

The innate immune system in delayed cutaneous allergic reactions to medications

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Purpose of review

Innate immune responses are attracting increasing interest from researchers in the field of drug allergy. This review discusses recent advances in the understanding of several innate immune components in delayed cutaneous hypersensitivity reactions to medications, with special attention on severe reactions.

Recent findings

The mechanism of activation of dendritic cells in response to drugs is being unravelled. Activated monocytes and macrophages have been found in affected skin of bullous diseases. Increased gene expression of monomyeloid cell products including several 'alarmins' or endogenous damage-associated molecular patterns has been described in Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN). Natural killer (NK) cells from patients respond to drugs *in vitro*. In-vivo, NK cells may contribute to severe diseases through the secretion of effector molecules such as granzysin. The innate receptor CD94/NKG2C is expressed by NK cells and cytotoxic T lymphocytes in SJS/TEN and triggers degranulation in response to human leukocyte antigen-E-expressing keratinocytes. T cells with innate activities have been detected in patients during severe acute reactions.

Summary

Humoral and cellular components of the innate immune response have been identified in association with delayed drug hypersensitivity reactions. Their participation in certain diseases may explain the variability of phenotypes in hypersensitivity reactions to medications.

Keywords

alarmins, CD94/NKG2C, drug hypersensitivity, granzysin, monomyeloid cells, natural killer cells

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Introduction

Drug hypersensitivity reactions [1] can be produced by any of the four mechanisms described by Gell and Coombs. Type IV or delayed reactions can now be categorized according to the cytokine pattern and preferential activation of immunocytes [2]. Although various organs may be affected, cutaneous manifestations are the most common. Among them, Drug Reaction with Eosinophilia and Systemic Symptoms (DRESS), and Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) are the most severe clinical entities. DRESS (also called drug induced hypersensitivity syndrome; DIHS) is characterized by a cutaneous rash with organ involvement, hematologic disorders, and herpes virus reactivation. SJS/TEN is characterized by the development of subepidermal blisters and further detachment of large extensions of epidermis, with a mortality rate ranging between 10 and 50% [3].

In-vivo and in-vitro studies provide evidence that T lymphocytes are involved in drug-induced delayed cutaneous diseases, being CD8 T cells the subpopulation most frequently acknowledged as the effector cells in severe reactions [2,4]. In order to elicit a specific immune response, drugs and other xenobiotics behave as haptens or prohaptens and are conjugated to cellular or serum proteins to be presented to T lymphocytes. This represents the hapten hypothesis, and most research efforts during the past two decades have aimed to find drug-specific T-cell receptors (TCRs). In addition, noncovalent associations of the drug to the major histocompatibility complex (MHC) molecule can also stimulate drug-specific T cells [5]. In this manner, the immune system reacts against nonself antigenic determinants defined by drugs, which may be coupled to self-structures. Surprisingly, most individuals tolerate drugs. Along these lines, in 1994 Polly Matzinger published her theory of the danger hypothesis [6], suggesting that the

immune system is not devoted to discriminating self from nonself but rather to distinguish dangerous versus harmless neoantigens. Both the hapten and danger hypotheses are concealed in our current view of the immune response. Basically, the immune system can be divided into two branches: the innate and the adaptive, both with cellular and humoral components. Adaptive immune cells (B and T lymphocytes) take care of the specificity of the response. Naive T and B cells need to receive an alarm signal from the innate immune system in order to differentiate into effector cells.

Although the adaptive immune system is easy to define as components with antigen-specific receptors or antibodies, the limits of the innate immune system are more difficult to delineate because cellular and humoral components of different origins may participate in the innate response. Besides epithelial barriers, innate cells are mainly of hematopoietic origin. Mononuclear and polymorphonuclear phagocytes, dendritic cells, natural killer (NK) cells, and innate lymphocytes such as B1 cells, $\gamma\delta$ T cells, and invariant NKT cells (iNKT) constitute the first line of defense against stress or external aggressions. Innate immune receptors are germ-line encoded. Some recognize common structures found in microorganisms through a series of membrane and intracellular receptors called pattern recognition receptors (PRRs). Toll-like receptors (TLRs) and Nod-like receptors (NLRs) among other PRRs recognize specific pathogen-associated molecular patterns or PAMPs [7]. Other sets of innate receptors expressed mainly by NK cells may recognize missing-self structures or stress-induced self proteins [8].

