data generated showed that Tregs blocked the efficient activation of naïve and memory nickel-
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34 514 specific T-cells. It will be interesting to see whether similar pathways (possibly when Tregs are
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36 515 depleted alongside checkpoint inhibition) are active in drug tolerant patients.

37 516 In *in vitro* T-cell priming assays with PBMC from healthy human donors, the depletion of FOXP3+ Tregs
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39 517 is important to detect CD4+ and CD8+ T-cell responses to drugs and haptenic chemicals (95, 148, 149).
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41 518 The reintroduction of Tregs to naïve T-cell priming assays block the activation of naïve T-cells by drugs
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43 519 in a cell concentration-dependent manner (114). Inducible effector Tregs (presumably drug peptide
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45 520 complex-responsive) are generated *in vitro* alongside effector CD4+ and CD8+ T-cells during the
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47 521 priming of naïve T-cells (unpublished data), further emphasizing their importance at regulating drug-
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49 522 specific immune responses. There is a potential for environmental and genetic factors to modulate
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51 523 the expression and activity of Tregs. For example, polymorphic variants of FOXP3 have been linked to
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53 524 various forms of autoimmune disease, while exposure to air pollution can methylate the FOXP3 locus,
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55 525 compromising Treg function (150-153). Thus, Tregs might be important in maintaining an effective
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57 526 level of tolerance in all drug-exposed patients.

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59 527 Little is known about the influence of Tregs and Treg dysregulation in the acute phase of a drug
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528 hypersensitivity reaction. In patients with toxic epidermal necrolysis, the most severe form of
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530 529 blistering skin eruption, Takahashi *et al* described a functional impairment of Tregs and a reduced
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531 530 capacity to suppress effector T-cell responses to drugs (154, 155). However, the key mechanisms

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3 531 implicit in Treg dysregulation were not defined. Recently, Wang et al. demonstrated that treatment
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5 532 with a TNF- α antagonist reduced skin healing time in patients with severe forms of toxic epidermal
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7 533 necrolysis (156). Drug treatment decreased TNF- α and granulysin levels in blister fluid and significantly
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9 534 increased Treg proportions in patients during the recovery phase. In patients with a different form of
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11 535 severe cutaneous hypersensitivity reaction, DRESS, CD14+ monocytes have been shown to mediate a
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13 536 gradual shift from a Treg to a Th17 phenotype during the course of the disease (157). In an
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15 537 independent study, lesional skin of patients with DRESS was found to be rich in FOXP3+ cells and the
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17 538 increase in Tregs positively correlated with the number of recorded days from the onset of the disease
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19 539 (158). Similarly, Hanafusa et al, found a switch in the population of dividing cells from CD8+ to FOXP3+
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21 540 Tregs in drug-treated PBMC from a patient with DRESS (159). Collectively, these data indicate that the
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23 541 Tregs are being activated and recruited to inflamed skin to attempt to control the strength and
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25 542 duration of the drug-specific effector T-cell response. Thus, it is important to develop strategies to
26
27 543 understand the role Tregs play in determining the outcome of drug exposure in patients.

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29 544 Recently, breaking tolerance through depletion of murine CD4+ T-cells was found to result in the
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31 545 development of abacavir hypersensitivity in a HLA-B*57:01 transgenic model (160). Abacavir exposure
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33 546 *per se* induced a CD8+ T-cell response; however, the mice maintained an anergic disease state. An
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35 547 adverse reaction in skin was only detected when CD4+ T-cells, which included Tregs, were depleted.
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37 548 The epidermis became heavily infiltrated with CD8+ T-cells and skin showed typical signs of tissue
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39 549 injury. The authors demonstrated through a series of detailed experiments that CD4+ T-cell depletion
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41 550 resulted in optimal dendritic cell co-stimulation and a break in regulation, predisposing the mice to
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43 551 tissue injury.

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46 47 553 **Cytokines**

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49 554 During T-cell priming, naïve CD4+ T-cells differentiate into one of several lineages, including Th1, Th2,
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51 555 Th17, Th22 and induced Tregs. Each T-cell population is characterized by the cytokines they secrete
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53 556 when activated. Importantly, the cytokine microenvironment during T-cell receptor triggering controls
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55 557 T-cell differentiation (Figure 6). The impact of the cytokine microenvironment on T-cell polarization
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57 558 can be demonstrated experimentally by culturing purified human T-cells with relevant cytokine
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59 559 cocktails (Th1, IL-12 & anti-IL-4; Th2, IL-4, anti-IL-12 & anti-IFN- γ ; Th17, IL-1 β , IL-6, IL-23 & TGF- β ; Th22,
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61 560 TNF- α & IL-6) for 7 days prior to non-specific mitogen stimulation. Activated T-cells secrete the
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63 561 polarized cytokines illustrated in Figure 6. The activation of CD8+ T-cells is also influenced by cytokines.
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65 562 In the absence of specific cytokine signals, CD8+ T-cells become anergic and unresponsive to antigen
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3 563 stimulation. The dominant cytokines that promote CD8+ T-cell activation are IL-12 and IFN- α/β (161,
4 564 162).

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7 565 There are many examples of disease induced cytokine imbalance (163-165) and this could have a
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9 566 major impact on the outcome of drug exposure. Diseases such as HIV and cystic fibrosis predispose
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11 567 individuals to drug hypersensitivity reactions. In patients with HIV the incidence of sulfonamide
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13 568 hypersensitivity is 10 times higher when compared with non-HIV infected patients (166). Cytokine
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15 569 imbalances such as Th1/Th2 switching are common features in patients with HIV as the disease
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17 570 progresses (167), but to date the impact of these changes on susceptibility to drug hypersensitivity
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19 571 has not been studied. Similarly, when patients with cystic fibrosis were compared to the general
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21 572 population, antibiotic reactions were found to be up to three times more common (100). The cystic
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23 573 fibrosis lung represents an area of chronic inflammation with high neutrophil numbers alongside
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25 574 elevated levels of cytokines such as IL-8, IL-1 β , IL-6, IL-17 and TNF- α (168-170). Obviously, this will
26
27 575 have a colossal impact on the outcome of T-cell receptor triggering through altered antigen
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29 576 presentation as well as differential polarization of the effector T-cell response. However, to date, it
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31 577 has not been possible to establish models/systems to explore this relationship directly.

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32 579 **Conclusions**

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34 580 It is becoming increasingly apparent that multiple tolerance pathways determine the outcome of
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36 581 antigen exposure through regulation of (i) naïve T-cell activation and (ii) the strength and duration of
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38 582 the effector T-cell response. Through the studies discussed herein we are beginning to understand
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40 583 that similar pathways are active in patients at the time of drug exposure and that immune regulation
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42 584 networks contribute towards the outcome of drug exposure: health benefit or a hypersensitivity
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44 585 reaction. Work is required to define how the distinct pathways contribute towards individual
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46 586 susceptibility. Such studies are urgent given the plethora of immune modulatory drugs that are in
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48 587 development, which once approved will be administered alongside traditional low molecular weight
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50 588 drugs. It will also be important to determine whether low molecular weight drugs modulate tolerance
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52 589 pathways in patients and whether this contributes to the successful desensitization of certain
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54 590 hypersensitive patients.

591 **Tables**592 **Table 1. HLA class I allele-associated drug hypersensitivity reactions with known drug HLA binding**
593 **interactions for T-cell activation**

Reaction phenotype	HLA allele	Known HLA (peptide) interaction ^a	Evidence of bioactivation ^b
Abacavir hypersensitivity	HLA-B*57:01 (7)	Direct non-covalent binding (9, 171)	Yes, aldehyde (172)
Allopurinol severe skin reactions	HLA-B*58:01 (21)	Direct labile metabolite binding (77)	No
Carbamazepine Stevens Johnson syndrome	HLA-B*15:02 (84)	Direct labile drug & metabolite binding (173, 174)	Yes, multiple metabolites (175, 176)
Carbamazepine skin reactions	HLA-A*31:01 (86)	Direct labile drug & metabolite binding (173, 174)	Yes, multiple metabolites (175, 176)
Dapsone drug reaction with eosinophilia and systemic symptoms	HLA-B*13:01 (85)	Direct labile & metabolite covalent binding (17, 177)	Yes, nitroso metabolite (178, 179)
Flucloxacillin liver injury	HLA-B*57:01 (83)	Direct labile & covalent binding (43, 55)	Not applicable (47)
Sulfamethoxazole skin reactions	HLA-B*38:02 (88)	Direct labile & metabolite covalent binding (4, 71, 72)	Yes, nitroso metabolite (180)
Co-amoxiclav liver injury	HLA-A*02:01 (87)	Direct covalent binding (42)	Not applicable (48)
Minocycline liver injury	HLA-B*35:02 (89)	Unknown	Yes, quinone iminium ion (181)
Terbinafine liver injury	HLA-A*33:01 (90)	Unknown	Yes, aldehyde metabolite (182)
Ticlopidine liver injury	HLA-A*33:03 (183)	Direct labile binding (184)	Yes, sulfenic acid (185)
Vancomycin drug reaction with eosinophilia and systemic symptoms	HLA-A*32:01 (186)	Unknown	No

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595 ^aalternative pathways feasible for all compounds, but to date have not been studied596 ^bformation of a metabolite does not indicate that they are involved in the reaction

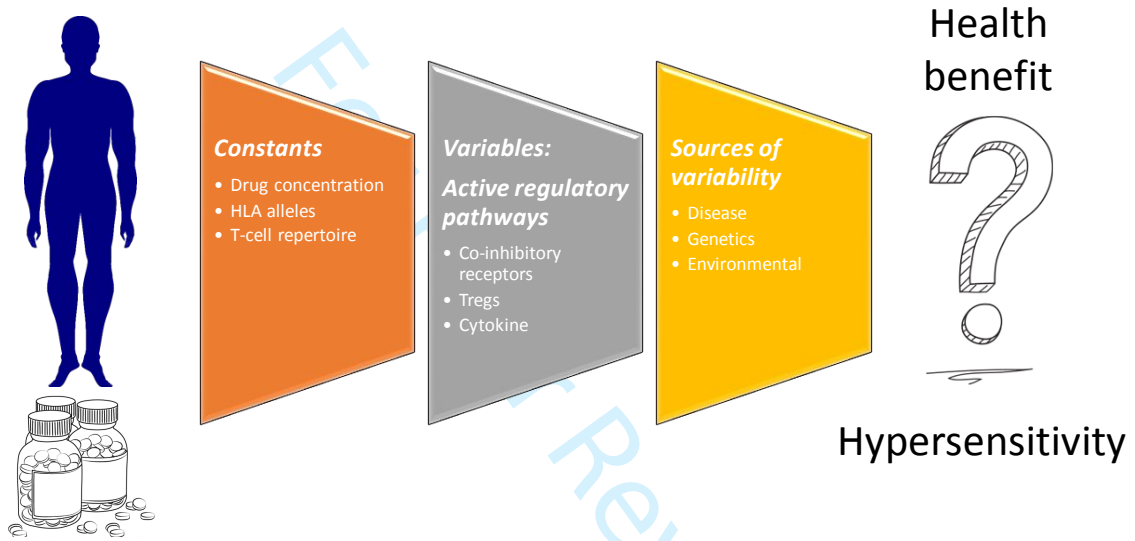
597 **Figures**

598 **Figure 1.** The drug hypersensitivity susceptibility conundrum proposes that if we assume exposure to
599 a drug and the availability of HLA proteins and T-cell receptors for drug peptide complexes are kept as
600 constants, then active immune regulatory pathways are the primary determinants of susceptibility.

