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Negative Regulation by PD-L1 during Drug-Specific Priming of IL-22–Secreting T Cells and the Influence of PD-1 on Effector T Cell Function

Andrew Gibson,*1 Monday Ogese,*1 Andrew Sullivan,* Eryi Wang,* Katy Saide,* Paul Whitaker,† Daniel Peckham,† Lee Faulkner,* B. Kevin Park,* and Dean J. Naisbitt*

Activation of PD-1 on T cells is thought to inhibit Ag-specific T cell priming and regulate T cell differentiation. Thus, we sought to measure the drug-specific activation of naive T cells after perturbation of PD-L1/2/PD-1 binding and investigate whether PD-1 signaling influences the differentiation of T cells. Priming of naive CD4+ and CD8+ T cells against drug Ags was found to be more effective when PD-L1 signaling was blocked. Upon restimulation, T cells proliferated more vigorously and secreted increased levels of IFN-γ, IL-13, and IL-22 but not IL-17. Naive T cells expressed low levels of PD-1; however, a transient increase in PD-1 expression was observed during drug-specific T cell priming. Next, drug-specific responses from in vitro primed T cell clones and clones from hypersensitive patients were measured and correlated with PD-1 expression. All clones were found to secrete IFN-γ, IL-5, and IL-13. More detailed analysis revealed two different cytokine signatures. Clones secreted either Fasl/IL-22 or granzyme B. The Fasl/IL-22–secreting clones expressed the skin-homing receptors CCR4, CCR10, and CLA and migrated in response to CCL17/CCL27. PD-1 was stably expressed at different levels on clones; however, PD-1 expression did not correlate with the strength of the Ag-specific proliferative response or the secretion of cytokines/cytolytic molecules. This study shows that PD-L1/PD-1 binding negatively regulates the priming of drug-specific T cells. ELISPORT analysis uncovered an Ag-specific Fasl/IL-22–secreting T cell subset with skin-homing properties.

Immunological drug reactions represent a major clinical problem because of their severity and unpredictable nature. In recent years, genome-wide association studies have identified specific HLA alleles as important susceptibility factors for certain reactions (1, 2). Drug Ag-specific CD4+ and/or CD8+ T cell responses are detectable in blood/tissue of patients presenting with mild and severe forms of skin (3–5) and liver injury (6, 7) and are therefore believed to participate in the disease pathogenesis. For a limited number of drugs, the drug-derived Ag has been shown to interact specifically with the protein encoded by the HLA risk allele to activate T cells. However, one must emphasize that, with the exception of abacavir hypersensitivity, the majority of individuals who carry known HLA risk alleles do not develop clinically relevant immunological reactions when exposed to a culprit drug. Thus, there is a need to characterize the immunological parameters that are superimposed on HLA-restricted T cell activation to determine why particular individuals develop drug hypersensitivity. Infection, especially reactivation of the herpes virus family (8, 9), has been put forward as an additional risk factor. Virus infection alone, however, does not fully explain the unpredictable nature of drug hypersensitivity.

Thus, our current study focuses on two model drug haptens, nitroso sulfamethoxazole (SMX-NO) and flucloxacillin, to investigate whether the programmed death (PD) pathway regulates the drug-specific priming of T cells from healthy, drug-naive blood donors. Both compounds have been shown previously to activate CD4+ and CD8+ T cells isolated from patients presenting with drug-induced tissue injury (SMX-NO, skin injury; flucloxacillin, liver injury) (4, 6, 10–14). SMX-NO is a cysteine-reactive drug metabolite that binds extensively to cellular protein, whereas flucloxacillin binds directly to lysine residues of serum proteins. This very different chemistry of Ag formation obviates compound-specific effects; as such, any regulation of T cell priming must involve signaling pathways downstream of the drug interaction with protein. Activation of the PD-1 receptor, which is transiently expressed on activated T cells (15, 16), leads to clustering between TCRs and the phosphatase SHP2, dephosphorylation of TCR signaling, and suppression of Ag-specific T cell responses (17). PD-1 has two ligands, PD-L1 (CD274) and PD-L2 (CD273); PD-L1 is expressed on a variety of immune cells, whereas PD-L2 expression is limited to dendritic cells, bone marrow–derived mast cells, and activated macrophages. The PD-1 pathway has already been shown to regulate autoimmunity in several experimental models. Furthermore, genome-wide association studies have identified single-nucleotide polymorphisms in the PD-1 gene in humans that are associated with a higher risk of developing autoimmune disease (18).