We are currently learning how the immune system is activated in delayed drug hypersensitivity reactions. In this review, we will discuss some of the recent advances in this area.

Dendritic cell activation in delayed drug-induced cutaneous reactions

Dendritic cells are the main antigen presenting cells (APCs). The activation status of the APC will determine whether a specific T cell will become activated upon encountering the antigen. Using sulfamethoxazole as a model, a recent publication provides formal proof of drug metabolism in in-vitro differentiated human dendritic cells as well as in mouse dendritic cells, and the subsequent formation of protein adducts able to stimulate specific T cells [9]. Although drug bioactivation is not always mandatory in drug hypersensitivity [5], these results together with previous data [10,11] provide evidence of a link between localized metabolism, dendritic cell activation, and drug immunogenicity.

In-vivo, two different dendritic cells subsets can be distinguished: myeloid dendritic cells (mDCs) and

Key points

- Plasmacytoid dendritic cells may be involved in the pathogenesis of drug reaction with eosinophilia and systemic symptoms.
- Genes encoding innate immune products including endogenous damage-associated molecular patterns are overexpressed in Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and may contribute to the pathogenesis of the disease.
- Myelomonocytic cells may contribute to keratinocyte killing by the secretion of TRAIL, TWEAK and myeloperoxidase.
- Drugs may shape natural killer (NK) cell–dendritic cell interactions in allergic patients.
- NK cells and cytotoxic T lymphocytes (CTLs) with NK-like activities are found in diseased skin in SJS/TEN and participate in epidermal necrosis through the secretion of granzyme and other cytolytic factors.
- The innate activating receptor CD94/NKG2C is expressed in circulating and skin-infiltrating NK cells and CTLs in SJS/TEN and triggers degranulation through the recognition of human leukocyte antigen-E in keratinocytes.

plasmacytoid DCs (pDCs), pDCs being the main population involved in viral responses. A transient decrease in peripheral pDCs, and increased numbers of dermal pDCs have been reported in DRESS patients [12]. As reactivation of herpes virus has been detected in most DRESS patients, massive pDC migration to the skin may be related to a local viral response. Recently, selective drug-induced activation of primary pDCs stimulated *ex vivo* with the culprit drug has also been reported [13].

The role of dendritic cells in drug allergy is discussed in more detail in an independent chapter in this issue of *Current Opinion in Allergy and Clinical Immunology*.

Monomyeloid cell products in Stevens-Johnson syndrome/toxic epidermal necrolysis

Microarray gene expression analysis of peripheral blood mononuclear cells (PBMCs) from patients with different clinical entities identified a set of genes that were differentially upregulated in the acute phase of bullous reactions with mucocutaneous involvement [14]. These genes are preferentially expressed in myelomonocytic cells and encode for proteins involved in inflammation, tissue remodeling and leukocyte extravasation. Among them, metalloproteases *MMP8* and *MMP9*, *LNC2* (lipocalin-2), *CD177*, *OLFM4* and *IL-10* were strongly upregulated in SJS/TEN. In this regard, increased MMP-9 activity has been reported in blister fluids [15] and lipocalin-2 has been

shown to prevent MMP-9 autodegradation [16], which may contribute to enhanced MMP-9 activity in patients with bullous diseases.

Monocytes were the main source of IL-10, which was upregulated 20-fold in SJS/TEN patients [14[•]]. IL-10 protein concentrations above normal serum values had been described previously in patients with TEN and were further increased in blister fluids [17,18]. Although IL-10 is typically regarded as an anti-inflammatory cytokine, IFN- γ -dependent pro-inflammatory effects of IL-10 have been described as well [19].