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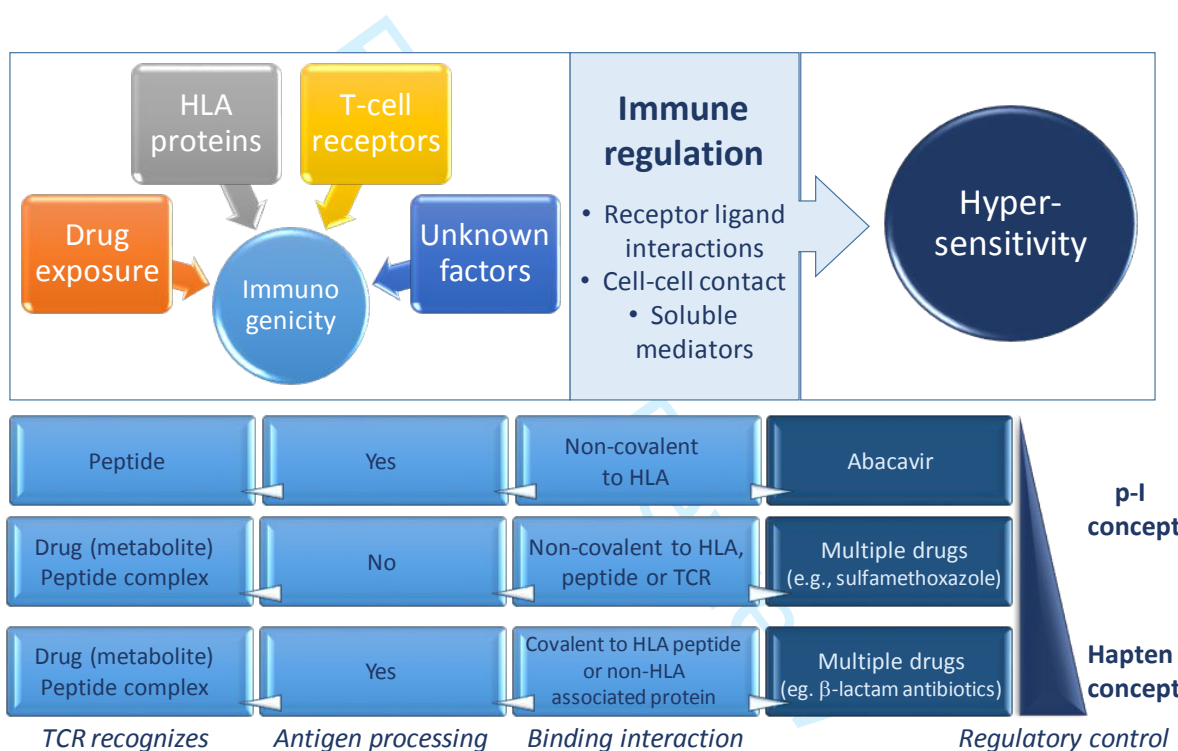
THE DRUG HYPERSENSITIVITY SUSCEPTIBILITY CONUNDRUM



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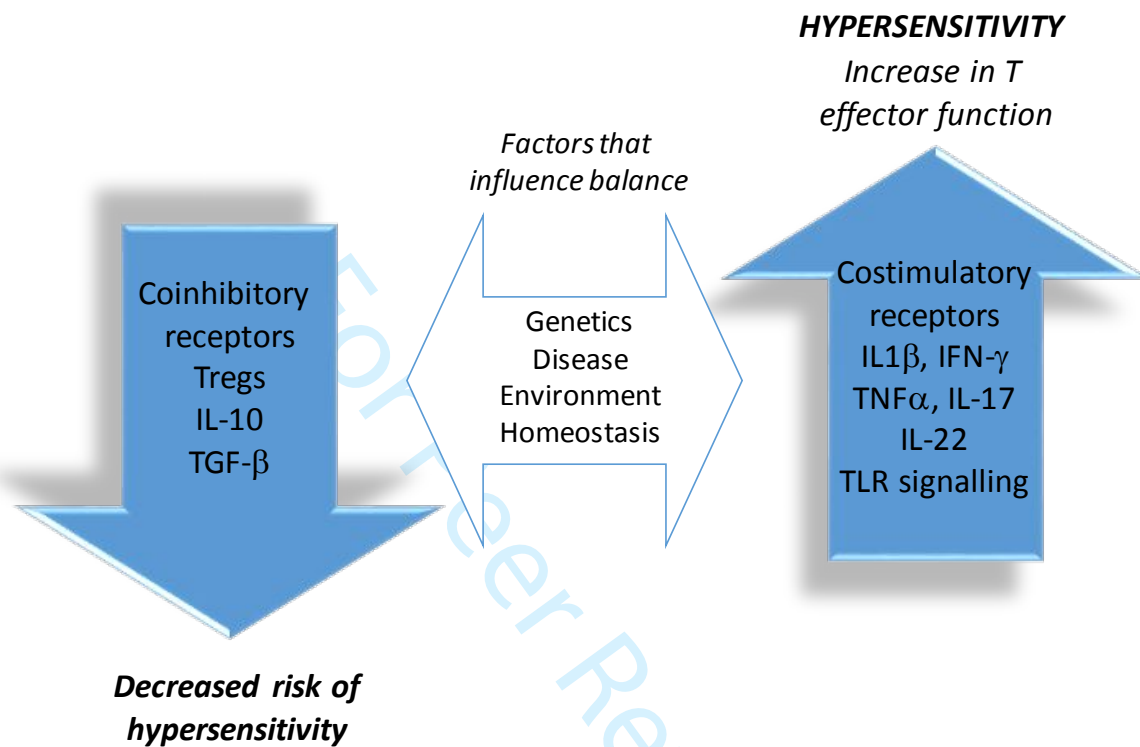
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3 **Figure 2.** The influence of drug- and patient-specific factors on drug immunogenicity and
4 hypersensitivity. Drug exposure and the availability of HLA proteins and T-cell receptors for drug
5 binding are essential for immunogenicity. However, these factors either together or in isolation do not
6 predict whether a patient will develop hypersensitivity. This is because immune regulatory pathways
7 control whether a pathogenic immune response will develop. These pathways may influence p-I and
8 hapten responses to different extents although this is yet to be proven even in the case of abacavir.
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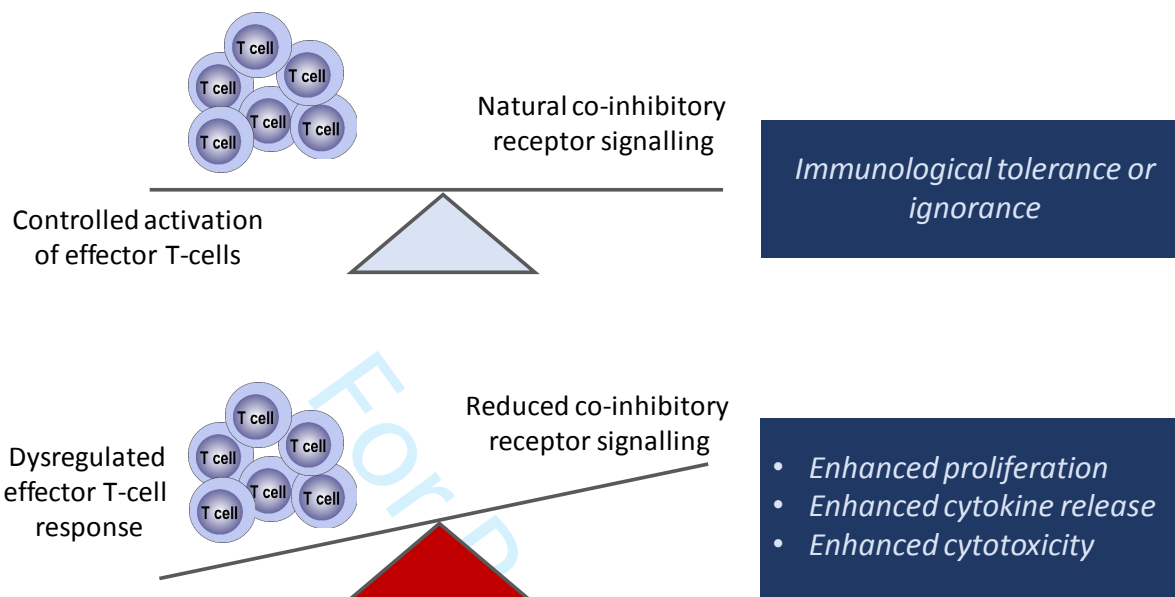
619 **Figure 3.** The balance between co-stimulatory and co-inhibitory pathways are the key determinant of
 620 whether drug exposure will result in hypersensitivity. This balance is influenced by genetic, disease
 621 and environmental factors. Thus, the balance will differ across individuals and within the same
 622 individual with time.

623



624 **Figure 4.** Co-inhibitory receptors regulate the strength of the drug-specific T-cell response and hence
 625 the balance between tolerance and hypersensitivity.

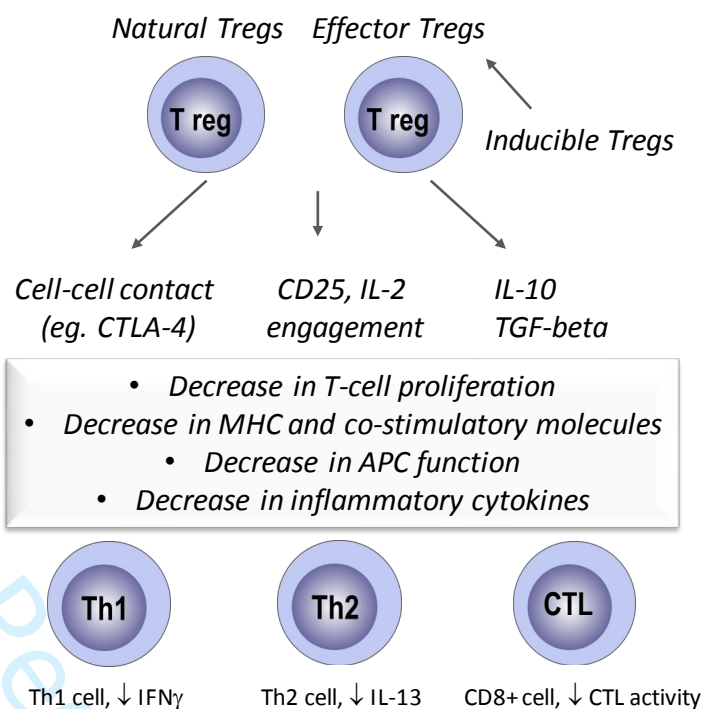
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628 **Figure 5.** Tregs regulate the strength of antigen-specific effector T-cell responses and hence may alter
 629 the balance between tolerance and hypersensitivity following drug exposure.

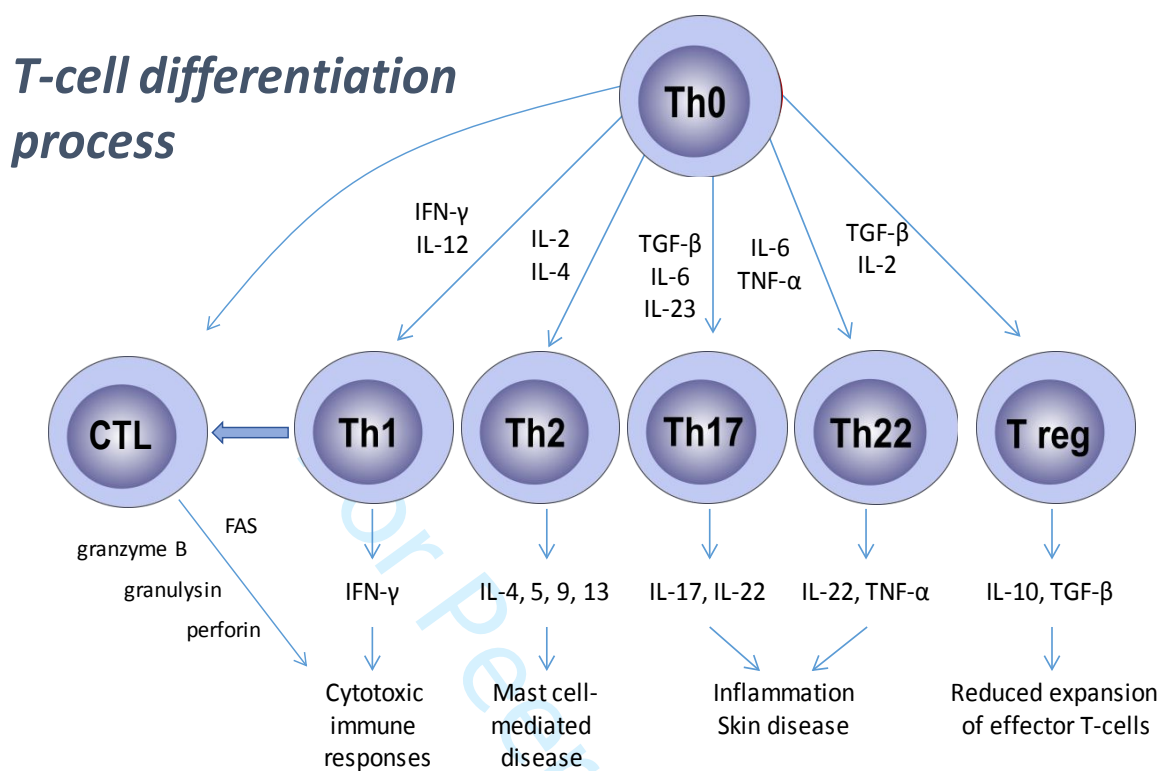
Tregs:
 Tregs produced in the thymus are termed **natural**
 Treg formed by differentiation of naïve T cells outside the thymus are called **adaptive or inducible**
 Exert function through
 • Cell contact
 • Cytokine secretion
 • Apoptosis of effector cells
 • Modulation of DC function
 Do they play a role in regulating drug hypersensitivity?



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631 **Figure 6.** Cytokine control of T-cell differentiation.