Although PD-1 has been classified as a marker of cell exhaustion (19, 20), recent studies from independent laboratories describe an alternative perspective. Duraissamy et al. (21) showed that most PD-1high human CD8+ T cells are effector memory cells rather than exhausted cells. Zelnisky et al. (22) showed that, although virus-specific CD8+ T cells upregulate PD-1 expression during acute
Infection, the majority of PD-1\textsuperscript{high} cells were highly cytotoxic and controlled virus replication. Finally, Reiley et al. (23) showed that PD-1\textsuperscript{high} CD4\textsuperscript{+} T cells were highly proliferative and appeared to maintain effector T cell responses during chronic infection. Consequently, in the current study, T cell clones were isolated directly from SMX-hypersensitive patient PBMC and from healthy drug-naive donors following in vitro priming to characterize the cytokine signature(s) of Ag-specific T cells and to study whether PD-1 expression/signaling governs the differentiation of T cells into effector/helper subsets.

Materials and Methods

**Human subjects**

A total of 120 ml venous blood was collected from drug-naive donors for T cell priming. Blood (60 ml) was also collected from four SMX-hypersensitive patients for cloning. Table I lists the clinical features of the adverse reactions. Approval for the study was acquired from the Liverpool local research ethics committee, and informed written consent was obtained.

**Cell separation**

PBMC were isolated using lymphoprep (Axis-shield; Dundee) by density gradient separation. CD14\textsuperscript{+} monocytes and different T cell populations were separated using magnetic beads and columns, according to the manufacturer’s instructions (Miltenyi Biotec, Bisley, U.K.). CD14\textsuperscript{+} cells were positively selected from total PBMC. For isolation of naive T cells, pan-negative T cell separation was performed using an anti-T cell Ab according to the manufacturer’s instructions (Miltenyi Biotec, Bisley, U.K.). CD14\textsuperscript{+} cells were positively selected from total PBMC. For isolation of naive T cells, pan-negative T cell separation was performed using an anti-T cell Ab according to the manufacturer’s instructions (Miltenyi Biotec, Bisley, U.K.). CD14\textsuperscript{+} cells were positively selected from total PBMC. For isolation of naive T cells, pan-negative T cell separation was performed using an anti-T cell Ab according to the manufacturer’s instructions (Miltenyi Biotec, Bisley, U.K.). CD14\textsuperscript{+} cells were positively selected from total PBMC.

**T cell priming assay**

CD14\textsuperscript{+} cells were cultured in medium (RPMI 1640, 100 μg/ml penicillin, 100 U/ml streptomycin, 25 μg/ml transferrin, 10% human AB serum [Innovative Research], 25 mM HEPES buffer, and 2 mM l-glutamine) supplemented with GM-CSF and IL-4 (800 U/ml; 37°C/5% CO\textsubscript{2}) for 7 d to generate dendritic cells. On the penultimate day, 25 ng/ml TNF-α and 1 μg/ml LPS were added as maturation factors. Dendritic cell phenotype (CD11a, CD11c, CD14, CD40, CD40L, CD83, CD86, CD274, MHC class II) was measured by flow cytometry.

Mature dendritic cells were plated (0.8 × 10\textsuperscript{5} per well) and cultured with naive CD3\textsuperscript{+} T cells (2.5 × 10\textsuperscript{4} per well; 24-well plated total volume 1.5 ml) and SMX-NO (50 μM) or flucloxacinil (2 mM) for 8 d. Anti-PD-L1 and/or PD-L2 Abs (BioLegend, London, U.K.; 100 ng/ml) were added to certain wells. Both Abs have previously been shown to block the receptors (24). Ab concentrations were optimized in dose-ranging studies around the concentration suggested by the supplier. Where indicated, TGF-β (5 ng/ml), IL-1β (10 ng/ml), and IL-23 (20 ng/ml) or TNF-α (50 ng/ml) and IL-6 (20 ng/ml) were added to the cultures to induce the differentiation of Th17 and Th22 cells, respectively. All experiments were performed at least three times using cells from different blood donors with no previous history of sulfonamide/flucloxacinil exposure.

**T cell readouts**

Primed T cells (1 × 10\textsuperscript{5}; 200 μl) were harvested and restimulated with autologous dendritic cells (4 × 10\textsuperscript{5}) and drug (SMX-NO [5–50 μM], flucloxacinil [0.5–2 mM]) and assessed for cytokine secretion (in duplicate cultures per condition) as well as proliferation (in triplicate cultures per condition). After 48 h, [3H]thymidine (0.5 μCi/well) was added to the proliferation plate. Incorporation radioactivity was counted after an additional 16-h incubation using a MicroBeta TriLux 1450 LSC beta counter (PerkinElmer, Cambridge, U.K.). Proliferation was also assessed using CFSE-labeled cells according to our recently published protocol (11). ELISPOT was used, according to the manufacturer’s instructions (Mabtech, Nacka Strand, Sweden), to visualize secreted cytokines (IFN-γ, IL-13, granzyme B, IL-17, and IL-22). Cell phenotype during priming and following restimulation was assessed by staining with CD3\textsuperscript{+}, CD4\textsuperscript{+}, CD4\textsuperscript{−}CD8\textsuperscript{+}, CD4\textsuperscript{+}IFN-γ, and CLA-FITC Abs.