The involvement of cells of myelomonocytic lineage in severe reactions is further supported by the finding of CD14⁺ monocytes and CD1a⁺ dendritic cells in blister fluid [20^{••}] and of CD68⁺ cells in skin biopsies of SJS/TEN patients [21[•]]. Double staining of CD68⁺ and myeloperoxidase (MPO) was shown in affected skin and MPO activity was demonstrated in blister fluids in SJS/TEN [21[•]]. Moreover, CD14⁺ monocytes and CD1a⁺ cells in blister fluids were able to produce the death receptor ligands TNF-related apoptosis-inducing ligand (TRAIL) and TNF-like weak inducer of apoptosis (TWEAK) which were found in significant amounts in blister fluids and were shown to induce apoptosis of in-vitro cultured keratinocytes [20^{••}].

High frequencies of circulating monomyeloid precursors have also been found in DRESS patients [22].

Alarmins or endogenous damage-associated molecular patterns in Stevens-Johnson syndrome/toxic epidermal necrolysis

Evidence is accumulating that tissue damage is recognized by the immune system at the cellular level via the receptor-mediated detection of proteins released in response to pathogen challenge or cell death. The molecules that alert the innate immune system to activate have recently been named 'alarmins' [23]. In turn, cells of innate and adaptive immunity can also secrete alarmins when they are activated by PAMPs or by other alarmins. Endogenous alarmins and exogenous PAMPs activate the same PRRs [24] and some authors consider them to be subgroups of a larger set: the damage-associated molecular patterns (DAMPs) [25]. Several of these 'alarmins' or endogenous DAMPs were detected to be upregulated in SJS/TEN. In particular, high levels of *LTF*, *RNASE2*, *S100A12*, *S100A8*, *S100A9*, *S100P*, *DEFA1A3*, and *DEFA4* transcripts were found in PBMCs during the acute phase of bullous reactions [14[•]]. Among them, S100A8/S100A9 complexes (previously known as calprotectin) have been detected in skin biopsies from SJS/TEN patients [26]. Interestingly, *RNASE2*, whose expression can be induced in human macrophages by

proinflammatory stimuli [27], has recently been shown to induce IL-10 secretion through TLR2 activation [28].

Other members of the 'alarmin' system whose expression increased dramatically during the acute phase of SJS/TEN were *DEFA1A3* and *DEFA4*, coding for α -defensins 1–3 and α -defensin 4 antimicrobial peptides. In addition to their antimicrobial activity, α -defensins have been involved in the regulation of inflammatory processes, including wound repair [29]. However, at high concentrations, defensins can be cytotoxic to mammalian cells [30]. Alpha-defensins, also known as human neutrophil peptides are major components of azurophilic granules in neutrophils. Nonetheless, expression has been reported in different hematopoietic cell subsets [30]. Intracellular flow cytometry studies in SJS/TEN patients verified high levels of α -defensin 1–3 protein expression in T lymphocytes during the acute phase of the disease [31[•]]. Specific mRNAs were found in purified CD4 and CD8 T cells from SJS patients as well as in blister fluid cells, in agreement with flow cytometry data. A higher intensity of α -defensin intracellular staining in CD3⁺ CD56⁺ PBMCs from patients during the acute phase was detected as compared with the mean fluorescence intensity of the total CD3⁺ population or their CD3⁺ CD56[−] counterparts. Interestingly, blister fluid T cells from most SJS/TEN patients are enriched in the CD3⁺CD56⁺ subpopulation [32,33[•],34^{••}]. Moreover, α -defensin 1–3 peptides were detected by immunohistochemistry within affected skin, and flow cytometry analysis of blister fluid cells demonstrated α -defensin production in skin-infiltrating mononuclear cells. Furthermore, high plasma levels of α -defensin 1,3 were found in SJS/TEN and were further increased in blister fluids from the same donors [31[•]].

