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

Short title: Regulatory pathways and drug hypersensitivity

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

Introduction

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

In 2002, Mallal et al. reported a strong association between the presence of HLA-B*57:01, HLA-DR7, and HLA-DQ3 and hypersensitivity to the HIV-1 reverse-transcriptase inhibitor abacavir (7). Subsequent studies demonstrated that (i) all skin test confirmed cases of abacavir hypersensitivity carry HLA-B*57:01 (8), (ii) abacavir interacts selectively with high affinity within the HLA-B57:01 peptide binding cleft through non-covalent interactions (9-11), and (iii) abacavir only activates CD8+ T-cells (12-14). It is important to note that the abacavir association differs from all other forms of HLA-linked hypersensitivity reaction. For example, drug-responsive CD4+ and CD8+ T-cells are observed in patients hypersensitive to drugs such as carbamazepine, dapsone, flucloxacillin who express the relevant HLA class I risk alleles, B*15:02, B*13:01 and B*57:01, respectively (15-17). These data indicate that although there is a preference for drug (parent drug, metabolite) peptide complex HLA T-cell receptor binding in patients, binding interactions are generally heterogeneous and this contributes to the complete adaptive drug-specific T-cell response. Throughout this manuscript we discuss the different forms of drug HLA interaction in detail highlighting similarities and differences in pathways that lead to T-cell activation. However, we subsequently use the general term “drug peptide complex” where appropriate to refer to any drug-derived structure that interacts with HLA proteins and T-cell receptors to trigger T-cell activation. This is because the formation of an HLA, drug, peptide and T-cell receptor complex is necessary for all pathways of T-cell activation. It is simply the nature of the complex and form of binding interaction that differs. As the number of associations between drug hypersensitivity and HLA allele expression increases (18-20), it is important to consider the additional patient factors that confer susceptibility. This is of particular importance because not all patients expressing a risk HLA are susceptible, while many patients lacking known risk alleles go on to develop hypersensitivity when exposed to culprit drugs.

Three factors are critical for the activation of T-cells with drugs; exposure to a drug peptide complex, the availability of a T-cell repertoire for a drug peptide complex and a protein encoded by HLA alleles for drug peptide complex binding. The argument is presented that although each factor detailed above is critical for drug immunogenicity; separately or together, they cannot be used to predict patient outcome following drug exposure. We hypothesize that when each factor is present, active immune regulatory pathways (co-inhibitory receptors, Tregs, cytokines) are key determinants of whether drug exposure will result in hypersensitivity. Since expression and activity of these regulatory pathways are altered by disease, the genetic make-up of the host and environmental factors, it is currently impossible to predict whether drug exposure will result in a health benefit, hypersensitivity or both (Figure 1).

Different manifestations of drug hypersensitivity

Drug-induced cutaneous reactions: Although skin rashes are common forms of drug hypersensitivity, serious and life-threatening reactions develop much less frequently. Examples of serious cutaneous hypersensitivity reactions include Stevens-Johnson syndrome, toxic epidermal necrolysis and drug reaction with eosinophilia and systemic symptoms (DRESS). Although less serious than the conditions listed above, acute generalised exanthematous pustulosis and maculopapular exanthema are also important adverse drug reactions. A broad spectrum of different drugs may cause cutaneous reactions including the sulfonamides, allopurinol, carbamazepine, dapsone and many of the penicillins (21-24) . Although there is some degree of pathophysiological overlap, there are some clinically defining features for each type of severe cutaneous adverse drug reaction and these are briefly discussed below.

The most common skin manifestation is maculopapular exanthema which accounts for approximately 95% of all cutaneous reactions (25). These are reported as eruptions starting on the trunk and upper extremities and progressively become more prevalent. These reactions are not life-threatening and almost always subside when the culprit drug has been withdrawn (26). Antibiotics and a number of tuberculosis medications such as rifampicin, isoniazid, pyrazinamide and ethambutol are common causes of maculopapular exanthema (27).

Acute generalised exanthematous pustulosis represents a more severe, usually drug-related skin reaction characterised by the presence of sterile pustules on an erythematous surface along with fever and neutrophilia in a patient. Furthermore, the involvement of activated neutrophils along with excessive production of cytokines IL-8 and IL-17 is characteristic of acute generalised exanthematous pustulosis, stimulating the recruitment to tissues and the induction of innate immune responses (28).

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

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

Drug-induced liver injury: The liver is the largest organ in humans; it is the major organ responsible for the metabolism and detoxification of drugs. Hepatocytes (parenchymal cells) make up about 85% of the liver while non-parenchymal cells, including liver sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells and biliary epithelial cells make up the remaining 15% and play important roles in maintaining the homeostasis of the liver. Drug-induced liver injury is a major reason for drug attrition and withdrawal of drugs in clinical trials or drugs already licenced for clinical use (35). Worldwide, the estimated annual incidence rate of drug-induced liver injury is 0.02% (36, 37). Hoofnagle and Björnsson have recently classified drug-induced liver injury into three categories (direct, indirect and idiosyncratic) according to frequency, predictability and reaction mechanisms (38). Direct liver injury is common and occurs rapidly when drugs are given at high doses (e.g., paracetamol). Indirect liver injury has an intermediate frequency, is partially predictable and occurs as an indirect action of the drug on liver or the immune system (e.g., monoclonal antibodies). Finally, idiosyncratic liver injury occurs in only a small number of individuals, is not predictable and involves activation of the patients adaptive immune system. The mean onset of idiosyncratic liver injury with certain drugs exceeds 100 days (39). Amoxicillin, clavulanic acid, NSAIDS, flucloxacillin, lapatinib, lumiracoxib, ximelagatran among other drugs have been implicated 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

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

Does drug exposure impact on susceptibility to hypersensitivity?

For this discussion, we assume that the initiating event for T-cell activation is either a drug or drug metabolite binding directly to the HLA T-cell receptor complex (through either covalent or non-covalent binding) or a drug or drug metabolite binding indirectly to non-HLA proteins (through covalent binding; the HLA binding epitope being a peptide derived from the modified protein, which may or may not contain the drug moiety).

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

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

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

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

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

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

Does the display of drug peptide complexes by human leukocyte antigen proteins impact on susceptibility to hypersensitivity?

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

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

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

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

Advances in high-throughput sequencing technologies has enabled the detailed analysis of global T-cell repertoires in patients with and without immunological diseases. Glanville et al. (91) recently defined the minimal requirements for T-cell receptor specificity through an analysis of T-cell receptor sequences using a panel of HLA binding peptides. Focussing on 5711 T-cell receptor Vβ chain sequences from CD4+ T-cells derived from 22 donors with mycobacterium tuberculosis, they identified 141 T-cell receptor specificity groups including 16 groups containing T-cell receptors from at least 3-4 individuals with shared alleles. The T-cell receptors shared HLA alleles from different donors for shared peptide presentation. These data indicate that a diverse array T-cell receptor sequences are available in any individual that interact with peptide ligands from a single protein antigen. Similar technologies should be applied to the study of drug hypersensitivity to explore whether shared drug peptide complex specificity clusters are present across different donors and whether this correlates with disease.

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

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

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

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

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

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

While immune cells survey the tissue microenvironment for drug-derived signals, a key task is to maintain tissue homeostasis. The outcome of immune surveillance may be unresponsiveness (the immune system does not detect the drug-derived signal), a conventional effector response (leading to hypersensitivity with a drug-derived signal) or tolerance (a state of immunological unresponsiveness to the drug-derived signal). Tolerance can be natural or induced and these terms are discussed in more detail below with reference to regulatory T-cells. In the context of drug hypersensitivity it is important to consider variation in natural tolerance and whether drug treatment actively induces or alters toleragenic pathways and indeed the potential for certain drug peptide complexes to bypass natural tolerance. The way the immune system regulates immune responses, and is able to adapt to change, is through the expression of an array of cell surface co-stimulatory and co-inhibitory signalling receptors (Figure 3). Co-stimulatory receptors collect 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 (Tregs) and stimulatory and inhibitory cytokines (e.g., IL-10, TGF-β) to preserve the regulatory environment to prevent unwanted immune responses against self and non-damaging agents and to prevent excessive responses to antigens when a T-cell response has been initiated. Factors that influence the balance between co-stimulatory and co-inhibitory signalling include the genetics of the host, disease and environmental factors.