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3 636 **Text box 1. Major Milestone Discoveries**
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- 5 637
- 6 638 • Drug, drug metabolite and drug-modified peptide HLA binding activates T-cells in patients with hypersensitivity
 - 7 639 • Development of assays with PBMC from healthy human donors to study naïve drug peptide complex T-cell priming *ex vivo*
 - 8 640
 - 9 641 • Individual HLA alleles are important determinants of disease susceptibility
 - 10 642 • Characterisation of HLA-allele-restricted drug-specific T-cell responses in patients with drug hypersensitivity.
 - 11 643
 - 12 644 • Co-inhibitory receptors impact on the ability of drug peptide complexes to activate naïve T-cells
 - 13 645
 - 14 646 • Discovery of an increased incidence of drug hypersensitivity reactions in patients receiving immune checkpoint inhibitor therapy
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26 649 **Text Box 2. Future Research Perspectives**
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29 650 Genome-wide association studies and functional assessment of patient T-cells have taught us that
30 651 drug peptide complexes interact selectively and specificity with HLA proteins to bring about
31 652 hypersensitivity reactions. It is now important to define, through detailed structural analysis, the way
32 653 in which drug peptide complexes bind to HLA proteins. The nature of the interaction will differ drug-
33 654 to-drug. It is also important to determine the contribution different forms of drug peptide complex
34 655 play in the disease pathogenesis as we know that parent drug, metabolite and drug-modified peptide-
35 656 responsive T-cells circulate in patients' blood and tissues.

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41 657 Of particular importance, is identification of the parameters that that influence susceptibility in
42 658 patients expressing known HLA risk alleles. Ongoing studies seem to suggest that drugs stimulate a
43 659 very restricted repertoire of T-cells in patients with Stevens Johnson syndrome. Might this be the case
44 660 in other forms of drug hypersensitivity? The balance between co-stimulatory and co-inhibitory
45 661 signalling during drug peptide complex-specific T-cell priming is also an important determinant of
46 662 susceptibility. Future research must focus on patients at the earliest stages of a reaction to delineate
47 663 the contribution individual pathways (e.g, receptor signalling, Tregs, cytokines) in play in
48 664 determination of the outcome of drug exposure. In this respect, important lessons will be learned
49 665 from patients receiving immune checkpoint inhibitor therapy for cancer treatment.

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3 1 **Immune dysregulation increases the incidence of delayed-type drug hypersensitivity reactions**
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8 3 **Short title:** Regulatory pathways and drug hypersensitivity
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27 12 **Conflict of Interest Statement:** The authors declare no conflicts of interest.
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34 writing the review and assessment of the final article. Each author agrees to be accountable for all
35 16 aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the
36 17 work are appropriately investigated and resolved.
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4 19 **Abstract:** Delayed-type, T-cell mediated, drug hypersensitivity reactions are a serious unwanted
5 20 manifestation of drug exposure that develops in a small percentage of the human population. Drugs
6 21 and drug metabolites are known to interact directly and indirectly (through irreversible protein
7 22 binding and processing to the derived adducts) with HLA proteins that present the drug-peptide
8 23 complex to T-cells. Multiple forms of drug hypersensitivity are strongly linked to expression of a single
9 24 HLA allele and there is increasing evidence that drugs and peptides interact selectively with the protein
10 25 encoded by the HLA allele. Despite this, many individuals expressing HLA risk alleles do not develop
11 26 hypersensitivity when exposed to culprit drugs suggesting a non-linear, multifactorial relationship in
12 27 which HLA risk alleles are one factor. This has prompted a search for additional susceptibility factors.
13 28 Herein, we argue that immune regulatory pathways are one key determinant of susceptibility. As
14 29 expression and activity of these pathways is influenced by disease, environmental and patient factors,
15 30 it is currently impossible to predict whether drug exposure will result in a health benefit,
16 31 hypersensitivity or both. Thus, a concerted effort is required to investigate how immune dysregulation
17 32 influences susceptibility towards drug hypersensitivity.
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35 Introduction

36 Drug hypersensitivity refers to objectively reproducible symptoms or signs initiated by exposure to a
37 drug at a dose normally tolerated by non-hypersensitive persons (1). Hypersensitivity is also
38 commonly referred to as a form of off-target toxicity, which means that the development of tissue
39 injury is not predictable from known pharmacology of the drug and there is no simple association
40 between the dose of the drug administered and the development of clinical signs and symptoms.
41 Delayed-type reactions vary in severity and can target individual organs such as liver and skin in
42 isolation or as part of a generalized hypersensitivity syndrome. Common to the cellular
43 pathophysiology of drug hypersensitivity is the presence of drug-specific T-lymphocytes in blood and
44 inflamed tissue (2-4). In fact, cutaneous hypersensitivity reactions (maculopapular, pustular, and
45 bullous) are classified according to the effector molecules secreted by T-cells when activated with drugs
46 (5, 6).

47 In 2002, Mallal et al. reported a strong association between the presence of HLA-B*57:01, HLA-DR7,
48 and HLA-DQ3 and hypersensitivity to the HIV-1 reverse-transcriptase inhibitor abacavir (7).
49 Subsequent studies demonstrated that (i) all skin test confirmed cases of abacavir hypersensitivity
50 carry HLA-B*57:01 (8), (ii) abacavir interacts selectively with high affinity within the HLA-B*57:01
51 peptide binding cleft through non-covalent interactions (9-11), and (iii) abacavir only activates CD8+
52 T-cells (12-14). It is important to note that the abacavir association differs from all other forms of HLA-
53 linked hypersensitivity reaction. For example, drug-responsive CD4+ and CD8+ T-cells are observed in
54 patients hypersensitive to drugs such as carbamazepine, dapsone, flucloxacillin who express the
55 relevant HLA class I risk alleles, B*15:02, B*13:01 and B*57:01, respectively (15-17). These data
56 indicate that although there is a preference for drug (parent drug, metabolite) peptide complex HLA
57 T-cell receptor binding in patients, binding interactions are generally heterogeneous and this
58 contributes to the complete adaptive drug-specific T-cell response. Throughout this manuscript we
59 discuss the different forms of drug HLA interaction in detail highlighting similarities and differences in
60 pathways that lead to T-cell activation. However, we subsequently use the general term "drug peptide
61 complex" where appropriate to refer to any drug-derived structure that interacts with HLA proteins
62 and T-cell receptors to trigger T-cell activation. This is because the formation of an HLA, drug, peptide
63 and T-cell receptor complex is necessary for all pathways of T-cell activation. It is simply the nature of
64 the complex and form of binding interaction that differs. As the number of associations between drug
65 hypersensitivity and HLA allele expression increases (18-20), it is important to consider the additional
66 patient factors that confer susceptibility. This is of particular importance because not all patients
67 expressing a risk HLA are susceptible, while many patients lacking known risk alleles go on to develop
68 hypersensitivity when exposed to culprit drugs.

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3 69 Three factors are critical for the activation of T-cells with drugs; exposure to a drug peptide complex,
4 70 the availability of a T-cell repertoire for a drug peptide complex and a protein encoded by HLA alleles
5 71 for drug peptide complex binding. The argument is presented that although each factor detailed above
6 72 is critical for drug immunogenicity; separately or together, they cannot be used to predict patient
7 73 outcome following drug exposure. We hypothesize that when each factor is present, active immune
8 74 regulatory pathways (co-inhibitory receptors, Tregs, cytokines) are key determinants of whether drug
9 75 exposure will result in hypersensitivity. Since expression and activity of these regulatory pathways are
10 76 altered by disease, the genetic make-up of the host and environmental factors, it is currently
11 77 impossible to predict whether drug exposure will result in a health benefit, hypersensitivity or both
12 78 (Figure 1).
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23 80 **Different manifestations of drug hypersensitivity**

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25 81 Drug-induced cutaneous reactions: Although skin rashes are common forms of drug hypersensitivity,
26 82 serious and life-threatening reactions develop much less frequently. Examples of serious cutaneous
27 83 hypersensitivity reactions include Stevens-Johnson syndrome, toxic epidermal necrolysis and drug
28 84 reaction with eosinophilia and systemic symptoms (DRESS). Although less serious than the conditions
29 85 listed above, acute generalised exanthematous pustulosis and maculopapular exanthema are also
30 86 important adverse drug reactions. A broad spectrum of different drugs may cause cutaneous reactions
31 87 including the sulfonamides, allopurinol, carbamazepine, dapsone and many of the penicillins (21-24) .
32 88 Although there is some degree of pathophysiological overlap, there are some clinically defining
33 89 features for each type of severe cutaneous adverse drug reaction and these are briefly discussed
34 90 below.
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43 91 The most common skin manifestation is maculopapular exanthema which accounts for approximately
44 92 95% of all cutaneous reactions (25). These are reported as eruptions starting on the trunk and upper
45 93 extremities and progressively become more prevalent. These reactions are not life-threatening and
46 94 ~~almost always often~~ subside ~~even with when~~ the ~~continued dosing with the~~ culprit drug ~~has been~~
47 95 ~~withdrawn~~ (26). Antibiotics and a number of tuberculosis medications such as rifampicin, isoniazid,
48 96 pyrazinamide and ethambutol are common causes of maculopapular exanthema (27).
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53 97 Acute generalised exanthematous pustulosis represents a more severe, usually drug-related skin
54 98 reaction characterised by the presence of sterile pustules on an erythematous surface along with fever
55 99 and ~~neutropenia-neutrophilia~~ in a patient. Furthermore, the involvement of activated neutrophils
56 100 along with excessive production of cytokines IL-8 and IL-17 is characteristic of acute generalised
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3 101 exanthematous pustulosis, stimulating the recruitment to tissues and the induction of innate immune
4 102 responses (28).

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7 103 DRESS is a severe skin reaction with an incidence of between 1:1000 and 1:10000 in patients exposed
8 104 to culprit drugs such as anticonvulsants, antimicrobials and antivirals (29). The reaction is
9 105 characterised by skin eruptions, fever as well as symptoms in other organs, such as hepatitis, nephritis
10 106 and thyroiditis (30). DRESS has been shown to be regulated by the cellular actions of eosinophils
11 107 mediated via the secretion of IL-5 from drug-specific T-cells (31). Furthermore DRESS is often
12 108 associated with reactivation of several viruses, including HHV-6, CMV and EBV (32) (33).

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18 109 Stevens-Johnson syndrome and toxic epidermal necrolysis define increasing degrees of severity of the
19 110 same skin disease and are often grouped together. The disease involves the mucosal membranes
20 111 including the eyes, mouth and genitals (30). The level of skin detachment can be used to categorise
21 112 the severity of the reaction. The clinical definition of Stevens-Johnson syndrome is when the
22 113 detachment of epidermal sheets remains on small areas and occurs on less than 10% of the body
23 114 surface area. Stevens-Johnson syndrome/toxic epidermal necrolysis overlap is when this value is
24 115 between 10-30% and toxic epidermal necrolysis patients experience large sheets of skin detachment
25 116 exceeding 30% of the body surface area (34).

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32 117 Drug-induced liver injury: The liver is the largest organ in humans; it is the major organ responsible for
33 118 the metabolism and detoxification of drugs. Hepatocytes (parenchymal cells) make up about 85% of
34 119 the liver while non-parenchymal cells, including liver sinusoidal endothelial cells, hepatic stellate cells,
35 120 Kupffer cells and biliary epithelial cells make up the remaining 15% and play important roles in
36 121 maintaining the homeostasis of the liver. Drug-induced liver injury is a major reason for drug attrition
37 122 and withdrawal of drugs in clinical trials or drugs already licenced for clinical use (35). Worldwide, the
38 123 estimated annual incidence rate of drug-induced liver injury is 0.02% (36, 37). Hoofnagle and
39 124 Björnsson have recently classified drug-induced liver injury into three categories (direct,
40 125 indirect and idiosyncratic) according to frequency, predictability and reaction mechanisms
41 126 (38). Direct liver injury is common and occurs rapidly when drugs are given at high doses (e.g.,
42 127 paracetamol). Indirect liver injury has an intermediate frequency, is partially predictable and
43 128 occurs as an indirect action of the drug on liver or the immune system (e.g., monoclonal
44 129 antibodies). Finally, idiosyncratic liver injury occurs in only a small number of individuals, is
45 130 not predictable and involves activation of the patients adaptive immune system. ~~and t~~The
46 131 mean onset of idiosyncratic liver injury tissue damage with certain drugs exceeds 100 days (39).
47 132 Amoxicillin, clavulanic acid, NSAIDs, flucloxacillin, lapatinib, lumiracoxib, ximelagatran among other
48 133 drugs have been implicated with various degrees of unpredictable/idiosyncratic liver injury. Several

1
2
3 134 forms of drug-induced liver injury are strongly associated with expression of specific HLA alleles (40).
4
5 135 This, alongside the delayed onset of clinical symptoms, is indicative of pathogenesis involving drug-
6
7 136 specific T-cells~~the adaptive immune system~~. Recent studies have identified and characterized drug-
8
9 137 responsive CD4+ and CD8+ T-cells from the peripheral blood of patients with tuberculosis medicine-,
10
11 138 co-amoxiclav- and flucloxacillin-induced liver injury (41-43). Furthermore, T-cells infiltrate~~have been~~
12
13 139 shown to infiltrate liver and kill hepatocytes through the release of cytolytic molecules (44, 45).