Independent gene expression profiling identified granulysin as a gene that is preferentially expressed in blister fluid cells [34^{••}]. Granulysin is a product of cytolytic granules of NK cells and cytotoxic T lymphocytes (CTLs). Surprisingly, granulysin has recently been described to be a TLR4 ligand and to act as an immune 'alarmin' [35[•]]. Given the proposed relevance of granulysin as a marker of SJS/TEN, we will discuss relevant findings relative to this protein in a separate section.

Granulysin in Stevens-Johnson syndrome/toxic epidermal necrolysis

Granulysin is a cytolytic molecule expressed constitutively in NK cells and upon activation in CTLs. It is synthesized as a 15 kDa protein, part of which is cleaved in its amino and carboxyl termini to yield a 9 kDa isoform. Both 15 and 9 kDa forms of granulysin are stored with perforin and granzymes in cytotoxic granules of NK cells and CTLs [36].

Chung *et al.* [34^{••}] found that granulysin transcript levels were the highest amongst the cytotoxic proteins in blister cells compared with PBMCs from patients with SJS/TEN. Moreover, protein levels were extremely high in blister fluids, and correlated with the severity of the disease. Blister fluids caused a 30% reduction in cell viability when incorporated to keratinocyte cell cultures and this effect was reversed when granulysin was depleted from blister fluids. Moreover, injection into mouse skin mimicked SJS/TEN lesions. These findings led to the proposal that granulysin is a key molecule responsible for keratinocyte cell death. In addition, increased levels were found in serum from patients in the 24–48 h prior to skin detachment. This prompted the suggestion of granulysin as a marker for early diagnosis of SJS/TEN [37].

As previously mentioned, granulysin activates APCs through TLR4 and acts as an immune alarmin, promoting the recruitment and activation of APCs. It has been proposed that different forms of granulysin may have distinct roles, with the 15 kDa form being more pro-inflammatory as it is 10 times more potent in the activation of APCs. Interestingly, degranulation of NK cells was necessary to fully recover activity in the activation of dendritic cells [35[•]].

Participation of natural killer cells and T cells with innate features in Stevens-Johnson syndrome/toxic epidermal necrolysis

NK cells have been shown to shape immune reactions through their interactions with dendritic cells. NK cells are commonly defined as CD3[−]CD56⁺ lymphocytes and can be divided into two subsets based on CD56 cell-surface density: CD56^{bright} and CD56^{dim} NK cells, each with different functional properties [38]. CD56^{dim} NK cells from amoxicillin-allergic patients upregulated perforin and granzyme B levels and showed increased cytotoxic ability against autologous iDCs when stimulated *in vitro* with the drug. In the same way, amoxicillin enhanced production of IFN- γ by CD56^{bright} NK cells [39[•]].

In a different study [40[•]], NK cells from drug-hypersensitivity patients were shown to degranulate *in vitro* upon stimulation with the culprit drug.

In-vivo, NK cells have been detected in blister fluids from SJS/TEN patients [33[•],34^{••}] and have been shown to express granulysin [34^{••}]. In the same paper it was demonstrated that blister cells killed autologous and allogenic Epstein-Barr Virus (EBV)-transformed B lymphoblasts either in the presence or absence of the culprit drug with the same efficiency. In addition, blister cells were able to kill allogenic keratinocytes in the absence of

the drug. These data demonstrate the presence of human leukocyte antigens (HLA)-unrestricted innate cytotoxic lymphocytes in the skin infiltrates. Moreover, primary NK cells directly isolated from blister fluids have been shown to degranulate in the presence of autologous keratinocytes [33[•]], further demonstrating the participation of NK cells in skin necrosis in SJS/TEN.