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

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

We have worked together with respiratory physicians to understand the chemical and cellular basis of β-lactam hypersensitivity in patients with cystic fibrosis. This patient population is an important study group as they have been monitored closely throughout childhood and adult life and as such they have almost complete drug histories as well as detailed records of the nature and timeframe of hypersensitivity reactions that occur more frequently when compared to the general population (100-102). Piperacillin is a commonly used β-lactam antibiotic for the treatment of recurrent respiratory infections. Patients receive repeated courses of the drug at the same dose (12g/day; iv injection) and duration (14 days). If one assumes that a patient receives 3 treatment courses a year, the overall mass of piperacillin a patient will be exposed to over a 20 year period would exceed 10kg. Thirty five percent of patients with cystic fibrosis develop delayed-type piperacillin hypersensitivity reactions characterized clinically with maculopapular or urticarial rashes, fever and arthralgia (100). Drug-responsive T-cells are detected in approximately 75% of hypersensitive patients, but not tolerant controls using the lymphocyte transformation test (60). Moreover, CD4+ and CD8+ T-cells that secrete proinflammatory cytokines, including IL-22 and cytolytic molecules, when exposed to piperacillin are present in inflamed skin (2). Drug-responsive T-cells are also detectable in drug tolerant patients (unpublished data) and drug-naïve donors (2, 96), but only when immune regulation has been perturbed *ex vivo* and the drug peptide adduct is presented by dendritic cells pre-treated with LPS to provide co-stimulation.

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

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

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

The immune regulatory network

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

Immune checkpoints

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

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

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

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

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

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

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

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

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

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

Regulatory T-cells (Tregs)

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

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

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

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

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

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

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

Cytokines

During T-cell priming, naïve CD4+ T-cells differentiate into one of several linages, including Th1, Th2, Th17, Th22 and induced Tregs. Each T-cell population is characterized by the cytokines they secrete when activated. Importantly, the cytokine microenvironment during T-cell receptor triggering controls T-cell differentiation (Figure 6). The impact of the cytokine microenvironment on T-cell polarization can be demonstrated experimentally by culturing purified human T-cells with relevant cytokine cocktails (Th1, IL-12 & anti-IL-4; Th2, IL-4, anti-IL-12 & anti-IFN-γ; Th17, IL-1β, IL-6, IL-23 & TGF-β; Th22, TNF-α & IL-6) for 7 days prior to non-specific mitogen stimulation. Activated T-cells secrete the polarized cytokines illustrated in Figure 6. The activation of CD8+ T-cells is also influenced by cytokines. In the absence of specific cytokine signals, CD8+ T-cells become anergic and unresponsive to antigen

stimulation. The dominant cytokines that promote CD8+ T-cell activation are IL-12 and IFN-α/β (161, 162).

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

Conclusions

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

Tables

Table 1. HLA class I allele-associated drug hypersensitivity reactions with known drug HLA binding 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
Carbmazepine Stevens Johnson syndrome	HLA-B*15:02 (84)	Direct labile drug & metabolite binding (173, 174)	Yes, multiple metabolites (175, 176)
Carbmazepine 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

[a]alternative pathways feasible for all compounds, but to date have not been studied

[b]formation of a metabolite does not indicate that they are involved in the reaction

Figures

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

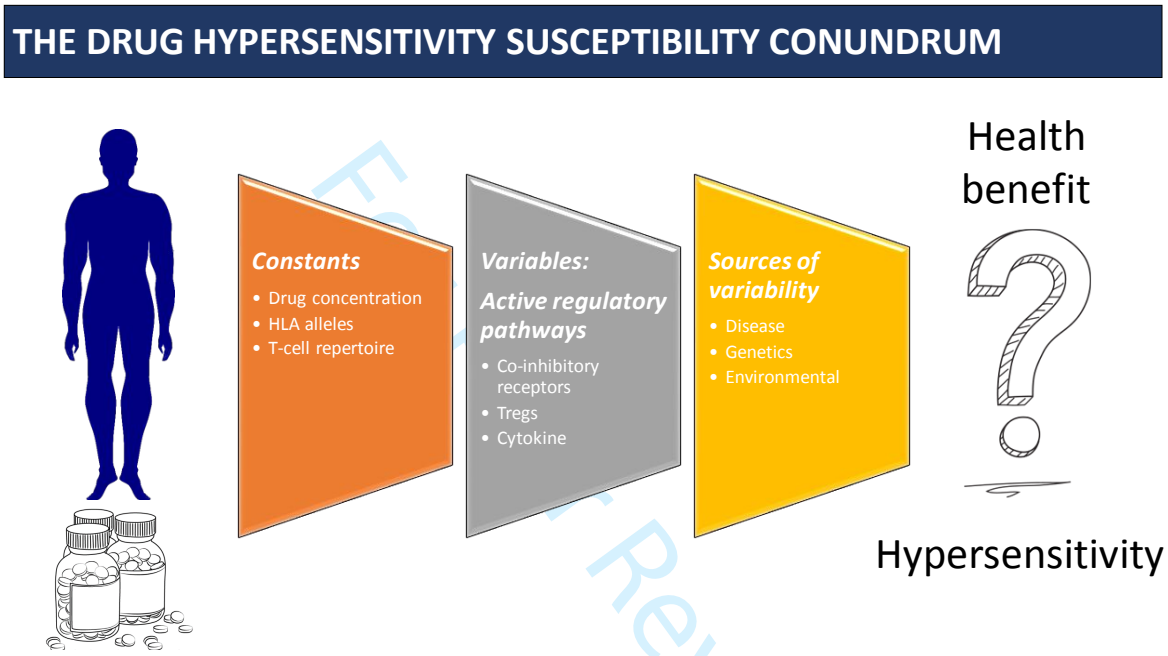


Figure 2. The influence of drug- and patient-specific factors on drug immunogenicity and hypersensitivity. Drug exposure and the availability of HLA proteins and T-cell receptors for drug binding are essential for immunogenicity. However, these factors either together or in isolation do not predict whether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

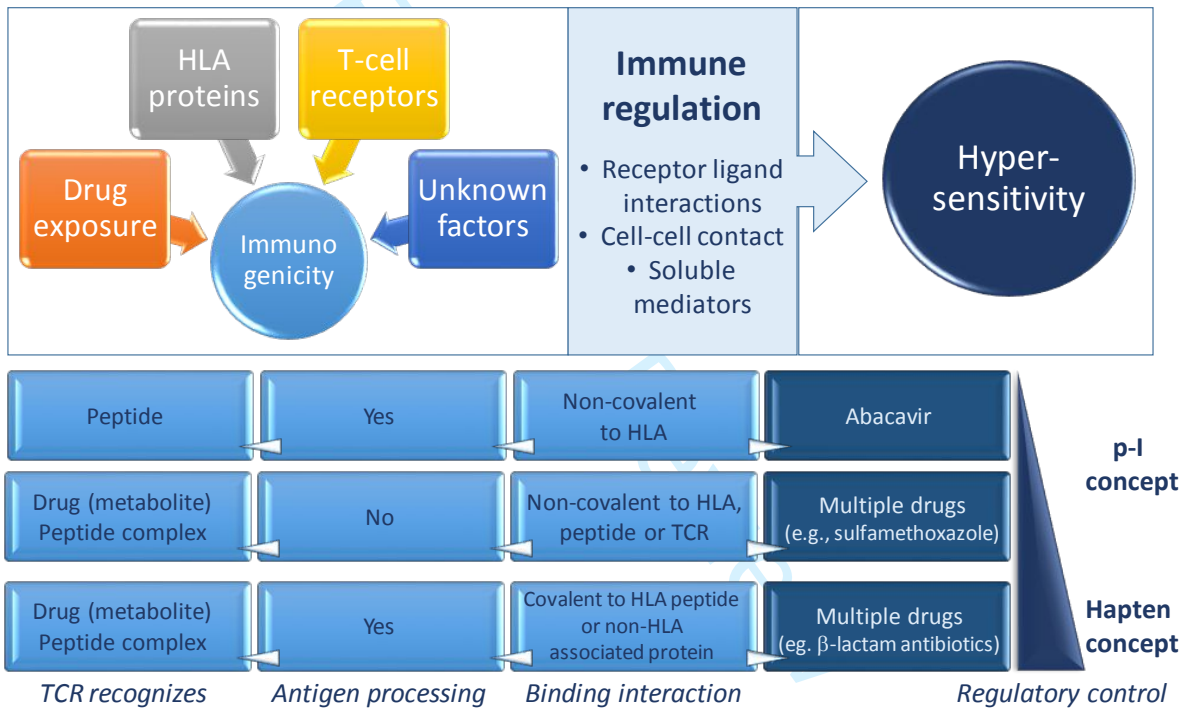


Figure 3. The balance between co-stimulatory and co-inhibitory pathways are the key determinant of whether drug exposure will result in hypersensitivity. This balance is influenced by genetic, disease and environmental factors. Thus, the balance will differ across individuals and within the same individual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