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15 140 ~~Drug-induced hematologic disorders: Agranulocytosis, aplastic anaemia, megaloblastic anaemia and~~
16
17 141 ~~thrombocytopenia are major forms of drug-induced hematologic disorders. Drugs can directly target~~
18
19 142 ~~progenitor cells in the bone marrow or peripheral blood cells in the systemic circulation (46, 47). These~~
20
21 143 ~~adverse drug reactions are rare but can result in significant mortality. Various classes of drugs have~~
22
23 144 ~~been linked with drug-induced hematologic disorders. Examples include antibacterial, anti-~~
24
25 145 ~~inflammatory, antithyroid, antimalarial, antiepileptic, antidepressant and antipsychotic drugs. Similar~~
26
27 146 ~~to skin and liver reactions, drug-induced hematologic disorders can be unpredictable. Genome-wide~~
28
29 147 ~~association studies have linked some variants of HLA-DQB1 and HLA-B allele to clozapine-induced~~
30
31 148 ~~agranulocytosis providing evidence for an immune pathogenesis. There is also evidence to suggest~~
32
33 149 ~~that drugs which cause hematologic disorders can activate (i) inflammasomes, (ii) B-cells to produce~~
34
35 150 ~~anti-drug antibodies and (iii) cytotoxic T-cells ((48-50) unpublished data).~~

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152 **Does drug exposure impact on susceptibility to hypersensitivity?**

37 153 For this discussion, we assume that the initiating event for T-cell activation is either a drug or drug
38
39 154 metabolite binding directly to the HLA T-cell receptor complex (through either covalent or non-
40
41 155 covalent binding) or a drug or drug metabolite binding indirectly to non-HLA proteins (through
42
43 156 covalent binding; the HLA binding epitope being a peptide derived from the modified protein, which
44
45 157 may or may not contain the drug moiety).

46 158 In consideration of the latter first, most research has been conducted on biological samples from
47
48 159 patients with β -lactam hypersensitivity. For adduct formation, the β -lactam ring is targeted by lysine
49
50 160 residues. Nucleophilic attack leads to ring opening and binding of the penicilloyl group to the lysine
51
52 161 residue (51). β -lactam antibiotics modify serum proteins such as serum albumin and multiple
53
54 162 intracellular proteins (52-56). Protein adducts are transported to antigen presenting cells via exosomal
55
56 163 transport (55, 57) and β -lactam-modified protein and peptide adducts have been shown to activate
57
58 164 patient T-cells (15, 58-62). Importantly, these adducts are formed in *all* drug exposed patients (53, 63-
59
60 165 65), those who develop skin and liver reactions as well as those that safely tolerate the drug.
166 Moreover, through the synthesis of β -lactam-modified peptides as standards for mass spectrometric

1
2
3 167 analysis, Meng et al (63) were able to quantify and compare the level of drug albumin binding in
4
5 168 hypersensitive and tolerant patients. No clear differences in the level of β -lactam antibiotic lysine
6
7 169 modification was detected between the two patient groups, and importantly, the level of modification
8
9 170 in all patients exceeded the threshold required for activation of β -lactam antibiotic-responsive T-cells.
10
11 171 Obviously, additional studies are required to explore whether hapten thresholds are exceeded in
12
13 172 patients receiving others β -lactam antibiotics and hapteneic drug metabolites. However, currently
14
15 173 available data suggests that although the formation of drug protein adducts may be an important, if
16
17 174 not critical factor for drug immunogenicity, the level of therapeutic drug exposure does not seem to
18
19 175 be a key determinant of patient outcome. One way to confirm this would be a detailed comparison of
20
21 176 the incidence of hypersensitivity reactions in patients receiving higher and lower β -lactam doses or
22
23 177 longer and shorter treatment courses, as long as this doesn't impact on clinical care.

24
25 178 An assortment of drug structures activate T-cells through a direct non-covalent interaction with HLA
26
27 179 and/or specific T-cell receptors. The p-I concept has been coined to explain this phenomenon and
28
29 180 differentiate this pathway of T-cell activation from the hapten concept. A number of pieces of
30
31 181 experimental evidence support this direct binding concept: first, the addition of parent drug to human
32
33 182 immune cell culture systems that express low levels of drug metabolizing enzymes leads to a T-cell
34
35 183 response characterized by proliferation and cytokine and cytolytic molecule release (66-68); second,
36
37 184 inhibition of protein processing within antigen presenting cells, which blocks T-cell responses to
38
39 185 protein antigens has no effect on the activation of T-cells with drugs (69, 70); and third, the kinetics of
40
41 186 T-cell activation with drugs is rapid, within minutes (14, 71), which is in stark contrast to classical
42
43 187 antigen presentation pathways that require several hours. Many drugs have been shown to activate
44
45 188 T-cells from hypersensitive patients via this pathway, including sulfamethoxazole (70), carbamazepine
46
47 189 (72, 73) and allopurinol (71). However, with the exception of abacavir, the nature of the drug peptide
48
49 190 HLA T-cell receptor interaction is yet to be defined. The selective interaction of abacavir with HLA-
50
51 191 B*57:01 alters the spatial arrangement of molecules within the peptide binding groove. This results in
52
53 192 the display of novel "altered" HLA-B*57:01 peptide sequences that seemingly go on to stimulate T-
54
55 193 cells that bring about abacavir hypersensitivity (9-11, 74). Adam et al. (74) demonstrated that
56
57 194 abacavir-responsive T-cells stemming from naïve and memory compartments are detectable in 100%
58
59 195 of donors expressing HLA-B*57:01. This led the authors to suggest that abacavir T-cell reactivity by-
60
196 passes normal co-stimulatory/regulatory requirements. However, we draw readers attention to the
197 fact that it has not been possible to explain why only half of HLA-B*57:01+ donors (who all possess
198 abacavir-responsive T-cells) exposed to abacavir develop hypersensitivity. It should also be noted that
199 p-I- and hapten-responsive T-cells are not always detected in isolation. For the β -lactam antibiotics

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3 200 (60, 75) and sulfonamides/sulfones (17, 76, 77), the only drug exemplars studied to date, drug p-i- and
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5 201 hapten-responsive T-cells are found together.

6
7 202 Drugs administered at a high mass dose more frequently cause hypersensitivity reactions, when
8
9 203 compared with drugs administered at lower doses (78). However, in humans, individual drugs tend to
10
11 204 be administered at similar doses using dosing regimens directed to achieve drug concentrations within
12
13 205 a therapeutic window for a sustained duration of time. Humans are therefore exposed to similar
14
15 206 plasma concentrations of the parent drug. A handful of studies describe associations between
16
17 207 metabolism (increased production of metabolite or increased exposure to parent drug) and the
18
19 208 incidence of drug hypersensitivity reactions (79). For example, CYP2C9*3, which decreases phenytoin
20
21 209 clearance is associated with an increased occurrence of anticonvulsant hypersensitivity (80, 81).
22
23 210 Similarly, impaired renal function and increased plasma levels of oxypurinol (the metabolite that
24
25 211 drives T-cell responses in hypersensitive patients (82)) correlate with the poor prognosis of
26
27 212 allopurinol-induced severe cutaneous hypersensitivity reactions (83). However, these findings seem
28
29 213 to be an exception, rather than a rule, as few other studies have reported associations between drug
30
31 214 disposition and hypersensitivity.

32
33 215 It is clear that a threshold level of drug exposure must be surpassed for the activation of T-cells. In
34
35 216 agreement with this, most drugs that have been withdrawn from the market or have received black
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37 217 box warnings due to liver injury are administered at daily doses greater than 50 mg per day (84, 85).
38
39 218 However, it is difficult to argue susceptibility to drug hypersensitivity is solely dependent upon plasma
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41 219 drug concentrations or the drug concentration at the site of T-cell activation. The vast majority of
42
43 220 patients tolerate therapeutics drug concentrations with little or no adverse effects. Thus, for the
44
45 221 purpose of this review we argue that everyone taking medicinal drugs may be exposed to therapeutic
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47 222 concentrations that are capable of forming HLA drug peptide complexes and delivering them to T-
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49 223 cells.

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51 225 **Does the display of drug peptide complexes by human leukocyte antigen proteins impact on**
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53 226 **susceptibility to hypersensitivity?**

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55 227 A plethora of studies, starting with abacavir discussed above, have identified astonishingly strong
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57 228 associations between HLA class I alleles and susceptibility to drug hypersensitivity reactions, which
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59 229 implies a direct effect of the gene product on the disease (86, 87) (Table 1 shows several HLA class I
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230 allele-associated drug hypersensitivity reactions with known drug peptide complex HLA binding
231 interactions for T-cell activation). This suggests that mechanistically, restriction of the fit of the drug
232 and peptide into HLA proteins is important for T-cell activation. HLA-B*57:01, which is associated with

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3 233 abacavir hypersensitivity, has a positive predictive value of 55 % and a negative predictive value of
4 234 100 % (8). This means that only individuals carrying the allele are at risk and 1 out of 2 carriers develop
5 235 hypersensitivity following abacavir exposure. Genetic screening prior to abacavir use is routine
6 236 practice and eradicates the appearance of hypersensitivity. Other forms of HLA class I associated
7 237 hypersensitivity (e.g., flucloxacillin [HLA-B*57:01] (88), allopurinol [HLA-B*58:01] (21), carbamazepine
8 238 [HLA-B*15:02] (89) and dapsone [HLA-B*13:01] (90)) display similar negative predictive values (99-
9 239 100%) in specific patient groups; however, the positive predictive value is much lower. This suggests
10 240 that the HLA allele is essential for drug peptide complex display, but other factors determine whether
11 241 drug exposure results in a T-cell response and hypersensitivity. In a final group of HLA class I associated
12 242 reactions (e.g., carbamazepine [HLA-A*31:01] (91), co-amoxiclav [HLA-A*02:01] (92),
13 243 sulfamethoxazole [HLA-B*38:02] (93), minocycline [HLA-B*35:02] (94) and terbinafine [HLA-A*33:01]
14 244 (95)), the carrier frequency in hypersensitive patients is 50% or lower. Thus, in these reactions, the
15 245 drug-peptide complex is displayed by a number of different HLA proteins to activate T-cells. Additional
16 246 forms of drug hypersensitivity are (i) linked to expression of HLA class II allele(s) or (ii) not known to
17 247 be associated with expression of a specific HLA allele despite the fact that drug-specific CD4+ and CD8+
18 248 T-cells are detectable. Importantly, it has not been possible to show that selective drug peptide
19 249 complex binding to HLA class II proteins, identified as risk factors, leads to the activation of CD4+ T-
20 250 cells (authors unpublished data).

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25 251 Drug-peptide complex HLA protein binding is without doubt critical for the development of drug
26 252 immunogenicity; however, from the above discussion it is clear that for most HLA allele associated
27 253 reactions, expression of the HLA protein alone does not determine whether drug exposure will result
28 254 in hypersensitivity.

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43 256 **Does expression of specific T-cell receptors impact on susceptibility to hypersensitivity?**

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45 257 Advances in high-throughput sequencing technologies has enabled the detailed analysis of global T-
46 258 cell repertoires in patients with and without immunological diseases. Glanville et al. (96) recently
47 259 defined the minimal requirements for T-cell receptor specificity through an analysis of T-cell receptor
48 260 sequences using a panel of HLA binding peptides. Focussing on 5711 T-cell receptor V β chain
49 261 sequences from CD4+ T-cells derived from 22 donors with mycobacterium tuberculosis, they identified
50 262 141 T-cell receptor specificity groups including 16 groups containing T-cell receptors from at least 3-4
51 263 individuals with shared alleles. The T-cell receptors shared HLA alleles from different donors for shared
52 264 peptide presentation. These data indicate that a diverse array T-cell receptor sequences are available
53 265 in any individual that interact with peptide ligands from a single protein antigen. Similar technologies
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3 266 should be applied to the study of drug hypersensitivity to explore whether shared drug peptide
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5 267 complex specificity clusters are present across different donors and whether this correlates with
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7 268 disease.