The activity of NK cells is regulated through the balance between activating and inhibitory receptors. Some of these receptors bind HLA-class I molecules [8]. In humans, MHC class I molecules comprise the classical (class Ia) HLA-A, HLA-B, and HLA-C, and the non-classical (class Ib) HLA-E, HLA-F, HLA-G, and HLA-H molecules. The best-characterized HLA-Ib molecule is HLA-E, which is the least polymorphic of all MHC-I molecules. HLA-E expression depends on overall HLA-Ia levels in a given cell. It was first identified as the ligand for the germ-line encoded heterodimeric receptors CD94/NKG2A and CD94/NKG2C expressed by NK cells and T cell subsets. NKG2A and NKG2C show highly homologous extracellular regions, but structural differences in their transmembrane and intracytoplasmic domains determine an inhibitory role for CD94/NKG2A and an activating function for CD94/NKG2C [41]. It has been demonstrated that under some circumstances, stimulation of the activating CD94/NKG2C receptor in T cells can circumvent activation of the TCR, thus endowing T cells with innate abilities and reprogramming NKG2C⁺ CTLs into natural killer-like cells [42,43]. Increased expression of CD94/NKG2C was detected in circulating NK cells and CD8 T cells of SJS/TEN patients as compared with healthy donors, and the frequency of NKG2C⁺ cells was much higher in activated blasts. CD94/NKG2C⁺ cells were also present in blister fluids and were found to kill HLA-E-expressing targets as well as HLA-E-expressing allogenic keratinocytes [33[•]].

Moreover, blister CTLs and NK cells degranulated in response to HLA-E-expressing targets and autologous keratinocytes in a CD94/NKG2C-dependent manner [33[•]]. Interestingly, NK cells were able to degranulate when triggered with autologous keratinocytes independently of HLA-E recognition and showed further degranulation when keratinocytes were induced to express HLA-E. A high expression of HLA-E was found in keratinocytes from SJS/TEN patients [33[•]]. Altogether, the data support a role for HLA-E recognition through CD94/NKG2C in the triggering of cytotoxic lymphocytes in SJS/TEN. Nonetheless, additional activating receptors in NK cells seem to be involved in the killing of keratinocytes and the presence of HLA-E-restricted T cells cannot be ruled out. Interestingly, high expression levels of granulysin have been reported in NKG2C⁺ lymphocytes [43]. It is tempting to speculate that

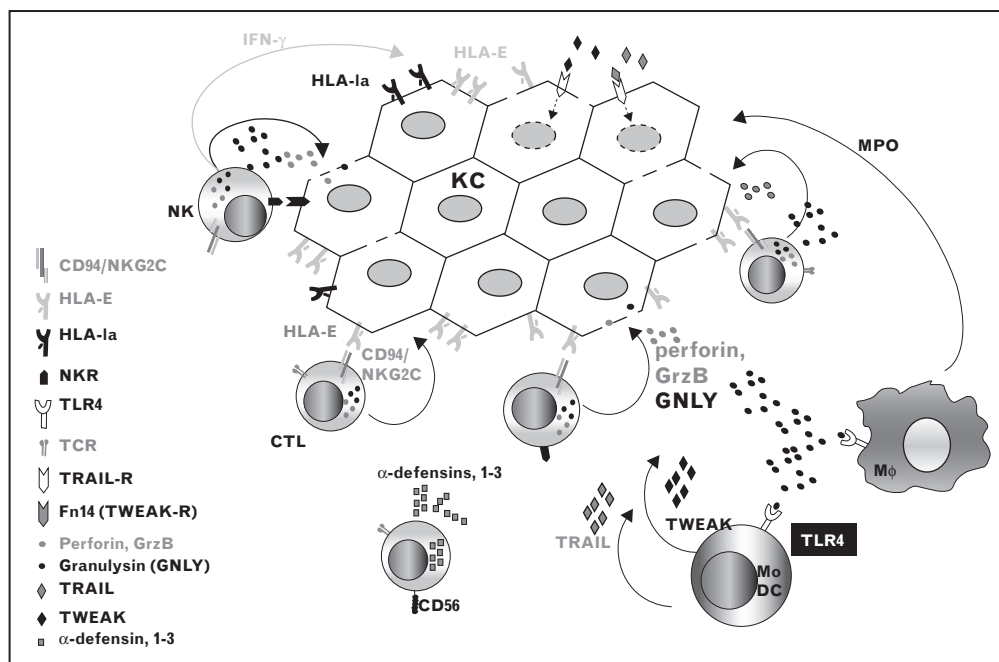
triggering of cytotoxic cells through CD94/NKG2C would lead to a substantial increase in granzysin and perforin levels in blister fluids. In this sense, it is of note that although depletion of either perforin, granzyme B, or Fas ligand (FasL) separately did not have an effect on the cytotoxic properties of blister fluids [34^{••}], these factors are released together upon cell activation and act synergistically to kill target cells. Although granzysin was the most abundant cytotoxic molecule found in blisters, perforin levels were also much higher than those found in serum from healthy donors and were equally related to the severity of the disease [34^{••}]. Along these lines, a recent report [44[•]] shows that cytotoxic cells expressing granzysin require perforin to cause apoptosis of targets, and that recombinant and cell-derived granzysin activate distinct apoptotic pathways in target cells. It is currently assumed that the mononuclear infiltrate in SJS/TEN-affected skin is scarce and would not explain the massive death of keratinocytes, and it has been suggested that cell-mediated cytotoxicity through the release of granzysin does not require cell contact. However, massive release of cytolytic factors by cytotoxic cells involves triggering of activating receptors through ligand recognition on targets. Moreover, the lytic pathways can be maintained, whereas CTL and