**Flow cytometry**

Cells were acquired using a FACSCanto II (BD Biosciences), and data were analyzed by Cylogic. For CFSE analysis, a minimum of 50,000 lymphocytes was acquired using forward scatter/side scatter characteristics.

**Statistics**

For in vitro priming of naive T cells, all experimental data show the mean of three triplicate incubations. All priming experiments were conducted on at least three separate occasions. To characterize cytokine signatures with clones, multiple clones (up to 17 per experiment) from different

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Clinical Details</th>
<th>Clones Tested (n)</th>
<th>SMX-NO–Specific Clones</th>
<th>CD4</th>
<th>CD8</th>
<th>Control</th>
<th>SMX-NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Female, age 30</td>
<td>Maculopapular rash day 2 of treatment; 7 y since reaction</td>
<td>336</td>
<td>21</td>
<td>100</td>
<td>—</td>
<td>6,840 ± 7,406</td>
<td>14,837 ± 13,224</td>
</tr>
<tr>
<td>2: Female, age 25</td>
<td>Maculopapular rash day 4 of treatment; 5 y since reaction</td>
<td>394</td>
<td>29</td>
<td>100</td>
<td>—</td>
<td>3,899 ± 5,522</td>
<td>23,970 ± 18,651</td>
</tr>
<tr>
<td>3: Female, age 34</td>
<td>Maculopapular rash day 6 of treatment; 8 y since reaction</td>
<td>216</td>
<td>6</td>
<td>100</td>
<td>—</td>
<td>1,058 ± 203</td>
<td>2,740 ± 469</td>
</tr>
<tr>
<td>4: Male, age 23</td>
<td>Maculopapular rash day 10 of treatment; 20 y since reaction</td>
<td>152</td>
<td>12</td>
<td>100</td>
<td>—</td>
<td>1,849 ± 1,659</td>
<td>5,086 ± 3,649</td>
</tr>
</tbody>
</table>

—, CD8\textsuperscript{+} cells not detected.
FIGURE 1. SMX-NO–specific priming of naive T cells with and without PD-L1/PD-L2 block. (A) Ag-specific T cell responses to SMX-NO measured by [³H]thymidine incorporation. (B) Ag-specific T cell responses measured by IFN-γ (B) and granzyme B (C) ELISPOT. Line graphs show mean ± SD of three experiments (*p < 0.05). (D) Flucloxacillin-specific priming of naive T cells with and without PD-L1. Ag-specific T cell responses measured by IFN-γ and granzyme B ELISPOT. Line graphs show mean ± SD of two experiments conducted in triplicate (*p < 0.05).
FIGURE 2. PD-1 expression on CD3+, CD4+, and CD8+ T cells. (A) PD-1 expression on dividing and non-dividing CD3+, CD4+, or CD8+ cells after restimulation with SMX-NO. (B) PD-1 expression on dividing and non-dividing cells during priming and after SMX-NO restimulation (± PD-L1 block). An aliquot of CFSE-labeled cells was taken throughout the culture period, and PD-1 expression was measured.
donors were analyzed. Experiments were conducted in duplicate or triplicate, depending on the availability of cells. Mean values and SDs were calculated, and statistical analysis was performed using paired t test (SigmaPlot 12 software).

**Results**

**PD-L1 block enhances the priming of naive T cells against drug-derived Ags**

For in vitro priming, naive CD3+ T cells from drug-naïve donors were cocultured with autologous mature dendritic cells in the presence of SMX-NO (±PD-L1/PD-L2 block). Dendritic cells were routinely stained for costimulatory receptors and characterized as CD1a<sup>negative</sup>CD1a<sup>high</sup>CD11<sup>high</sup>CD14<sup>negative</sup>CD40<sup>high</sup>CD80<sup>high</sup>CD83<sup>low</sup>CD86<sup>high</sup>MHC class II<sup>high</sup> and PD-L1<sup>high</sup> (data not shown). As described previously, SMX-NO does not significantly alter the maturation status of mature dendritic cells. Upon restimulation, dose-dependent Ag-specific proliferation was clearly detectable (Fig. 1A; p < 0.05 SMX-NO 12.5–50 μM). Inclusion of PD-L1 blockade markedly enhanced the proliferative response (p < 0.05; at each SMX-NO concentration). PD-L2 block, however, gave proliferative responses comparable to those without PD-ligand block (Fig. 1A). Blockade of PD-L1 and PD-L2 together produced enhanced proliferation compared with medium alone (p < 0.05 SMX-NO 12.5–50 μM), but less so than for PD-L1 block.