8
9 269 Our knowledge of how T-cell receptor sequences impact on drug hypersensitivity is in its infancy.
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11 270 Through global expression level analysis and assessment of the third complementary-determining
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13 271 region length distribution of the T-cell receptor profile in patients with carbamazepine-induced
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15 272 Stevens-Johnson syndrome, Ko et al. (97) identified VB-11-ISGSY as a dominant clonotype shared
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17 273 amongst different hypersensitive, but not drug-tolerant, donors. Furthermore, carbamazepine-
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19 274 specific cytotoxic T-cells could be primed from PBMC of healthy human donors that were carriers of
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21 275 both HLA-B*15:02 and VB-11-IsGSY. More recently, the same group working on the same patient
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23 276 cohort reported the detection of a public T-cell receptor composed of paired TCR α CDR3 "VFDNTDKLI"
24
25 277 and TCR β CDR3 "ASSLAGELF" clonotypes and that similar receptor clusters are found in the blister
26
27 278 fluid cells and peripheral blood (98). These data suggest that the correct combination of HLA, drug
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29 279 peptide complex and T-cell receptor may be important drivers for carbamazepine-induced Stevens-
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31 280 Johnson syndrome. Unpublished data analysing blister fluid from a different cohort of patients with
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33 281 Stevens Johnson syndrome after administration of multiple drugs also show an enrichment of T-cells
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35 282 that display a selective repertoire of T-cell receptor sequences at the most early phase of the adverse
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37 283 event (Vocanson, personal communication). However, the T-cell receptor identified differs across
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39 284 patients, even those exposed to the same culprit drug. Moreover, a dominant clonotype was not
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41 285 detected in all patients.

42
43 286 The proposal that susceptibility to drug hypersensitivity relates to expression of a single T-cell
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45 287 clonotype contrasts with published literature showing the polyclonal expansion of T-cells by certain
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47 288 drugs. Abacavir, which interacts non-covalently with HLA-B*57:01, activates T-cells in 100% of human
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49 289 donors that carry the risk allele (even though only half develop hypersensitivity when exposed to
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51 290 abacavir) (99). Analysis of T-cell receptors expressed on abacavir-responsive T-cells did not reveal
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53 291 skewed patterns (9). This is consistent with abacavir activating an array of different T-cell receptors.
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55 292 Similarly, nitroso sulfamethoxazole, a cysteine-reactive metabolite of sulfamethoxazole has been
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57 293 shown to prime naïve CD4+ and CD8+ T-cells from 59/60 healthy human donors (100, 101).
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59 294 Spectratyping revealed that nitroso-sulfamethoxazole-specific T-cell responses were controlled by
60
295 public T-cell receptors present in all individuals alongside private T-cell repertoires specific to each
296 individual (102). Finally, elegant studies by Azoury et al. (103, 104) utilized immunodominant β -
297 lactam-modified peptides derived from albumin to calculate the frequency of naïve CD4+ T-cells that
298 recognize the drug peptide complex. The haptened peptides were recognized by naïve T-cells from
299 13/14 human donors.

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3 300 These data, although utilizing a limited number of drugs, cover three forms of drug HLA binding
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5 301 derivative (parent drug, drug metabolite and haptened peptide) and show that PBMC from each
6
7 302 and every one of us contain naïve T-cells capable of recognizing and responding to drugs. Although
8
9 303 certain HLA drug peptide complexes may associate preferentially with specific T-cell receptors and this
10
11 304 may impact on the development of hypersensitivity: as has been described with HLA-B*15:02 and
12
13 305 patients with carbamazepine-induced Stevens Johnson syndrome. It needs to be emphasized that the
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15 306 Caucasian population very rarely express HLA-B*15:02; they do however still develop carbamazepine
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17 307 hypersensitivity. The only explanation for this is that carbamazepine interacts with multiple HLA
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19 308 proteins and T-cell receptors to bring about hypersensitivity reactions.

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21 309

22 310 To summarize the discussion thus far, most, if not all, drug-treated patients have a T-cell repertoire
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24 311 for drug peptide complexes and are exposed to drugs in sufficient quantities to activate the T-cells.
25
26 312 Although expression of a specific HLA protein is important, for many forms of hypersensitivity, HLA
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28 313 risk allele expression *per se* does not predict the outcome of drug exposure. Therefore, for the
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30 314 remainder of this article we focus on the hypothesis that immune regulatory pathways are key
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32 315 determinants of whether drug exposure in genetically predisposed individuals will result in
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34 316 hypersensitivity. Figure 2 illustrates that drug exposure, expression of HLA alleles and T-cell receptors
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36 317 are all important determinants of immunogenicity, whereas regulatory pathways are determinants of
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38 318 hypersensitivity. The pathways of drug-specific T-cell activation are also depicted with reference to
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40 319 the possible different requirements for immune regulation.

41 320 While immune cells survey the tissue microenvironment for drug-derived signals, a key task is to
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43 321 maintain tissue homeostasis. The outcome of immune surveillance may be unresponsiveness (the
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45 322 immune system does not detect the drug-derived signal), a conventional effector response (leading
46
47 323 to hypersensitivity with a drug-derived signal) or tolerance (a state of immunological
48
49 324 unresponsiveness to the drug-derived signal). Tolerance can be natural or induced and these terms
50
51 325 are discussed in more detail below with reference to regulatory T-cells. In the context of drug
52
53 326 hypersensitivity it is important to consider variation in natural tolerance and whether drug treatment
54
55 327 actively induces or alters toleragenic pathways and indeed the potential for certain drug peptide
56
57 328 complexes to bypass natural tolerance. The way the immune system ~~does~~ regulates immune
58
59 329 responses, and is able to adapt to change, is through the expression of an array of cell surface co-
60
330 stimulatory and co-inhibitory signalling receptors (Figure 3). Co-stimulatory receptors collect
331
332 information from stressed or damaged cells and tissue and determine whether an effector response
should be directed towards an antigen. The co-inhibitory receptors act alongside regulatory T-cells

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2
3 333 (Tregs) and stimulatory and inhibitory cytokines (e.g., IL-10, TGF- β) to preserve the regulatory
4 334 environment to prevent unwanted immune responses against self and non-damaging agents and to
5 335 prevent excessive responses to antigens when a T-cell response has been initiated. Factors that
6 336 influence the balance between co-stimulatory and co-inhibitory signalling include the genetics of the
7 337 host, disease and environmental factors.

11
12 338 It is possible that each and every one of us may develop a hypersensitivity reaction following drug
13 339 treatment if the balance between co-stimulation and co-inhibition is skewed at the time of exposure.
14 340 This represents a frightening concept for Pharma and healthcare professionals, since the factors that
15 341 control this balance are difficult to predict and will vary across individuals and within an individual
16 342 when they are exposed to different immunomodulatory environments (e.g., infections or damaging
17 343 agents). For this reason, although it might be possible to work towards a framework to predict the
18 344 intrinsic immunogenicity of a drug, prediction of the number of individuals that will ultimately develop
19 345 a clinical drug hypersensitivity reaction is very difficult.

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29 347 **Clinical evidence to exclude drug exposure, the availability of a T-cell repertoire or a single genetic**
30 348 **factor as key determinants that impact on susceptibility to drug hypersensitivity**

31
32 349 We have worked together with respiratory physicians to understand the chemical and cellular basis
33 350 of β -lactam hypersensitivity in patients with cystic fibrosis. This patient population is an important
34 351 study group as they have been monitored closely throughout childhood and adult life and as such they
35 352 have almost complete drug histories as well as detailed records of the nature and timeframe of
36 353 hypersensitivity reactions that occur more frequently when compared to the general population (105-
37 354 107). Piperacillin is a commonly used β -lactam antibiotic for the treatment of recurrent respiratory
38 355 infections. Patients receive repeated courses of the drug at the same dose (12g/day; iv injection) and
39 356 duration (14 days). If one assumes that a patient receives 3 treatment courses a year, the overall mass
40 357 of piperacillin a patient will be exposed to over a 20 year period would exceed 10kg. Thirty five percent
41 358 of patients with cystic fibrosis develop delayed-type piperacillin hypersensitivity reactions
42 359 characterized clinically with maculopapular or urticarial rashes, fever and arthralgia (105). Drug-
43 360 responsive T-cells are detected in approximately 75% of hypersensitive patients, but not tolerant
44 361 controls using the lymphocyte transformation test (65). Moreover, CD4+ and CD8+ T-cells that secrete
45 362 proinflammatory cytokines, including IL-22 and cytolytic molecules, when exposed to piperacillin are
46 363 present in inflamed skin (2). Drug-responsive T-cells are also detectable in drug tolerant patients
47 364 (unpublished data) and drug-naïve donors (2, 101), but only when immune regulation has been

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3 365 perturbed *ex vivo* and the drug peptide adduct is presented by dendritic cells pre-treated with LPS to
4
5 366 provide co-stimulation.
6

7 367 The mean time to onset of piperacillin hypersensitivity is the ninth day of the ninth treatment course
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9 368 (i.e., the average patient will tolerate eight separate courses of piperacillin), which might lead one to
10
11 369 assume that susceptibility is linked to accumulation of, or repeated exposure to, the drug peptide
12
13 370 complex. However, over a 20 year assessment period at the St. James Cystic Fibrosis Unit (Leeds, UK)
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15 371 patients have been diagnosed with hypersensitivity after every treatment course (1-15; personnel
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17 372 communication, Dr Paul Whitaker). These clinical data are impossible to rationalize in terms of drug
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19 373 exposure/accumulation, the availability of a T-cell repertoire for the drug peptide complex or indeed
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21 374 a single genetic factor such as HLA.

22
23 375 As depicted in figure 2, the pathway of T-cell activation for drugs such as allopurinol and
24
25 376 carbamazepine are very different to that of β -lactam antibiotics. It is possible that reactions with these
26
27 377 drugs occur after T-cell responses develop in the presence of other classical peptide antigens (i.e., the
28
29 378 drug peptide complex cross-reacts with the peptide antigen). In this case, the drug will not always
30
31 379 activate a *de novo* response for hypersensitivity to develop and the regulatory requirements for
32
33 380 activation will be lower. The caveat to this argument however is that both of these drugs have been
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35 381 shown to prime naïve T-cells using autologous dendritic cells to present the drug peptide complex in
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37 382 an appropriate immunological form (108).

383 **The immune regulatory network**

384 Several mechanisms have evolved to regulate T-cell responses and prevent the development of
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40 385 autoimmune disease and other inflammatory conditions. The best known mechanisms of peripheral
41
42 386 tolerance include thymic selection of T-cells, the suppressive activity of Tregs (109) and the increased
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44 387 expression of cell surface receptors, the so-called immune checkpoints (110, 111). The importance of
45
46 388 immune regulation and power of the regulatory network has been demonstrated clinically through
47
48 389 the application of immune checkpoint inhibitors for the treatment of cancer (112). Furthermore,
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50 390 mutations in FOXP3, the regulatory transcription factor for Tregs, results in dysfunctional Tregs and
51
52 391 the development of autoimmune disease and allergy (113). IPEX syndrome – a loss of function
53
54 392 mutation in FOXP3 (and other regulatory pathways such as CTLA4) - is the most extreme clinical
55
56 393 scenario. IPEX syndrome is often fatal presenting clinically for a variety of autoimmune-like
57
58 394 syndromes. It would be interesting to investigate whether patients with IPEX syndrome also develop
59
60 395 more drug hypersensitivity reactions. Tregs are now easy to expand *ex vivo* and have been used in
61
62 396 Phase I clinical trials for the treatment of autoimmune disease to prevent transplant rejection (114).

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3 397 In the following sections we briefly discuss the major immune regulatory pathways and how
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5 398 dysregulation of these pathways may impact on drug hypersensitivity.
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9 400 **Immune checkpoints**

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11 401 Immune checkpoints are a series of receptor ligand interactions between T-cells and antigen
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13 402 presenting/tissue cells which specifically co-ordinate the secondary co-stimulatory signal required for
14
15 403 immune activity following TCR binding. Checkpoint proteins negatively regulate the activation of naïve
16
17 404 T-cells. Furthermore, checkpoint receptor expression is upregulated on T-cells when they are
18
19 405 activated, providing a negative feedback loop to restrict the effector response. PD-1 and CTLA-4, which
20
21 406 are expressed on T-cells, are the most studied immune checkpoints. PD-1 interacts with ligands PD-L1
22
23 407 and PD-L2, which activates tyrosine phosphatases that inactivate tyrosine kinase-mediated activation
24
25 408 signals (115). CTLA-4 binds to ligands CD80 and CD86 on antigen presenting cells displaying antigen.
26
27 409 T-cell inhibition is achieved through competitive antagonism of CD28 signalling and direct delivery of
28
29 410 an intracellular signal (116). Other less well characterized immune checkpoints include TIM-3
30
31 411 (suppresses Th1/Th17 CD4+ responses (117)) and LAG-3 (contributes towards Treg activity and directly
32
33 412 suppresses CD8+ T-cells (118)). The complex interaction between immune checkpoints and naïve and
34
35 413 memory T-cell subsets and how intra- and inter-individual variation impacts on susceptibility to
36
37 414 adverse immunological reactions is ill-defined.