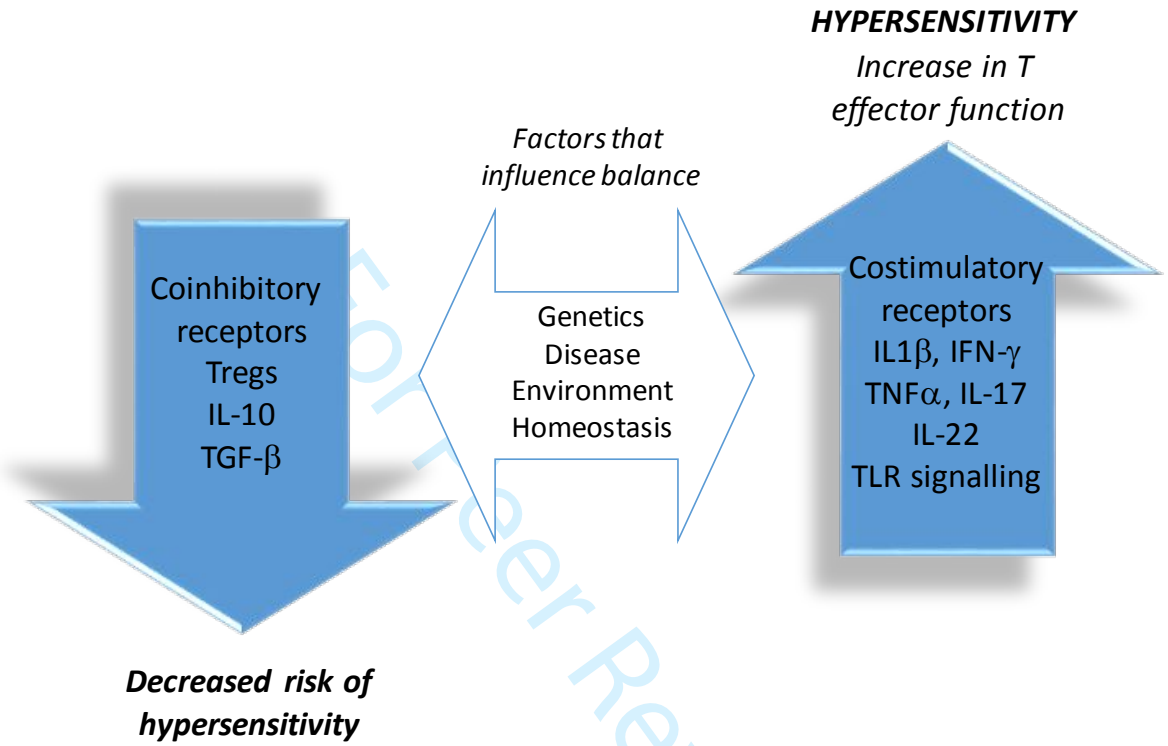


Figure 4. Co-inhibitory receptors regulate the strength of the drug-specific T-cell response and hence the balance between tolerance and hypersensitivity.

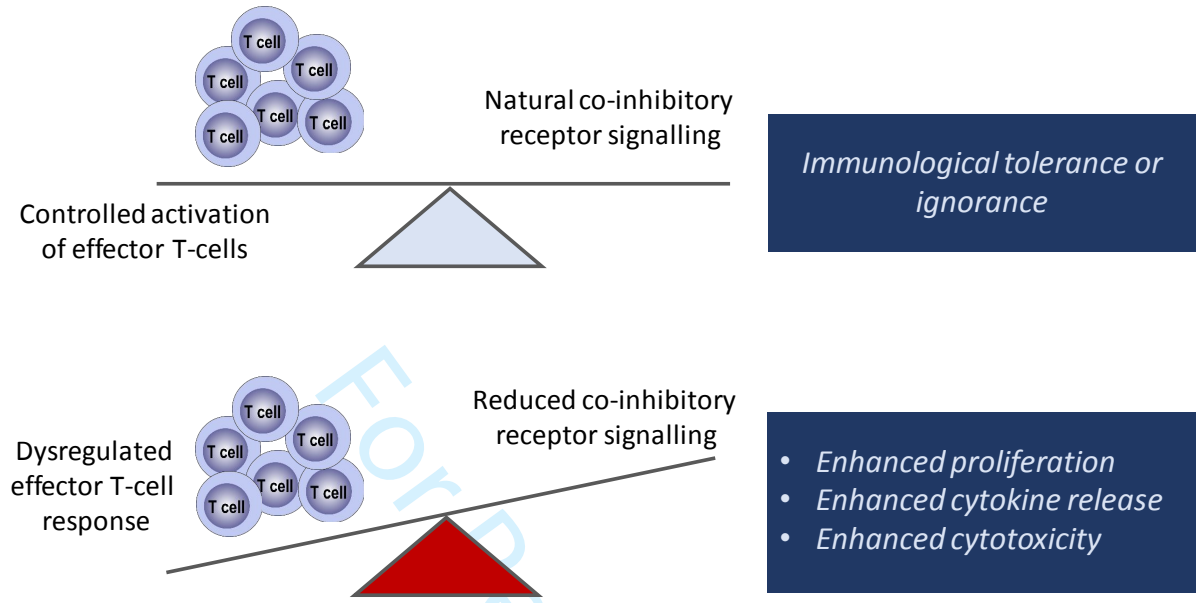


Figure 5. Tregs regulate the strength of antigen-specific effector T-cell responses and hence may alter the balance between tolerance and hypersensitivity following drug exposure.

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?

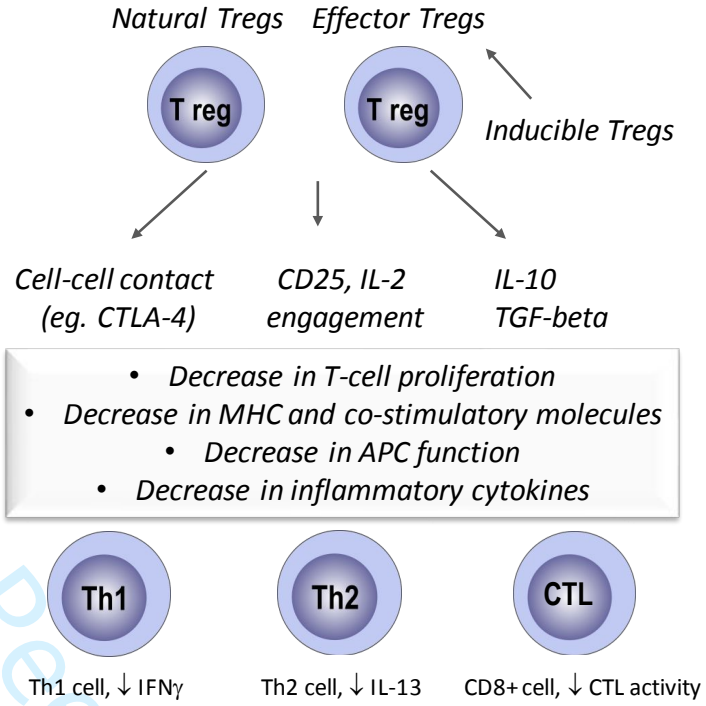
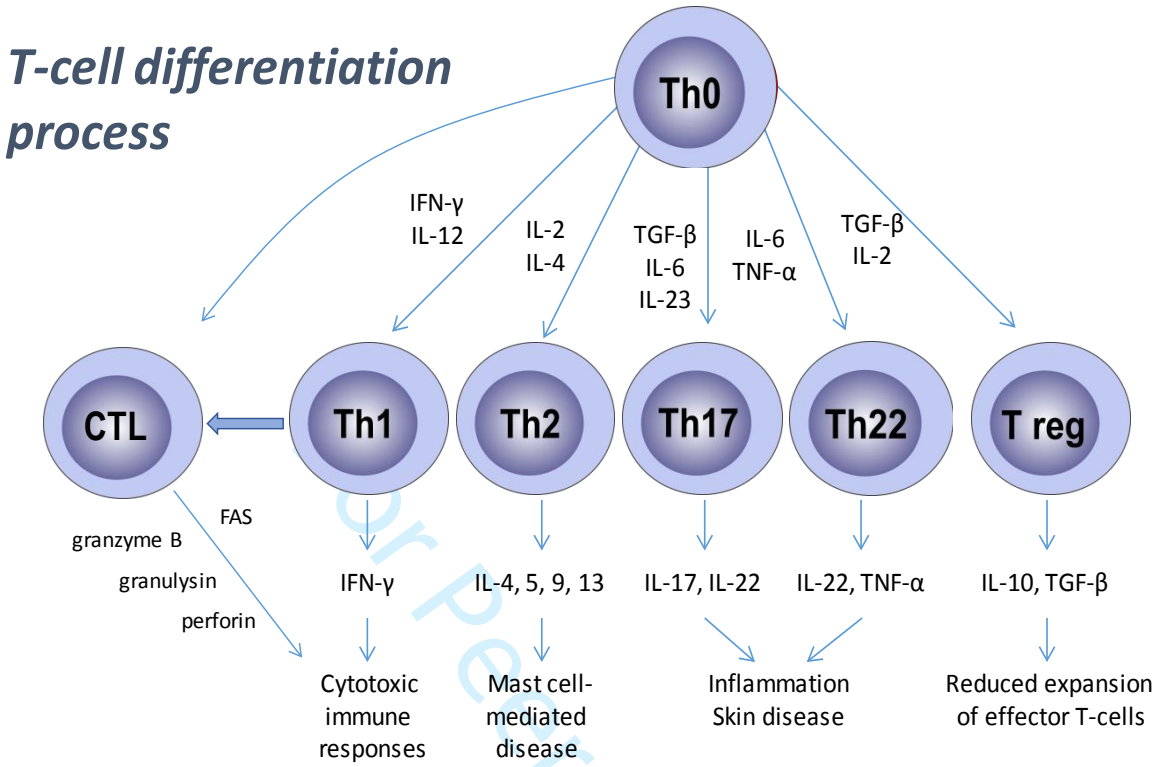


Figure 6. Cytokine control of T-cell differentiation.