NK cells kill multiple targets [45,46]. Even though both the 15 and 9kDa isoforms of granzysin were detected in total cell lysates, only the 15 kDa isoform was detected by western blotting in blister fluids. This result led to the proposal that spontaneous secretion of 15 kDa granzysin is primarily responsible for keratinocyte cell death. However, although 15 kDa granzysin is constitutively released by cytotoxic cells, a substantial amount remains unprocessed as the 15 kDa isoform in the cytolytic granules and is released through the granule exocytosis pathway when NK cells kill target cells. Surprisingly, the 9kDa isoform was barely detected by western blotting in the supernatants of degranulated NK cells [36].

Conclusion

Humoral and cellular components of the innate immune response have been associated mainly with blistering reactions induced by medications (Fig. 1). Amongst the humoral components, several 'alarmins' including α -defensins and granzysin may contribute to the activation of immune cells. Monomyeloid cell products such as TWEAK, TRAIL and MPO may cooperate with cytotoxic lymphocyte-derived factors in inducing

Figure 1 Humoral and cellular components of the innate immune system cooperate to induce keratinocyte cell damage in drug hypersensitivity reactions



A simplified schematic representation of innate immune responses in the skin of severe hypersensitivity reactions to medications. IFN- γ , which is produced by natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), contributes to the enhanced expression of human leukocyte antigen (HLA) class Ia and HLA-E molecules in keratinocytes. HLA-E recognition by innate receptors stimulates NK cells and CTLs with innate features to degranulate and release perforin, granzyme B, and granzysin, which induce keratinocyte cell death. Additionally, granzysin contributes to the recruitment and activation of M ϕ , monocytes and dendritic cells (DCs). MPO, TWEAK, and TRAIL, together with IFN- γ , contribute to keratinocyte cell damage. The α -defensins may also contribute to keratinocyte killing and to the recruitment and activation of monomyeloid cells. GNLY, granzysin; KC, keratinocyte; M ϕ , macrophage; Mo, monocyte; MPO, myeloperoxidase.

keratinocyte death. The cellular components include T cells with innate cell functions such as high expression of α -defensins or CD94/NKG2C-activated killing of targets. Further research is necessary to understand the interactions between dendritic cells and NK cells and comparative studies are needed to dissect these mechanisms in different clinical entities such as DRESS and SJS/TEN.

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