38 415 In recent years, we have investigated whether receptor blockade with immune checkpoint inhibitors
39
40 416 remove the immune brakes and enhance the priming of naïve T-cells by drugs. Naïve T-cells were
41
42 417 cultured *in vitro* with drug and autologous dendritic cells in the presence and absence of immune
43
44 418 checkpoint inhibitors targeting PD-1, CTLA-4 and Tim-3 for 14 days to allow priming to occur. Drug
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46 419 exposure was associated with an increase in expression of all three immune checkpoints on dividing
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48 420 T-cells during the culture period, presumably a regulatory event to keep the drug-specific response in
49
50 421 check (119). After the 14 day culture period, the primed T-cells were restimulated with drug and a
51
52 422 second batch of autologous dendritic cells and the strength of the T-cell response was assessed. PD-1
53
54 423 and CTLA-4 block enhanced the priming of naïve T-cells to drugs, whereas Tim-3 block had no effect
55
56 424 (102, 119). A similar effect (enhanced priming of naïve T-cells to drugs) has been demonstrated *in vivo*
57
58 425 with PBMC from patients receiving immune checkpoint inhibitor therapy (unpublished data).
59
60 426 Furthermore, it is becoming apparent that patients receiving immune checkpoint inhibitor therapy
427
428 427 develop more frequent drug hypersensitivity reactions. Ford et al. (120) recently described the
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430 428 development of sulfasalazine (a combination of salicylic acid and sulfapyridine)-induced cutaneous
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430 429 hypersensitivity in 4 patients with metastatic melanoma that had previous been treated with the anti

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3 430 PD-1 inhibitor pembrolizumab or the anti CTLA-4 inhibitor ipilimumab. Presumably the T-cell response
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5 431 and subsequent hypersensitivity reaction was induced by the sulfonamide component of sulfasalazine
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7 432 when natural immune checkpoints had been suppressed. Phillips et al. have recently reported on the
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9 433 treatment outcomes of 285 patients that developed cutaneous adverse events attributed to immune
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11 434 checkpoint inhibitor therapy (121). It would be interesting to consider the number of these patients
12
13 435 receiving concomitant therapy with low molecular weight drugs.

14 436 A report of the post-approval safety of the B-raf inhibitor vemurafenib described seven patients that
15
16 437 developed serious cutaneous hypersensitivity reactions and importantly, six of these patients received
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18 438 anti-PD-1 antibody therapy prior to starting vemurafenib (122). Phase II studies of ipilimumab plus or
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20 439 minus dacarbazine therapy concluded that ipilimumab monotherapy had a manageable adverse
21
22 440 events profile (123), while dual therapy provided no improvement in efficacy and was not tolerable
23
24 441 due to serious liver injury (124). Dacarbazine use alone is only associated with rare cases of liver injury
25
26 442 (125). The immune checkpoint inhibitor again seems to alter the co-stimulatory/co-inhibitory balance,
27
28 443 permitting the development of dacarbazine-induced liver injury in almost all treated patients. Finally,
29
30 444 it has been reported that polymorphisms in regulatory targets of immune responses such as CTLA-4
31
32 445 and IL-10 could modulate susceptibility to nonsteroidal anti-inflammatory drug (126) and efavirenz
33
34 446 (127) hypersensitivity reactions. Collectively, these data indicate that immune checkpoints act to
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36 447 regulate the strength of the drug-specific T-cell response and hence impact on the balance between
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38 448 tolerance and hypersensitivity (Figure 4). These interactions will become increasingly relevant as the
39
40 449 focus on combination therapies for the treatment of various malignancies increases. Combination
41
42 450 therapies in oncology started by using two checkpoint inhibitors in combination (α CTLa-4/ α PD-1)
43
44 451 which illustrated increased efficacy but also an increased incidence of toxicity with a severe toxicity
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46 452 incidence of 56% of patients (128). Latterly there have been an increasing number of trials combining
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48 453 checkpoint inhibitors with additional systemic anticancer therapies including chemotherapy
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50 454 (KEYNOTE189 [ClinicalTrials.gov number, NCT02578680], IMpassion150 [ClinicalTrials.gov
51
52 455 number, NCT03125902]) and tyrosine kinase inhibitors (KEYNOTE426 [ClinicalTrials.gov
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54 456 number, NCT02853331]). This has culminated in the use of all three agents in some anticancer regimes
55
56 457 eg atezolizumab, bevacizumab, carboplatin and paclitaxel used in combination for the treatment of
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58 458 non-small cell lung cancer (NSCLC) within IMPower150 (ClinicalTrials.gov number, NCT02366143).
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60 459 Given the propensity for immune checkpoint inhibitors to interact and display phenotypically typical
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62 460 hypersensitivity reactions the ability to predict individuals at risk of hypersensitivity or particular drug
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64 461 combinations which carry an increased risk is increasingly important. It also remains to be seen if there
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66 462 is a characterizable dose-toxicity relationship or whether there is a temporal relationship to
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68 463 hypersensitivity. It is known that as monoclonal antibodies, immune checkpoint inhibitors have long

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3 464 half-lives (6.1-25 days) (129) and receptor occupancy exists for weeks. However it is currently unclear
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5 465 if there is a dynamic relationship with hypersensitivity and the duration of risk.
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7 466 Tim-3 is an immune checkpoint receptor that interacts with its ligand galectin 9 to modulate Th1 CD4+
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9 467 T-cell responses. The expression of Tim-3 has recently been shown to be significantly reduced on
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11 468 peripheral blood CD4+ T-cells in the acute phase of drug-induced maculopapular exanthema (130),
12
13 469 a classical Th1-mediated iatrogenic disease. Furthermore, galectin 9 expression and release was
14
15 470 reduced on dendritic cells. These data indicate that the Tim-3 immune checkpoint also contributes to
16
17 471 the maintenance of drug tolerance and the prevention of hypersensitivity reactions.

18 472 Contact allergy is a CD8+ T-cell mediated delayed-type hypersensitivity reaction brought about by low
19
20 473 molecular weight haptens. Unlike drug hypersensitivity, where for the most part murine models do
21
22 474 not exist, contact allergy can be reproduced easily in mice through direct application of the hapten to
23
24 475 skin. In recent years, contact allergy has been used to explore how Toll-like receptors, the
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26 476 inflammasome and endogenous danger signals impact on the hapten specific CD8+ T-cell response
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28 477 and skin inflammation (131-135). Most recently, Gamradt et al., (136) discovered that intrinsic control
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30 478 mechanisms such as immune regulatory (PD-1 and TIM-3) signalling determine whether the cytotoxic
31
32 479 CD8+ T-cells will be reactivated and hence prevent tissue injury. Blocking of immune checkpoints *in*
33
34 480 *vivo* lead to severe contact hypersensitivity responses with low hapten doses.

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36 481 Immune checkpoint blockade has been used in mice to attempt to develop animal models of drug-
37
38 482 induced liver injury with a delayed onset (137-140). Treatment of mice with therapeutic doses of
39
40 483 human liver injury inducing compounds such as amodiaquine, isoniazid and nevirapine did not result
41
42 484 in significant tissue damage. However, when the drugs were administered in the presence of PD-1 and
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44 485 CTLA-4 block, mild, but significant, delayed onset liver injury was observed. Liver injury was associated
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46 486 with hepatic recruitment of immune cells including CD8+ T-cells, suggesting that they participate in
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48 487 the pathogenesis. Although this work represents an important step forward – an *in vivo* model is now
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50 488 available to begin to study drug-induced delayed-typed liver injury - additional studies are required to
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52 489 determine why the liver injury does not progress to the serious forms of tissue damage seen in human
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54 490 patients.

55
56 491 From the above discussion one can begin to visualize how immune checkpoint signalling impacts on
57
58 492 the co-regulatory/co-stimulatory network that determines whether an effector response will ensue
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60 493 following antigen exposure as well as the strength and duration of the response. As one pathway is
61
62 494 blocked other pathways exert an increased influence in an attempt to maintain tolerance. As we move
63
64 495 forward combined immune checkpoint therapy will become more commonplace. This will result in an

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3 496 increase in serious autoimmune side effects. However, it is highly likely that drug hypersensitivity
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5 497 reactions will also become more prevalent.
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9 499 **Regulatory T-cells (Tregs)**

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12 500 Tregs regulate or suppress other cells in the immune system. They control the immune response to
13
14 501 self and foreign antigens and help prevent autoimmune disease and allergy. Natural Tregs are
15
16 502 identified by expression of the regulatory transcription factor FOXP3. Natural Tregs express CD4+ and
17
18 503 CD25+ (141); however, CD25+ is also expressed on other forms of T-cell including activated T-cells.
19
20 504 Thus, there was a search for additional classification markers. CD127+ has been identified as a marker
21
22 505 that is only expressed at low levels on Tregs and can be used alongside CD4+, CD25+ and FOXP3 to
23
24 506 identify natural Tregs (142, 143). Tregs can also be classified according to the expression of a naïve T-
25
26 507 cell marker CD45RA (144). CD45RA+FOXP3^{low}CD4+ (CTLA-4^{low}, CD25^{high}, CD127^{low}) cells are referred to
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28 508 as naïve or inducible Tregs. These cells exhibit weak suppressive activity until they differentiate
29
30 509 following antigen-mediated T-cell receptor engagement. They differentiate into effector Tregs
31
32 510 (CD45RA-FOXP3^{high}CD4+) that display a range of additional markers including CTLA-4, CD25+, PD-1,
33
34 511 TIM-3 and secretory molecules such as IL-10 and TGF- β . These cells display a strong inhibitory activity
35
36 512 and increase in number in blood with age. Tregs exert their suppressive function through a range of
37
38 513 pathways ((144-146) Figure 5). These include the inhibition of antigen presenting cells through
39
40 514 expression of immune checkpoint receptors, the release of cytokines such as IL-10 and TGF- β that
41
42 515 decrease dendritic cell function and the production of pro-inflammatory cytokines and restriction of
43
44 516 IL-2 for effector T-cells through CD25+ ligation.

45
46 517 A plethora of studies have shown that FOXP3+ Tregs suppress hypersensitivity reactions to chemical
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48 518 contact allergens in mice by blocking effector CD8+ T-cell responses (147-149). Gomez de Agüero et
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50 519 al (150) reported that Langerhans cells (cutaneous dendritic cells) are critical in the regulatory process
51
52 520 through inducing the depletion of antigen-responsive T-cells and by activating FOXP3+ Tregs.
53
54 521 Furthermore, *in vivo* expansion of Treg populations has been shown to induce long-term suppression
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56 522 of contact hypersensitivity (151). In humans, Cavani et al (152) hasve reported that CD25+ regulatory
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58 523 T-cells maintain tolerance to the contact metal allergen nickel in non-hypersensitive individuals. T-
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60 524 cells showed a limited capacity to proliferate in the presence of nickel *ex vivo*. However, T-cell
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526 525 activation was strongly increased when Tregs were depleted from the PBMC population. Collectively,
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528 526 the data generated showed that Tregs blocked the efficient activation of naïve and memory nickel-
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528 527 specific T-cells. It will be interesting to see whether similar pathways (possibly when Tregs are
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528 528 depleted alongside checkpoint inhibition) are active in drug tolerant patients.

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3 529 In *in vitro* T-cell priming assays with PBMC from healthy human donors, the depletion of FOXP3+ Tregs
4 is important to detect CD4+ and CD8+ T-cell responses to drugs and haptenic chemicals (100, 153,
5 530 154). The reintroduction of Tregs to naïve T-cell priming assays block the activation of naïve T-cells by
6 531 drugs in a cell concentration-dependent manner (119). Inducible effector Tregs (presumably drug
7 532 peptide complex-responsive) are generated *in vitro* alongside effector CD4+ and CD8+ T-cells during
8 533 the priming of naïve T-cells (unpublished data), further emphasizing their importance at regulating
9 534 drug-specific immune responses. There is a potential for environmental and genetic factors to
10 535 modulate the expression and activity of Tregs. For example, polymorphic variants of FOXP3 have been
11 536 linked to various forms of autoimmune disease, while exposure to air pollution can methylate the
12 537 FOXP3 locus, compromising Treg function (155-158). Thus, Tregs might be important in maintaining
13 538 an effective level of tolerance in all drug-exposed patients.
14 539

15 540 Little is known about the influence of Tregs and Treg dysregulation in the acute phase of a drug
16 541 hypersensitivity reaction. In patients with toxic epidermal necrolysis, the most severe form of
17 542 blistering skin eruption, Takahashi *et al* described a functional impairment of Tregs and a reduced
18 543 capacity to suppress effector T-cell responses to drugs (159, 160). However, the key mechanisms
19 544 implicit in Treg dysregulation were not defined. Recently, Wang *et al.* demonstrated that treatment
20 545 with a TNF- α antagonist reduced skin healing time in patients with severe forms of toxic epidermal
21 546 necrolysis (161). Drug treatment decreased TNF- α and granulysin levels in blister fluid and significantly
22 547 increased Treg proportions in patients during the recovery phase. In patients with a different form of
23 548 severe cutaneous hypersensitivity reaction, DRESS, CD14+ monocytes have been shown to mediate a
24 549 gradual shift from a Treg to a Th17 phenotype during the course of the disease (162). In an
25 550 independent study, lesional skin of patients with DRESS was found to be rich in FOXP3+ cells and the
26 551 increase in Tregs positively correlated with the number of recorded days from the onset of the disease
27 552 (163). Similarly, Hanafusa *et al*, found a switch in the population of dividing cells from CD8+ to FOXP3+
28 553 Tregs in drug-treated PBMC from a patient with DRESS (164). Collectively, these data indicate that the
29 554 Tregs are being activated and recruited to inflamed skin to attempt to control the strength and
30 555 duration of the drug-specific effector T-cell response. Thus, it is important to develop strategies to
31 556 understand the role Tregs play in determining the outcome of drug exposure in patients.