Text box 1. Major Milestone Discoveries

- Drug, drug metabolite and drug-modified peptide HLA binding activates T-cells in patients with hypersensitivity
- Development of assays with PBMC from healthy human donors to study naïve drug peptide complex T-cell priming *ex vivo*
- Individual HLA alleles are important determinants of disease susceptibility
- Characterisation of HLA-allele-restricted drug-specific T-cell responses in patients with drug hypersensitivity.
- Co-inhibitory receptors impact on the ability of drug peptide complexes to activate naïve T-cells
- Discovery of an increased incidence of drug hypersensitivity reactions in patients receiving immune checkpoint inhibitor therapy

Text Box 2. Future Research Perspectives

Genome-wide association studies and functional assessment of patient T-cells have taught us that drug peptide complexes interact selectively and specificity with HLA proteins to bring about hypersensitivity reactions. It is now important to define, through detailed structural analysis, the way in which drug peptide complexes bind to HLA proteins. The nature of the interaction will differ drug-to-drug. It is also important to determine the contribution different forms of drug peptide complex play in the disease pathogenesis as we know that parent drug, metabolite and drug-modified peptide-responsive T-cells circulate in patients' blood and tissues.

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

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

Short title: Regulatory pathways and drug hypersensitivity

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

Introduction

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

In 2002, Mallal et al. reported a strong association between the presence of HLA-B*57:01, HLA-DR7, and HLA-DQ3 and hypersensitivity to the HIV-1 reverse-transcriptase inhibitor abacavir (7). Subsequent studies demonstrated that (i) all skin test confirmed cases of abacavir hypersensitivity carry HLA-B*57:01 (8), (ii) abacavir interacts selectively with high affinity within the HLA-B57:01 peptide binding cleft through non-covalent interactions (9-11), and (iii) abacavir only activates CD8+ T-cells (12-14). It is important to note that the abacavir association differs from all other forms of HLA-linked hypersensitivity reaction. For example, drug-responsive CD4+ and CD8+ T-cells are observed in patients hypersensitive to drugs such as carbamazepine, dapsone, flucloxacillin who express the relevant HLA class I risk alleles, B*15:02, B*13:01 and B*57:01, respectively (15-17). These data indicate that although there is a preference for drug (parent drug, metabolite) peptide complex HLA T-cell receptor binding in patients, binding interactions are generally heterogeneous and this contributes to the complete adaptive drug-specific T-cell response. Throughout this manuscript we discuss the different forms of drug HLA interaction in detail highlighting similarities and differences in pathways that lead to T-cell activation. However, we subsequently use the general term “drug peptide complex” where appropriate to refer to any drug-derived structure that interacts with HLA proteins and T-cell receptors to trigger T-cell activation. This is because the formation of an HLA, drug, peptide and T-cell receptor complex is necessary for all pathways of T-cell activation. It is simply the nature of the complex and form of binding interaction that differs. As the number of associations between drug hypersensitivity and HLA allele expression increases (18-20), it is important to consider the additional patient factors that confer susceptibility. This is of particular importance because not all patients expressing a risk HLA are susceptible, while many patients lacking known risk alleles go on to develop hypersensitivity when exposed to culprit drugs.

Three factors are critical for the activation of T-cells with drugs; exposure to a drug peptide complex, the availability of a T-cell repertoire for a drug peptide complex and a protein encoded by HLA alleles for drug peptide complex binding. The argument is presented that although each factor detailed above is critical for drug immunogenicity; separately or together, they cannot be used to predict patient outcome following drug exposure. We hypothesize that when each factor is present, active immune regulatory pathways (co-inhibitory receptors, Tregs, cytokines) are key determinants of whether drug exposure will result in hypersensitivity. Since expression and activity of these regulatory pathways are altered by disease, the genetic make-up of the host and environmental factors, it is currently impossible to predict whether drug exposure will result in a health benefit, hypersensitivity or both (Figure 1).

Different manifestations of drug hypersensitivity

Drug-induced cutaneous reactions: Although skin rashes are common forms of drug hypersensitivity, serious and life-threatening reactions develop much less frequently. Examples of serious cutaneous hypersensitivity reactions include Stevens-Johnson syndrome, toxic epidermal necrolysis and drug reaction with eosinophilia and systemic symptoms (DRESS). Although less serious than the conditions listed above, acute generalised exanthematous pustulosis and maculopapular exanthema are also important adverse drug reactions. A broad spectrum of different drugs may cause cutaneous reactions including the sulfonamides, allopurinol, carbamazepine, dapsone and many of the penicillins (21-24) . Although there is some degree of pathophysiological overlap, there are some clinically defining features for each type of severe cutaneous adverse drug reaction and these are briefly discussed below.

The most common skin manifestation is maculopapular exanthema which accounts for approximately 95% of all cutaneous reactions (25). These are reported as eruptions starting on the trunk and upper extremities and progressively become more prevalent. These reactions are not life-threatening and almost always ~~often~~ subside ~~even with~~ when the ~~continued dosing with the~~ culprit drug has been withdrawn (26). Antibiotics and a number of tuberculosis medications such as rifampicin, isoniazid, pyrazinamide and ethambutol are common causes of maculopapular exanthema (27).

Acute generalised exanthematous pustulosis represents a more severe, usually drug-related skin reaction characterised by the presence of sterile pustules on an erythematous surface along with fever and ~~neutropenia~~ neutrophilia in a patient. Furthermore, the involvement of activated neutrophils along with excessive production of cytokines IL-8 and IL-17 is characteristic of acute generalised

exanthematous pustulosis, stimulating the recruitment to tissues and the induction of innate immune responses (28).

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

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

Drug-induced liver injury: The liver is the largest organ in humans; it is the major organ responsible for the metabolism and detoxification of drugs. Hepatocytes (parenchymal cells) make up about 85% of the liver while non-parenchymal cells, including liver sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells and biliary epithelial cells make up the remaining 15% and play important roles in maintaining the homeostasis of the liver. Drug-induced liver injury is a major reason for drug attrition and withdrawal of drugs in clinical trials or drugs already licenced for clinical use (35). Worldwide, the estimated annual incidence rate of drug-induced liver injury is 0.02% (36, 37). Hoofnagle and Björnsson have recently classified drug-induced liver injury into three categories (direct, indirect and idiosyncratic) according to frequency, predictability and reaction mechanisms (38). Direct liver injury is common and occurs rapidly when drugs are given at high doses (e.g., paracetamol). Indirect liver injury has an intermediate frequency, is partially predictable and occurs as an indirect action of the drug on liver or the immune system (e.g., monoclonal antibodies). Finally, idiosyncratic liver injury occurs in only a small number of individuals, is not predictable and involves activation of the patients adaptive immune system. ~~and t~~The mean onset of idiosyncratic liver injury ~~tissue damage~~ with certain drugs exceeds 100 days (39). Amoxicillin, clavulanic acid, NSAIDS, flucloxacillin, lapatinib, lumiracoxib, ximelagatran among other drugs have been implicated 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 pathogenesis involving drug-specific T-cells~~the adaptive immune system~~. Recent studies have identified and characterized drug-responsive CD4+ and CD8+ T-cells from the peripheral blood of patients with tuberculosis medicine-, co-amoxiclav- and flucloxacillin-induced liver injury (41-43). Furthermore, T-cells ~~infiltrate~~ have been shown to infiltrate liver and kill hepatocytes through the release of cytolytic molecules (44, 45).

~~Drug-induced hematologic disorders: Agranulocytosis, aplastic anaemia, megaloblastic anaemia and thrombocytopenia are major forms of drug-induced hematologic disorders. Drugs can directly target progenitor cells in the bone marrow or peripheral blood cells in the systemic circulation~~ (46, 47)~~. These adverse drug reactions are rare but can result in significant mortality. Various classes of drugs have been linked with drug-induced hematologic disorders. Examples include antibacterial, anti-inflammatory, antithyroid, antimalarial, antiepileptic, antidepressant and antipsychotic drugs. Similar to skin and liver reactions, drug-induced hematologic disorders can unpredictable. Genome wide association studies have linked some variants of HLA-DQB1 and HLA-B allele to clozapine-induced agranulocytosis providing evidence for an immune pathogenesis. There is also evidence to suggest that drugs which cause hematologic disorders can activate (i) inflammasomes, (ii) B-cells to produce anti-drug antibodies and (iii) cytotoxic T-cells (~~(48-50)~~ ~~unpublished data).~~

Does drug exposure impact on susceptibility to hypersensitivity?