32 557 Recently, breaking tolerance through depletion of murine CD4+ T-cells was found to result in the
33 558 development of abacavir hypersensitivity in a HLA-B*57:01 transgenic model (165). Abacavir exposure
34 559 *per se* induced a CD8+ T-cell response; however, the mice maintained an anergic disease state. An
35 560 adverse reaction in skin was only detected when CD4+ T-cells, which included Tregs, were depleted.
36 561 The epidermis became heavily infiltrated with CD8+ T-cells and skin showed typical signs of tissue
37 562 injury. The authors demonstrated through a series of detailed experiments that CD4+ T-cell depletion

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3 563 resulted in optimal dendritic cell co-stimulation and a break in regulation, predisposing the mice to
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5 564 tissue injury.

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9 566 **Cytokines**

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11 567 During T-cell priming, naïve CD4⁺ T-cells differentiate into one of several lineages, including Th1, Th2,
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13 568 Th17, Th22 and induced Tregs. Each T-cell population is characterized by the cytokines they secrete
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15 569 when activated. Importantly, the cytokine microenvironment during T-cell receptor triggering controls
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17 570 T-cell differentiation (Figure 6). The impact of the cytokine microenvironment on T-cell polarization
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19 571 can be demonstrated experimentally by culturing purified human T-cells with relevant cytokine
20
21 572 cocktails (Th1, IL-12 & anti-IL-4; Th2, IL-4, anti-IL-12 & anti-IFN- γ ; Th17, IL-1 β , IL-6, IL-23 & TGF- β ; Th22,
22
23 573 TNF- α & IL-6) for 7 days prior to non-specific mitogen stimulation. Activated T-cells secrete the
24
25 574 polarized cytokines illustrated in Figure 6. The activation of CD8⁺ T-cells is also influenced by cytokines.
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27 575 In the absence of specific cytokine signals, CD8⁺ T-cells become anergic and unresponsive to antigen
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29 576 stimulation. The dominant cytokines that promote CD8⁺ T-cell activation are IL-12 and IFN- α/β (166,
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31 577 167).

32
33 578 There are many examples of disease induced cytokine imbalance (168-170) and this could have a
34
35 579 major impact on the outcome of drug exposure. Diseases such as HIV and cystic fibrosis predispose
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37 580 individuals to drug hypersensitivity reactions. In patients with HIV the incidence of sulfonamide
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39 581 hypersensitivity is 10 times higher when compared with non-HIV infected patients (171). Cytokine
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41 582 imbalances such as Th1/Th2 switching are common features in patients with HIV as the disease
42
43 583 progresses (172), but to date the impact of these changes on susceptibility to drug hypersensitivity
44
45 584 has not been studied. Similarly, when patients with cystic fibrosis were compared to the general
46
47 585 population, antibiotic reactions were found to be up to three times more common (105). The cystic
48
49 586 fibrosis lung represents an area of chronic inflammation with high neutrophil numbers alongside
50
51 587 elevated levels of cytokines such as IL-8, IL-1 β , IL-6, IL-17 and TNF- α (173-175). Obviously, this will
52
53 588 have a colossal impact on the outcome of T-cell receptor triggering through altered antigen
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55 589 presentation as well as differential polarization of the effector T-cell response. However, to date, it
56
57 590 has not been possible to establish models/systems to explore this relationship directly.

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59 591

60 592 **Conclusions**

593 It is becoming increasingly apparent that multiple tolerance pathways determine the outcome of
594 594 antigen exposure through regulation of (i) naïve T-cell activation and (ii) the strength and duration of

1
2
3 595 the effector T-cell response. Through the studies discussed herein we are beginning to understand
4
5 596 that similar pathways are active in patients at the time of drug exposure and that immune regulation
6
7 597 networks contribute towards the outcome of drug exposure: health benefit or a hypersensitivity
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9 598 reaction. Work is required to define how the distinct pathways contribute towards individual
10
11 599 susceptibility. Such studies are urgent given the plethora of immune modulatory drugs that are in
12
13 600 development, which once approved will be administered alongside traditional low molecular weight
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15 601 drugs. It will also be important to determine whether low molecular weight drugs modulate tolerance
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17 602 pathways in patients and whether this contributes to the successful desensitization of certain
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19 603 hypersensitive patients.
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For Peer Review

604 **Tables**605 **Table 1. HLA class I allele-associated drug hypersensitivity reactions with known drug HLA binding**
606 **interactions for T-cell activation**

Reaction phenotype	HLA allele	Known HLA (peptide) interaction ^a	Evidence of bioactivation ^b
Abacavir hypersensitivity	HLA-B*57:01 (7)	Direct non-covalent binding (9, 176)	Yes, aldehyde (177)
Allopurinol severe skin reactions	HLA-B*58:01 (21)	Direct labile metabolite binding (82)	No
Carbamazepine Stevens Johnson syndrome	HLA-B*15:02 (89)	Direct labile drug & metabolite binding (178, 179)	Yes, multiple metabolites (180, 181)
Carbamazepine skin reactions	HLA-A*31:01 (91)	Direct labile drug & metabolite binding (178, 179)	Yes, multiple metabolites (180, 181)
Dapsone drug reaction with eosinophilia and systemic symptoms	HLA-B*13:01 (90)	Direct labile & metabolite covalent binding (17, 182)	Yes, nitroso metabolite (183, 184)
Flucloxacillin liver injury	HLA-B*57:01 (88)	Direct labile & covalent binding (43, 60)	Not applicable (52)
Sulfamethoxazole skin reactions	HLA-B*38:02 (93)	Direct labile & metabolite covalent binding (4, 76, 77)	Yes, nitroso metabolite (185)
Co-amoxiclav liver injury	HLA-A*02:01 (92)	Direct covalent binding (42)	Not applicable (53)
Minocycline liver injury	HLA-B*35:02 (94)	Unknown	Yes, quinone iminium ion (186)
Terbinafine liver injury	HLA-A*33:01 (95)	Unknown	Yes, aldehyde metabolite (187)
Ticlopidine liver injury	HLA-A*33:03 (188)	Direct labile binding (189)	Yes, sulfenic acid (190)
Vancomycin drug reaction with eosinophilia and systemic symptoms	HLA-A*32:01 (191)	Unknown	No

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608 ^aalternative pathways feasible for all compounds, but to date have not been studied609 ^bformation of a metabolite does not indicate that they are involved in the reaction

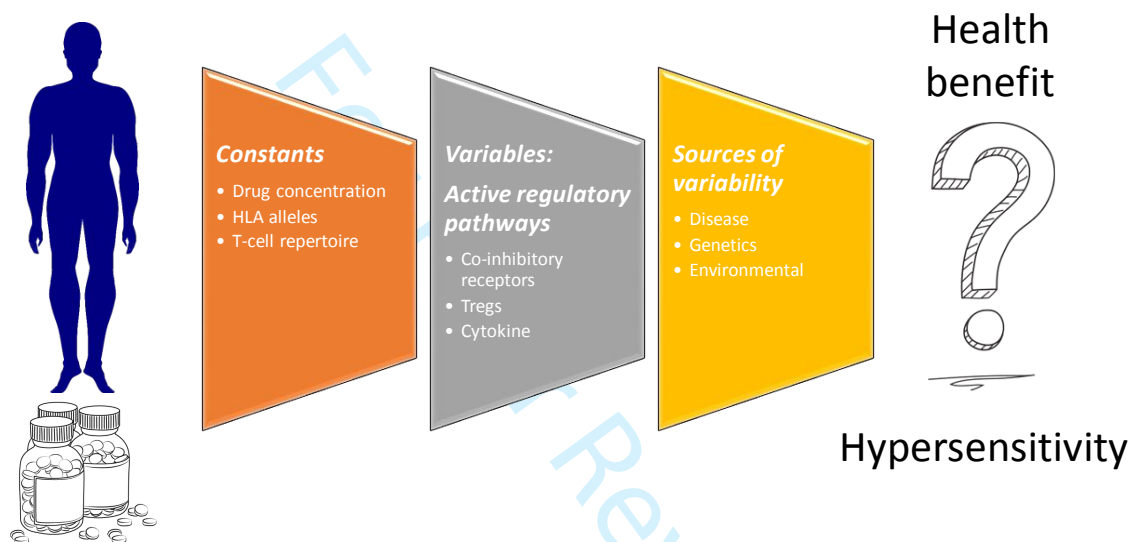
610 **Figures**

611 **Figure 1.** The drug hypersensitivity susceptibility conundrum proposes that if we assume exposure to
 612 a drug and the availability of HLA proteins and T-cell receptors for drug peptide complexes are kept as
 613 constants, then active immune regulatory pathways are the primary determinants of susceptibility.

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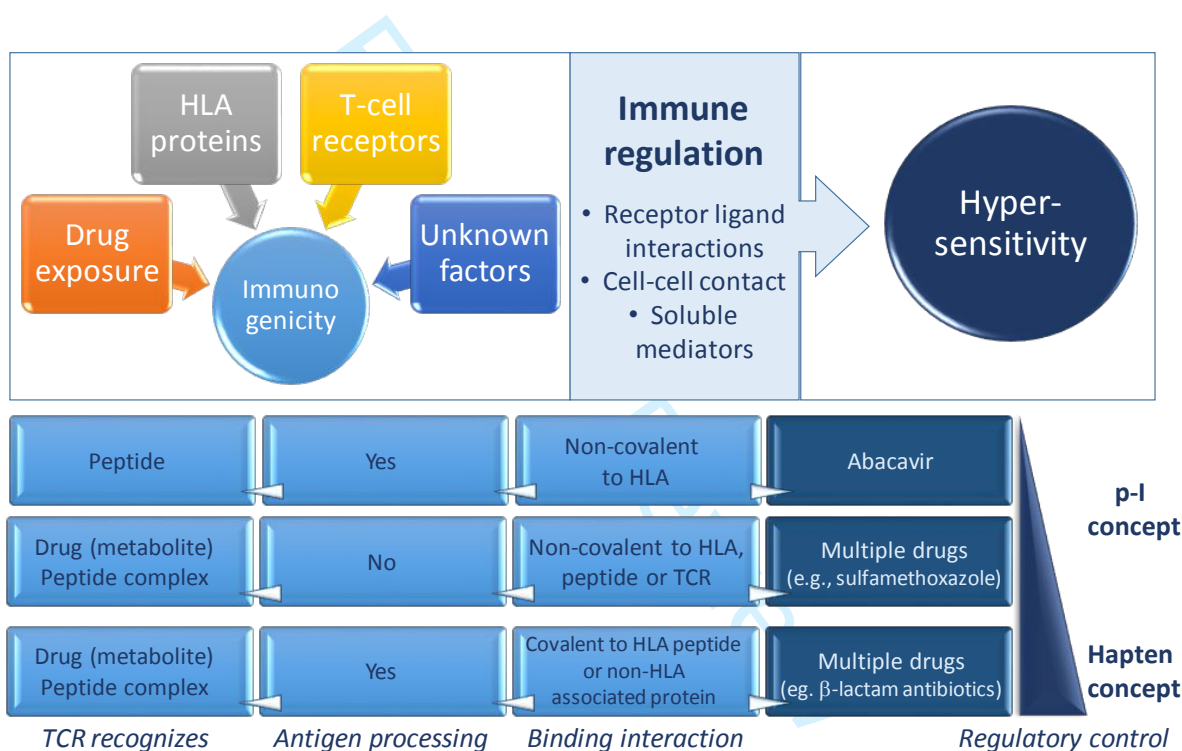
THE DRUG HYPERSENSITIVITY SUSCEPTIBILITY CONUNDRUM



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619 **Figure 2.** The influence of drug- and patient-specific factors on drug immunogenicity and
 620 hypersensitivity. Drug exposure and the availability of HLA proteins and T-cell receptors for drug
 621 binding are essential for immunogenicity. However, these factors either together or in isolation do not
 622 predict whether a patient will develop hypersensitivity. This is because immune regulatory pathways
 623 control whether a pathogenic immune response will develop. These pathways may influence p-I and
 624 hapten responses to different extents although this is yet to be proven even in the case of abacavir.
 625 The bottom component of the figure highlights the nature of the drug immune receptor binding
 626 interaction, the requirement for antigen processing and the derivative that T-cell receptors interact
 627 with for hapten and p-I reactions.



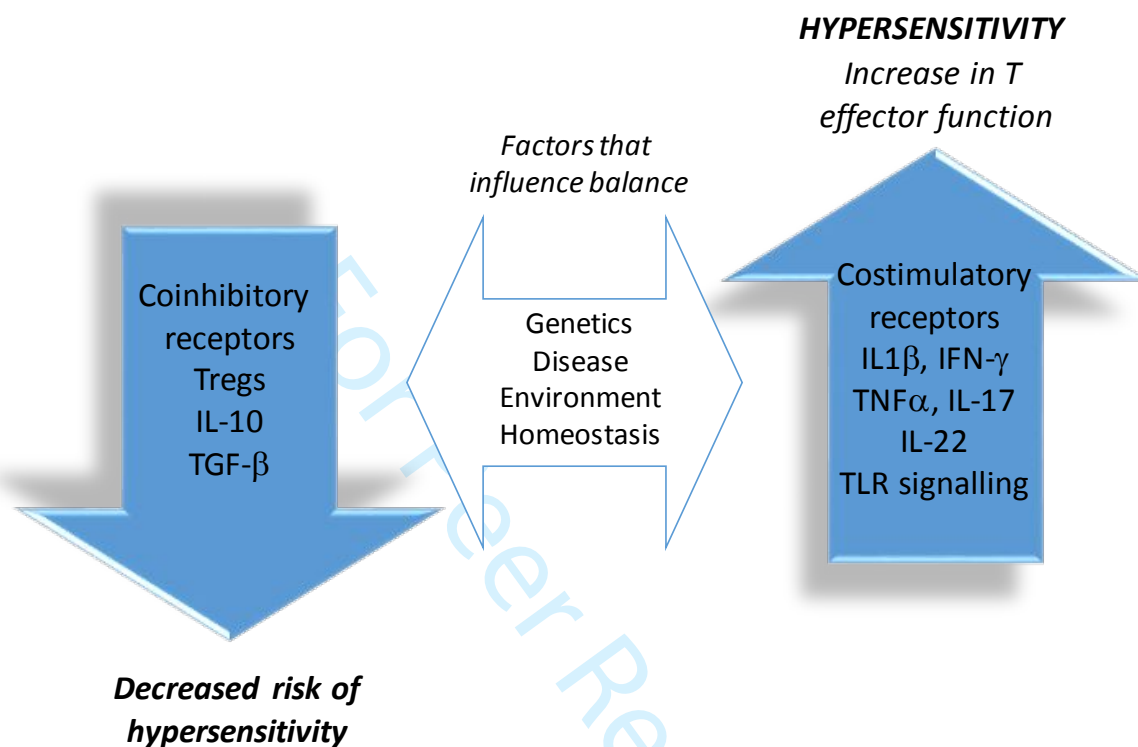
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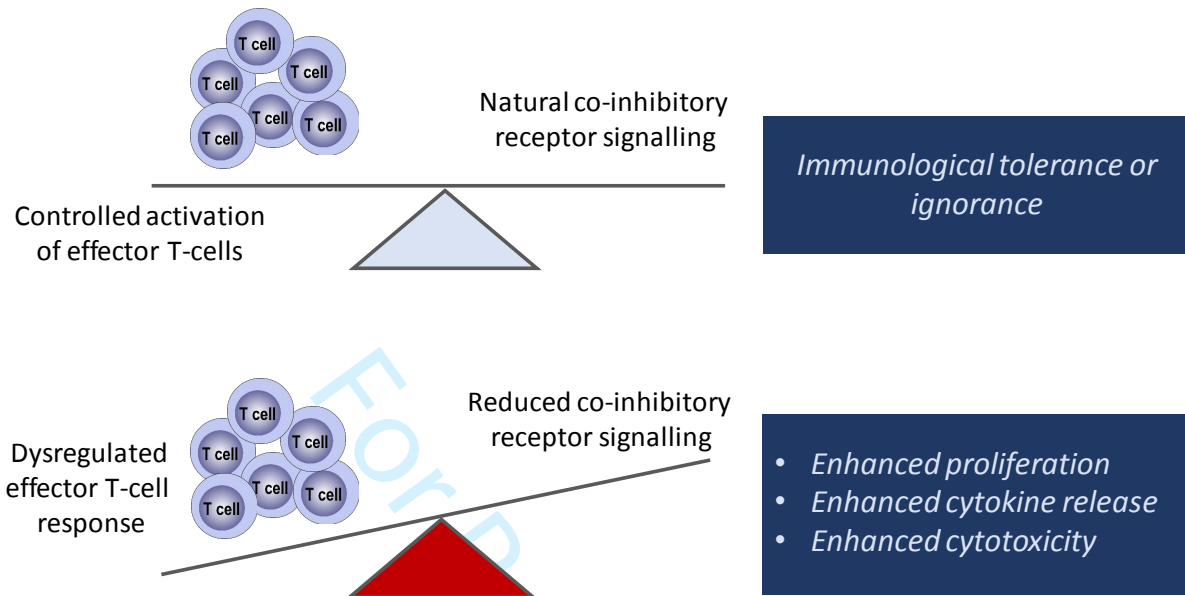
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3 **Figure 3.** The balance between co-stimulatory and co-inhibitory pathways are the key determinant of
4
5 633 whether drug exposure will result in hypersensitivity. This balance is influenced by genetic, disease
6
7 634 and environmental factors. Thus, the balance will differ across individuals and within the same
8
9 635 individual with time.

636



637 **Figure 4.** Co-inhibitory receptors regulate the strength of the drug-specific T-cell response and hence
638 the balance between tolerance and hypersensitivity.

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641 **Figure 5.** Tregs regulate the strength of antigen-specific effector T-cell responses and hence may alter
 642 the balance between tolerance and hypersensitivity following drug exposure.

Tregs:

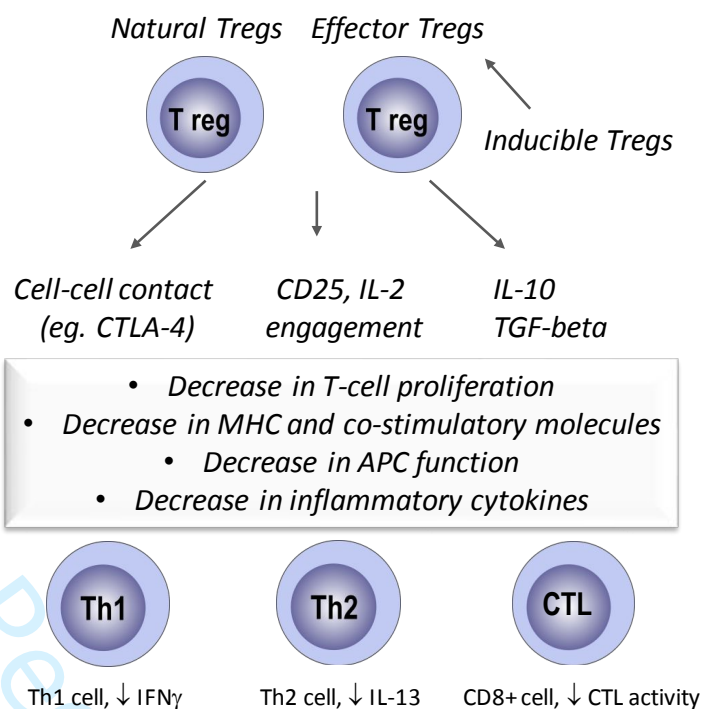
Tregs produced in the thymus are termed **natural**

Treg formed by differentiation of naïve T cells outside the thymus are called **adaptive** or **inducible**

Exert function through

- Cell contact
- Cytokine secretion
- Apoptosis of effector cells
- Modulation of DC function

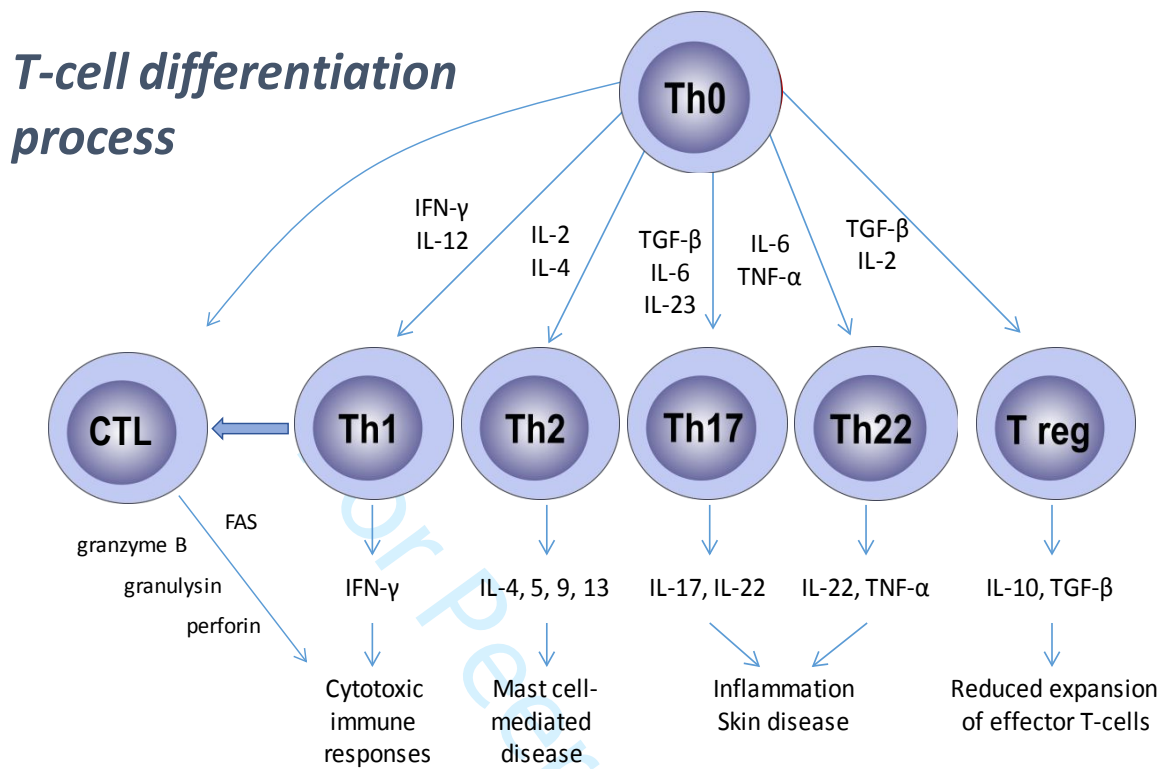
Do they play a role in regulating drug hypersensitivity?



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644 **Figure 6.** Cytokine control of T-cell differentiation.

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3 649 **Text box 1. Major Milestone Discoveries**
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- 5 650
- 6 651 • Drug, drug metabolite and drug-modified peptide HLA binding activates T-cells in
7 patients with hypersensitivity
 - 8 652 • Development of assays with PBMC from healthy human donors to study naïve drug
9 peptide complex T-cell priming *ex vivo*
 - 10 653
 - 11 654 • Individual HLA alleles are important determinants of disease susceptibility
 - 12 655 • Characterisation of HLA-allele-restricted drug-specific T-cell responses in patients with
13 drug hypersensitivity.
 - 14 656
 - 15 657 • Co-inhibitory receptors impact on the ability of drug peptide complexes to activate naïve
16 T-cells
 - 17 658
 - 18 659 • Discovery of an increased incidence of drug hypersensitivity reactions in patients
19 receiving immune checkpoint inhibitor therapy
 - 20 660
 - 21 661

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26 662 **Text Box 2. Future Research Perspectives**
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28
29 663 Genome-wide association studies and functional assessment of patient T-cells have taught us that
30 664 drug peptide complexes interact selectively and specificity with HLA proteins to bring about
31 hypersensitivity reactions. It is now important to define, through detailed structural analysis, the way
32 665 in which drug peptide complexes bind to HLA proteins. The nature of the interaction will differ drug-
33 to-drug. It is also important to determine the contribution different forms of drug peptide complex
34 666 play in the disease pathogenesis as we know that parent drug, metabolite and drug-modified peptide-
35 responsive T-cells circulate in patients' blood and tissues.
36 667

37 668 Of particular importance, is identification of the parameters that that influence susceptibility in
38 patients expressing known HLA risk alleles. Ongoing studies seem to suggest that drugs stimulate a
39 669 very restricted repertoire of T-cells in patients with Stevens Johnson syndrome. Might this be the case
40 in other forms of drug hypersensitivity? The balance between co-stimulatory and co-inhibitory
41 670 signalling during drug peptide complex-specific T-cell priming is also an important determinant of
42 susceptibility. Future research must focus on patients at the earliest stages of a reaction to delineate
43 671 the contribution individual pathways (e.g, receptor signalling, Tregs, cytokines) in play in
44 determination of the outcome of drug exposure. In this respect, important lessons will be learned
45 672 from patients receiving immune checkpoint inhibitor therapy for cancer treatment.
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