For this discussion, we assume that the initiating event for T-cell activation is either a drug or drug metabolite binding directly to the HLA T-cell receptor complex (through either covalent or non-covalent binding) or a drug or drug metabolite binding indirectly to non-HLA proteins (through covalent binding; the HLA binding epitope being a peptide derived from the modified protein, which may or may not contain the drug moiety).

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

analysis, Meng et al (63) were able to quantify and compare the level of drug albumin binding in hypersensitive and tolerant patients. No clear differences in the level of β-lactam antibiotic lysine modification was detected between the two patient groups, and importantly, the level of modification in all patients exceeded the threshold required for activation of β-lactam antibiotic-responsive T-cells. Obviously, additional studies are required to explore whether hapten thresholds are exceeded in patients receiving others β-lactam antibiotics and hapteneic drug metabolites. However, currently available data suggests that although the formation of drug protein adducts may be an important, if not critical factor for drug immunogenicity, the level of therapeutic drug exposure does not seem to be a key determinant of patient outcome. One way to confirm this would be a detailed comparison of the incidence of hypersensitivity reactions in patients receiving higher and lower β-lactam doses or longer and shorter treatment courses, as long as this doesn't impact on clinical care.

An assortment of drug structures activate T-cells through a direct non-covalent interaction with HLA and/or specific T-cell receptors. The p-I concept has been coined to explain this phenomenon and differentiate this pathway of T-cell activation from the hapten concept. A number of pieces of experimental evidence support this direct binding concept: first, the addition of parent drug to human immune cell culture systems that express low levels of drug metabolizing enzymes leads to a T-cell response characterized by proliferation and cytokine and cytolytic molecule release (66-68); second, inhibition of protein processing within antigen presenting cells, which blocks T-cell responses to protein antigens has no effect on the activation of T-cells with drugs (69, 70); and third, the kinetics of T-cell activation with drugs is rapid, within minutes (14, 71), which is in stark contrast to classical antigen presentation pathways that require several hours. Many drugs have been shown to activate T-cells from hypersensitive patients via this pathway, including sulfamethoxazole (70), carbamazepine (72, 73) and allopurinol (71). However, with the exception of abacavir, the nature of the drug peptide HLA T-cell receptor interaction is yet to be defined. The selective interaction of abacavir with HLA-B*57:01 alters the spatial arrangement of molecules within the peptide binding groove. This results in the display of novel "altered" HLA-B*57:01 peptide sequences that seemingly go on to stimulate T-cells that bring about abacavir hypersensitivity (9-11, 74). Adam et al. (74) demonstrated that abacavir-responsive T-cells stemming from naïve and memory compartments are detectable in 100% of donors expressing HLA-B*57:01. This led the authors to suggest that abacavir T-cell reactivity by-passes normal co-stimulatory/regulatory requirements. However, we draw readers attention to the fact that it has not been possible to explain why only half of HLA-B*57:01+ donors (who all possess abacavir-responsive T-cells) exposed to abacavir develop hypersensitivity. It should also be noted that p-I- and hapten-responsive T-cells are not always detected in isolation. For the β-lactam antibiotics

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

Drugs administered at a high mass dose more frequently cause hypersensitivity reactions, when compared with drugs administered at lower doses (78). However, in humans, individual drugs tend to be administered at similar doses using dosing regimens directed to achieve drug concentrations within a therapeutic window for a sustained duration of time. Humans are therefore exposed to similar plasma concentrations of the parent drug. A handful of studies describe associations between metabolism (increased production of metabolite or increased exposure to parent drug) and the incidence of drug hypersensitivity reactions (79). For example, CYP2C9*3, which decreases phenytoin clearance is associated with an increased occurrence of anticonvulsant hypersensitivity (80, 81). Similarly, impaired renal function and increased plasma levels of oxypurinol (the metabolite that drives T-cell responses in hypersensitive patients (82)) correlate with the poor prognosis of allopurinol-induced severe cutaneous hypersensitivity reactions (83). However, these findings seem to be an exception, rather than a rule, as few other studies have reported associations between drug disposition and hypersensitivity.

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

Does the display of drug peptide complexes by human leukocyte antigen proteins impact on susceptibility to hypersensitivity?

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

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

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

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

Advances in high-throughput sequencing technologies has enabled the detailed analysis of global T-cell repertoires in patients with and without immunological diseases. Glanville et al. (96) recently defined the minimal requirements for T-cell receptor specificity through an analysis of T-cell receptor sequences using a panel of HLA binding peptides. Focussing on 5711 T-cell receptor Vβ chain sequences from CD4+ T-cells derived from 22 donors with mycobacterium tuberculosis, they identified 141 T-cell receptor specificity groups including 16 groups containing T-cell receptors from at least 3-4 individuals with shared alleles. The T-cell receptors shared HLA alleles from different donors for shared peptide presentation. These data indicate that a diverse array T-cell receptor sequences are available in any individual that interact with peptide ligands from a single protein antigen. Similar technologies

should be applied to the study of drug hypersensitivity to explore whether shared drug peptide complex specificity clusters are present across different donors and whether this correlates with disease.

Our knowledge of how T-cell receptor sequences impact on drug hypersensitivity is in its infancy. Through global expression level analysis and assessment of the third complementary-determining region length distribution of the T-cell receptor profile in patients with carbamazepine-induced Stevens-Johnson syndrome, Ko et al. (97) identified VB-11-ISGSY as a dominant clonotype shared amongst different hypersensitive, but not drug-tolerant, donors. Furthermore, carbamazepine-specific cytotoxic T-cells could be primed from PBMC of healthy human donors that were carriers of both HLA-B*15:02 and VB-11-IsGSY. More recently, the same group working on the same patient cohort reported the detection of a public T-cell receptor composed of paired TCRα CDR3 "VFDNTDKLI" and TCRβ CDR3 "ASSLAGELF" clonotypes and that similar receptor clusters are found in the blister fluid cells and peripheral blood (98). These data suggest that the correct combination of HLA, drug peptide complex and T-cell receptor may be important drivers for carbamazepine-induced Stevens-Johnson syndrome. Unpublished data analysing blister fluid from a different cohort of patients with Stevens Johnson syndrome after administration of multiple drugs also show an enrichment of T-cells that display a selective repertoire of T-cell receptor sequences at the most early phase of the adverse event (Vocanson, personal communication). However, the T-cell receptor identified differs across patients, even those exposed to the same culprit drug. Moreover, a dominant clonotype was not detected in all patients.

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

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

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

While immune cells survey the tissue microenvironment for drug-derived signals, a key task is to maintain tissue homeostasis. The outcome of immune surveillance may be unresponsiveness (the immune system does not detect the drug-derived signal), a conventional effector response (leading to hypersensitivity with a drug-derived signal) or tolerance (a state of immunological unresponsiveness to the drug-derived signal). Tolerance can be natural or induced and these terms are discussed in more detail below with reference to regulatory T-cells. In the context of drug hypersensitivity it is important to consider variation in natural tolerance and whether drug treatment actively induces or alters toleragenic pathways and indeed the potential for certain drug peptide complexes to bypass natural tolerance. The way the immune system ~~does~~ regulates immune responses, and is able to adapt to change, is through the expression of an array of cell surface co-stimulatory and co-inhibitory signalling receptors (Figure 3). Co-stimulatory receptors collect 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

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

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

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

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

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

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

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

The immune regulatory network

Several mechanisms have evolved to regulate T-cell responses and prevent the development of autoimmune disease and other inflammatory conditions. The best known mechanisms of peripheral tolerance include thymic selection of T-cells, the suppressive activity of Tregs (109) and the increased expression of cell surface receptors, the so-called immune checkpoints (110, 111). The importance of immune regulation and power of the regulatory network has been demonstrated clinically through the application of immune checkpoint inhibitors for the treatment of cancer (112). Furthermore, mutations in FOXP3, the regulatory transcription factor for Tregs, results in dysfunctional Tregs and the development of autoimmune disease and allergy (113). IPEX syndrome – a loss of function mutation in FOXP3 (and other regulatory pathways such as CTLA4) - is the most extreme clinical scenario. IPEX syndrome is often fatal presenting clinically for a variety of autoimmune-like syndromes. It would be interesting to investigate whether patients with IPEX syndrome also develop more drug hypersensitivity reactions. Tregs are now easy to expand *ex vivo* and have been used in Phase I clinical trials for the treatment of autoimmune disease to prevent transplant rejection (114).

In the following sections we briefly discuss the major immune regulatory pathways and how dysregulation of these pathways may impact on drug hypersensitivity.

Immune checkpoints

Immune checkpoints are a series of receptor ligand interactions between T-cells and antigen presenting/tissue cells which specifically co-ordinate the secondary co-stimulatory signal required for immune activity following TCR binding. Checkpoint proteins negatively regulate the activation of naïve T-cells. Furthermore, checkpoint receptor expression is upregulated on T-cells when they are activated, providing a negative feedback loop to restrict the effector response. PD-1 and CTLA-4, which are expressed on T-cells, are the most studied immune checkpoints. PD-1 interacts with ligands PD-L1 and PD-L2, which activates tyrosine phosphatases that inactivate tyrosine kinase-mediated activation signals (115). CTLA-4 binds to ligands CD80 and CD86 on antigen presenting cells displaying antigen. T-cell inhibition is achieved through competitive antagonism of CD28 signalling and direct delivery of an intracellular signal (116). Other less well characterized immune checkpoints include TIM-3 (suppresses Th1/Th17 CD4+ responses (117)) and LAG-3 (contributes towards Treg activity and directly suppresses CD8+ T-cells (118)). The complex interaction between immune checkpoints and naïve and memory T-cell subsets and how intra- and inter-individual variation impacts on susceptibility to adverse immunological reactions is ill-defined.

In recent years, we have investigated whether receptor blockade with immune checkpoint inhibitors remove the immune brakes and enhance the priming of naïve T-cells by drugs. Naïve T-cells were cultured *in vitro* with drug and autologous dendritic cells in the presence and absence of immune checkpoint inhibitors targeting PD-1, CTLA-4 and Tim-3 for 14 days to allow priming to occur. Drug exposure was associated with an increase in expression of all three immune checkpoints on dividing T-cells during the culture period, presumably a regulatory event to keep the drug-specific response in check (119). After the 14 day culture period, the primed T-cells were restimulated with drug and a second batch of autologous dendritic cells and the strength of the T-cell response was assessed. PD-1 and CTLA-4 block enhanced the priming of naïve T-cells to drugs, whereas Tim-3 block had no effect (102, 119). A similar effect (enhanced priming of naïve T-cells to drugs) has been demonstrated *in vivo* with PBMC from patients receiving immune checkpoint inhibitor therapy (unpublished data). Furthermore, it is becoming apparent that patients receiving immune checkpoint inhibitor therapy develop more frequent drug hypersensitivity reactions. Ford et al. (120) recently described the development of sulfasalazine (a combination of salicylic acid and sulfapyridine)-induced cutaneous hypersensitivity in 4 patients with metastatic melanoma that had previous been treated with the anti

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

A report of the post-approval safety of the B-raf inhibitor vemurafenib described seven patients that developed serious cutaneous hypersensitivity reactions and importantly, six of these patients received anti-PD-1 antibody therapy prior to starting vemurafenib (122). Phase II studies of ipilimumab plus or minus dacarbazine therapy concluded that ipilimumab monotherapy had a manageable adverse events profile (123), while dual therapy provided no improvement in efficacy and was not tolerable due to serious liver injury (124). Dacarbazine use alone is only associated with rare cases of liver injury (125). The immune checkpoint inhibitor again seems to alter the co-stimulatory/co-inhibitory balance, permitting the development of dacarbazine-induced liver injury in almost all treated patients. Finally, it has been reported that polymorphisms in regulatory targets of immune responses such as CTLA-4 and IL-10 could modulate susceptibility to nonsteroidal anti-inflammatory drug (126) and efavirenz (127) hypersensitivity reactions. Collectively, these data indicate that immune checkpoints act to regulate the strength of the drug-specific T-cell response and hence impact on the balance between tolerance and hypersensitivity (Figure 4). These interactions will become increasingly relevant as the focus on combination therapies for the treatment of various malignancies increases. Combination therapies in oncology started by using two checkpoint inhibitors in combination (αCTLa-4/αPD-1) which illustrated increased efficacy but also an increased incidence of toxicity with a severe toxicity incidence of 56% of patients (128). Latterly there have been an increasing number of trials combining checkpoint inhibitors with additional systemic anticancer therapies including chemotherapy (KEYNOTE189 [ClinicalTrials.gov number, NCT02578680], IMpassion150 [ClinicalTrials.gov number, NCT03125902]) and tyrosine kinase inhibitors (KEYNOTE426 [ClinicalTrials.gov number, NCT02853331]). This has culminated in the use of all three agents in some anticancer regimes eg atezolizumab, bevacizumab, carboplatin and paclitaxel used in combination for the treatment of non-small cell lung cancer (NSCLC) within IMPower150 (ClinicalTrials.gov number, NCT02366143). Given the propensity for immune checkpoint inhibitors to interact and display phenotypically typical hypersensitivity reactions the ability to predict individuals at risk of hypersensitivity or particular drug combinations which carry an increased risk is increasingly important. It also remains to be seen if there is a characterizable dose-toxicity relationship or whether there is a temporal relationship to hypersensitivity. It is known that as monoclonal antibodies, immune checkpoint inhibitors have long

half-lives (6.1-25 days) (129) and receptor occupancy exists for weeks. However it is currently unclear if there is a dynamic relationship with hypersensitivity and the duration of risk.

Tim-3 is an immune checkpoint receptor that interacts with its ligand galectin 9 to modulate Th1 CD4+ T-cell responses. The expression of Tim-3 has recently been shown to be significantly reduced on peripheral blood CD4+ T-cells in the acute phase of drug-induced manculopapular exanthema (130), a classical Th1-mediated iatrogenic disease. Furthermore, galectin 9 expression and release was reduced on dendritic cells. These data indicate that the Tim-3 immune checkpoint also contributes to the maintenance of drug tolerance and the prevention of hypersensitivity reactions.

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

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

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

increase in serious autoimmune side effects. However, it is highly likely that drug hypersensitivity reactions will also become more prevalent.

Regulatory T-cells (Tregs)

Tregs regulate or suppress other cells in the immune system. They control the immune response to self and foreign antigens and help prevent autoimmune disease and allergy. Natural Tregs are identified by expression of the regulatory transcription factor FOXP3. Natural Tregs express CD4+ and CD25+ (141); however, CD25+ is also expressed on other forms of T-cell including activated T-cells. Thus, there was a search for additional classification markers. CD127+ has been identified as a marker that is only expressed at low levels on Tregs and can be used alongside CD4+, CD25+ and FOXP3 to identify natural Tregs (142, 143). Tregs can also be classified according to the expression of a naïve T-cell marker CD45RA (144). CD45RA+FOXP3^{low}CD4+ (CTLA-4^{low}, CD25^{high}, CD127^{low}) cells are referred to as naïve or inducible Tregs. These cells exhibit weak suppressive activity until they differentiate following antigen-mediated T-cell receptor engagement. They differentiate into effector Tregs (CD45RA-FOXP3^{high}CD4+) that display a range of additional markers including CTLA-4, CD25+, PD-1, TIM-3 and secretory molecules such as IL-10 and TGF-β. These cells display a strong inhibitory activity and increase in number in blood with age. Tregs exert their suppressive function through a range of pathways ((144-146) Figure 5). These include the inhibition of antigen presenting cells through expression of immune checkpoint receptors, the release of cytokines such as IL-10 and TGF-β that decrease dendritic cell function and the production of pro-inflammatory cytokines and restriction of IL-2 for effector T-cells through CD25+ ligation.

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

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

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

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

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

Cytokines

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

There are many examples of disease induced cytokine imbalance (168-170) and this could have a major impact on the outcome of drug exposure. Diseases such as HIV and cystic fibrosis predispose individuals to drug hypersensitivity reactions. In patients with HIV the incidence of sulfonamide hypersensitivity is 10 times higher when compared with non-HIV infected patients (171). Cytokine imbalances such as Th1/Th2 switching are common features in patients with HIV as the disease progresses (172), but to date the impact of these changes on susceptibility to drug hypersensitivity has not been studied. Similarly, when patients with cystic fibrosis were compared to the general population, antibiotic reactions were found to be up to three times more common (105). The cystic fibrosis lung represents an area of chronic inflammation with high neutrophil numbers alongside elevated levels of cytokines such as IL-8, IL-1β, IL-6, IL-17 and TNF-α (173-175). Obviously, this will have a colossal impact on the outcome of T-cell receptor triggering through altered antigen presentation as well as differential polarization of the effector T-cell response. However, to date, it has not been possible to establish models/systems to explore this relationship directly.

Conclusions

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

the effector T-cell response. Through the studies discussed herein we are beginning to understand that similar pathways are active in patients at the time of drug exposure and that immune regulation networks contribute towards the outcome of drug exposure: health benefit or a hypersensitivity reaction. Work is required to define how the distinct pathways contribute towards individual susceptibility. Such studies are urgent given the plethora of immune modulatory drugs that are in development, which once approved will be administered alongside traditional low molecular weight drugs. It will also be important to determine whether low molecular weight drugs modulate tolerance pathways in patients and whether this contributes to the successful desensitization of certain hypersensitive patients.

Tables

Table 1. HLA class I allele-associated drug hypersensitivity reactions with known drug HLA binding 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
Carbmazepine Stevens Johnson syndrome	HLA-B*15:02 (89)	Direct labile drug & metabolite binding (178, 179)	Yes, multiple metabolites (180, 181)
Carbmazepine 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

[a]alternative pathways feasible for all compounds, but to date have not been studied

[b]formation of a metabolite does not indicate that they are involved in the reaction

Figures

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

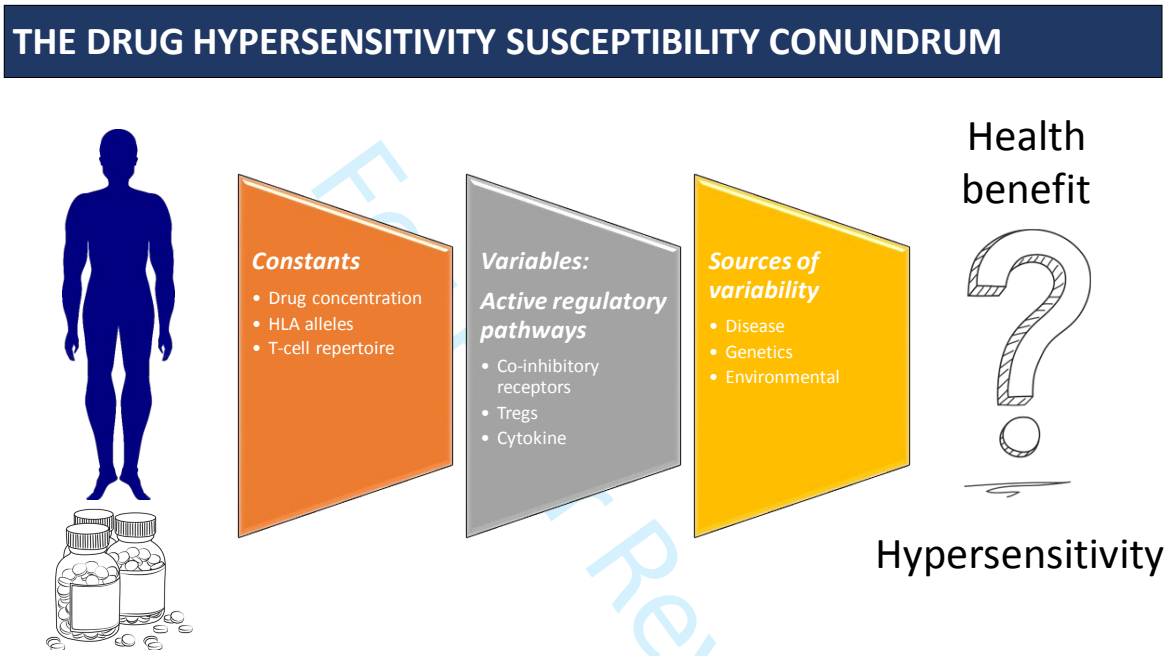


Figure 2. The influence of drug- and patient-specific factors on drug immunogenicity and hypersensitivity. Drug exposure and the availability of HLA proteins and T-cell receptors for drug binding are essential for immunogenicity. However, these factors either together or in isolation do not predict whether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

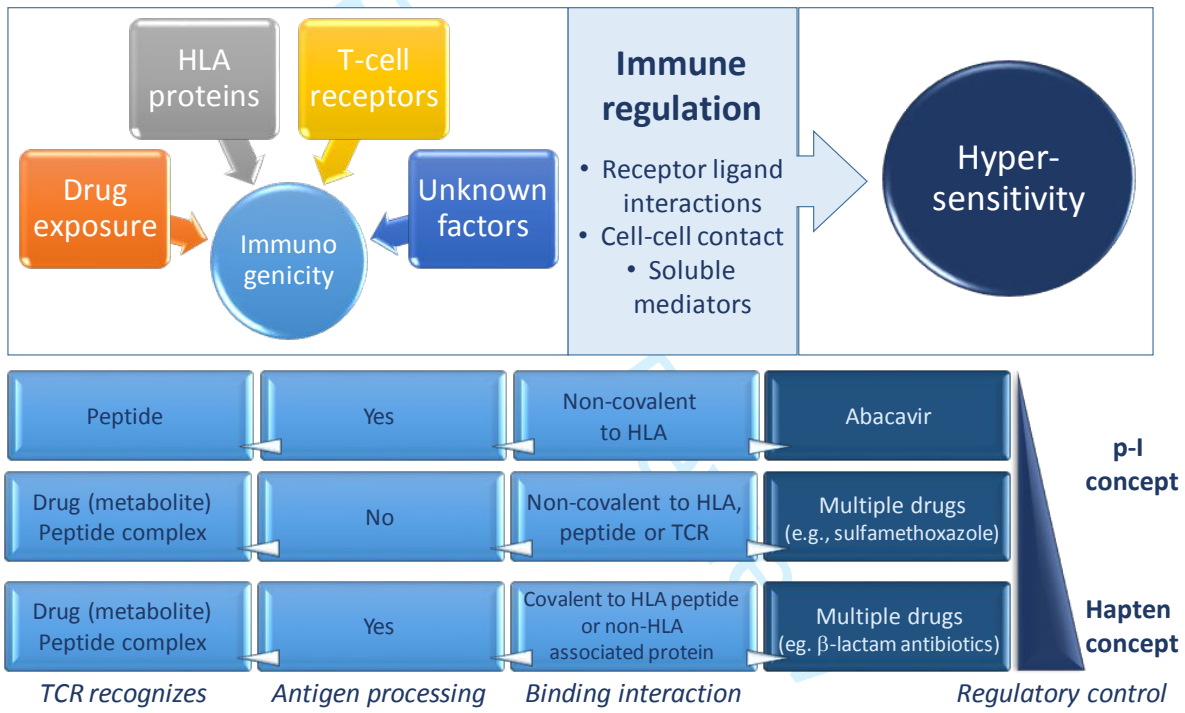


Figure 3. The balance between co-stimulatory and co-inhibitory pathways are the key determinant of whether drug exposure will result in hypersensitivity. This balance is influenced by genetic, disease and environmental factors. Thus, the balance will differ across individuals and within the same individual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

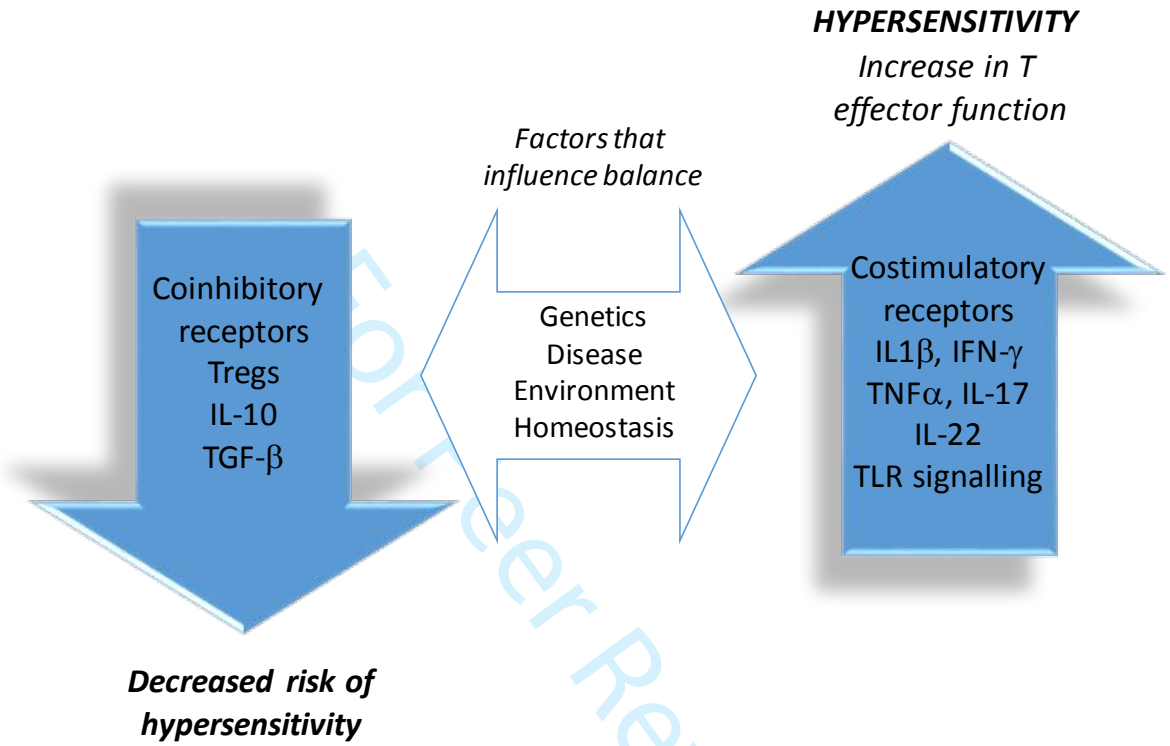


Figure 4. Co-inhibitory receptors regulate the strength of the drug-specific T-cell response and hence the balance between tolerance and hypersensitivity.

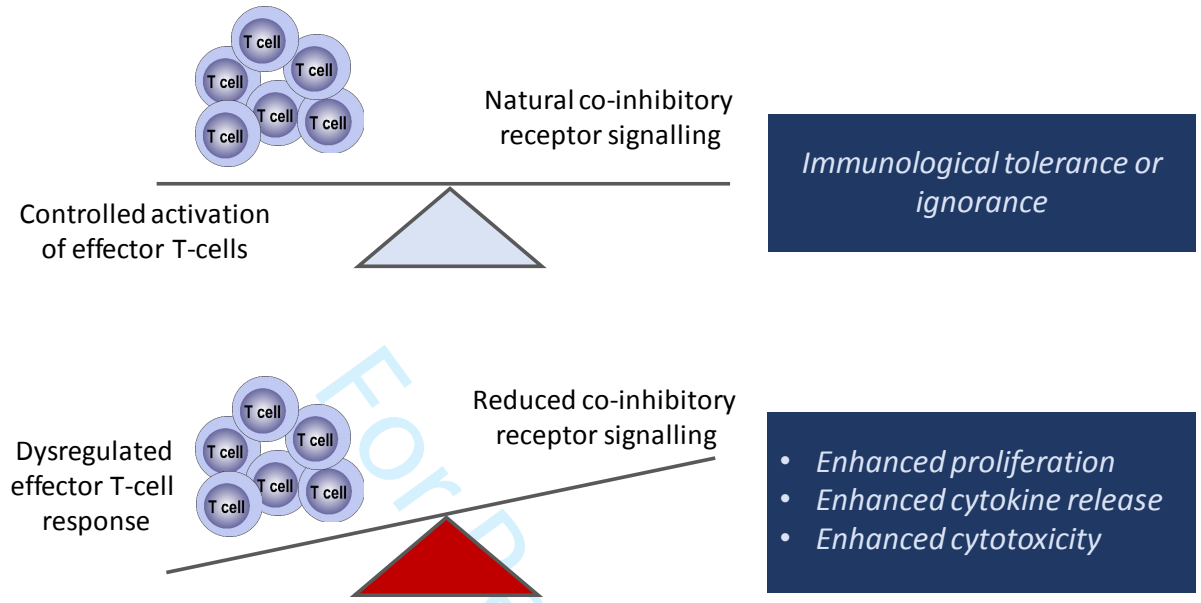


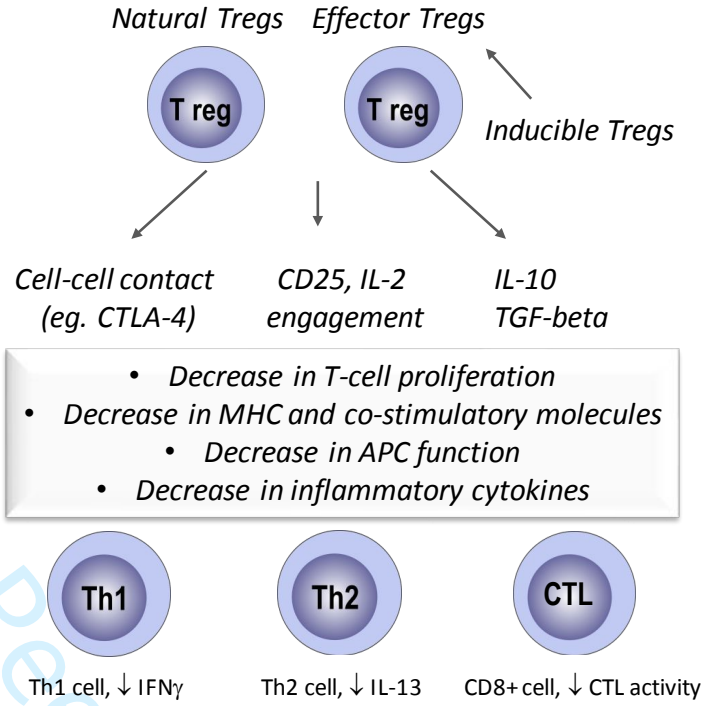
Figure 5. Tregs regulate the strength of antigen-specific effector T-cell responses and hence may alter the balance between tolerance and hypersensitivity following drug exposure.

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?



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

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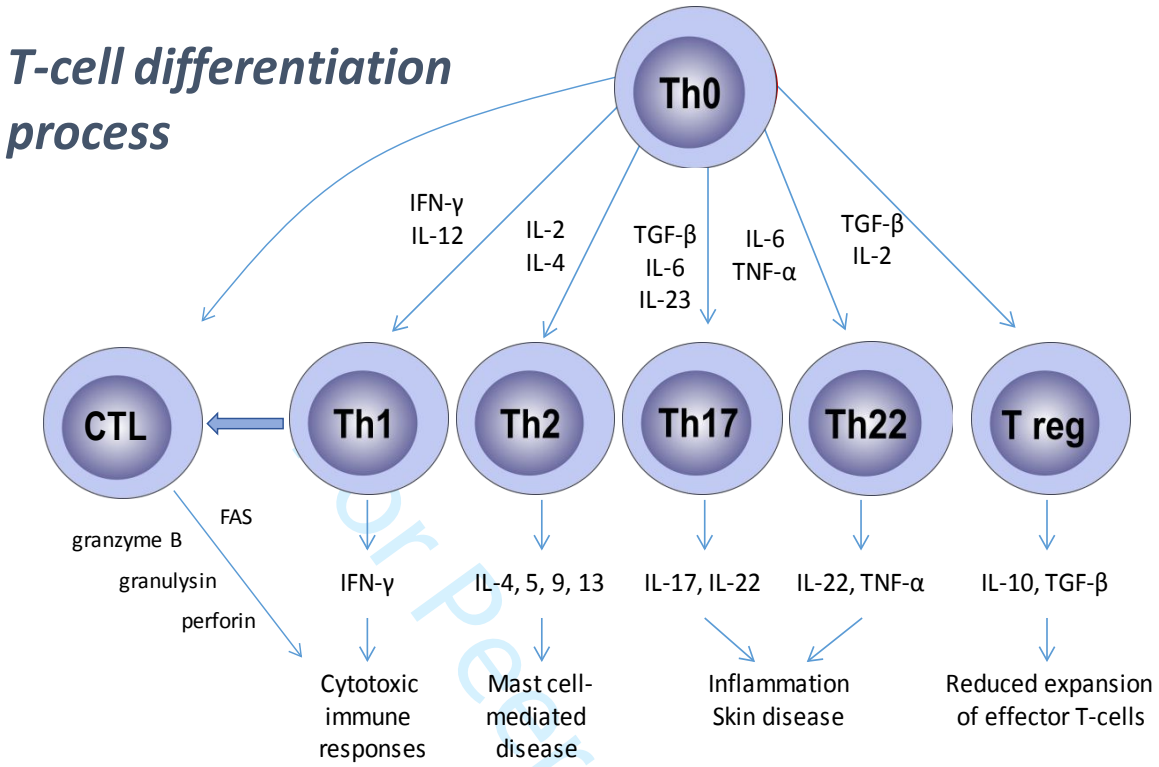

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Text box 1. Major Milestone Discoveries

- Drug, drug metabolite and drug-modified peptide HLA binding activates T-cells in patients with hypersensitivity
- Development of assays with PBMC from healthy human donors to study naïve drug peptide complex T-cell priming *ex vivo*
- Individual HLA alleles are important determinants of disease susceptibility
- Characterisation of HLA-allele-restricted drug-specific T-cell responses in patients with drug hypersensitivity.
- Co-inhibitory receptors impact on the ability of drug peptide complexes to activate naïve T-cells
- Discovery of an increased incidence of drug hypersensitivity reactions in patients receiving immune checkpoint inhibitor therapy

Text Box 2. Future Research Perspectives

Genome-wide association studies and functional assessment of patient T-cells have taught us that drug peptide complexes interact selectively and specificity with HLA proteins to bring about hypersensitivity reactions. It is now important to define, through detailed structural analysis, the way in which drug peptide complexes bind to HLA proteins. The nature of the interaction will differ drug-to-drug. It is also important to determine the contribution different forms of drug peptide complex play in the disease pathogenesis as we know that parent drug, metabolite and drug-modified peptide-responsive T-cells circulate in patients' blood and tissues.

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

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