Additional SMX-NO–priming experiments were performed using IFN-γ and granzyme B secretion as readouts. IFN-γ (Fig. 1B) and granzyme B (Fig. 1C) were released from SMX-NO–primed cells following restimulation, and the response was enhanced when the anti–PD-L1 Ab was included in the coculture.

To explore whether PD-L1 regulates priming against other drug Ags, naive T cells from HLA-B*57:01+ donors were cocultured with autologous dendritic cells and flucloxacillin (2 mM; ±PD-L1/PD-L2 block). In initial experiments, activation of naive T cells with flucloxacillin was only detected with PD-L1 block (Fig. 1D, *left-hand side*); IFN-γ and granzyme B release above that of background was discernible with flucloxacillin. The granzyme B ELISPOT was then repeated on two further occasions with naive T cells from different donors (using triplicate samples per culture condition). Low levels of granzyme B secretion were detectable when T cells primed in the absence of PD-L1 block were stimulated with flucloxacillin, and this was significantly enhanced using T cells primed in the presence of PD-L1 block (Fig. 1D, *right-hand side*).

To confirm Ag specificity, SMX-NO– and flucloxacillin-primed T cells were cultured with the alternative drug prior to analysis of proliferation and/or cytokine secretion. SMX-NO– and flucloxacillin-primed cells were not activated with flucloxacillin and SMX-NO, respectively (results not shown).

**PD-1 expression is enhanced on drug-primed dividing T cells**

CFSE staining was also used to measure PD-1 on dividing and nondividing T cells. In initial experiments, PD-1 expression was found to be significantly upregulated on dividing CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells 48 h after SMX-NO restimulation (Fig. 2A). In subsequent experiments, PD-1 expression was measured during SMX-NO priming and for 72 h after restimulation. Little or no expression of PD-1 was detected on naive CD4<sup>+</sup> and CD8<sup>+</sup> cells. After priming, a small population of PD-1–positive cells was seen on day 7, both in the presence and absence of PD-L1 block. After restimulation with SMX-NO (day 9), PD-1 expression was rapidly upregulated on 20–40% of CD4<sup>+</sup> and CD8<sup>+</sup> cells in a transient fashion (Fig. 2B, *columns 1 and 3*). PD-1 reverted back to prere-stimulation levels within 48–72 h. Based on the intensity of PD-1 staining detected by flow cytometry, it would seem that activated CD4<sup>+</sup> T cells expressed higher levels of PD-1, when CD4<sup>+</sup> and CD8<sup>+</sup> T cells were compared. Collectively, these data show that CD4<sup>+</sup> and CD8<sup>+</sup> T cells are activated during drug-specific priming, and the activated cells express high levels of PD-1 in a transient fashion.

In the presence of PD-L1 block, the increase in PD-1 expression was sustained (Fig. 2B, *columns 2 and 4*). Greater than 30% of CD4<sup>+</sup> cells stained positive for PD-1 72 h after restimulation.

**Cytokine signatures secreted from drug-primed T cells**

Cutaneous reactions to drugs have been classified previously according to the phenotype and function of Ag-specific T cells. However, the discovery of new T cell subsets (e.g., Th17, Th22 cells) may render this classification somewhat obsolete. Thus, to study whether PD-1 expression/signaling governs the function of effector/helper T cell subsets, it was first important to characterize...
the cytokine signature(s) of Ag-specific T cells following SMX-NO priming under different polarizing conditions.

In initial experiments, naive CD3+ T cells were cultured with dendritic cells and SMX-NO in the absence of polarizing cytokines. These cells were then harvested, restimulated with SMX-NO, and assayed for IFN-γ, IL-13, IL-17, and IL-22 secretion. SMX-NO–specific secretion of IFN-γ, IL-13, and IL-22 was observed. However, IL-17 release was not detected (Fig. 3).

When the priming assay was repeated under Th22-polarizing conditions, a substantial number of IL-22–secreting colonies was observed for a second time. In contrast, SMX-NO–specific IL-17 secretion was not detected when naive T cells were primed with SMX-NO under Th17- or Th22-polarizing conditions (Supplemental Fig. 2).

Generation of CD4+ T cell clones and characterization of cytokine secretion profiles

To characterize the functionality of drug-responsive T cells and the way in which PD-1 signaling influences effector T cell responses, clones isolated following in vitro priming and from hypersensitive patient PBMC were studied.

Two hundred and eighty-three T cell clones were generated from drug-naive donors following SMX-NO priming in the presence of PD-L1 block. Nineteen CD4+ clones were found to proliferate in the presence of SMX-NO (no drug, 1651 ± 410 cpm; SMX-NO, 4880 ± 913 cpm). SMX-NO–responsive clones secreted IFN-γ, IL-5, and IL-13 following activation (Fig. 4). For comparison, SMX-NO–responsive clones were generated following priming in the absence of PD-L1 block. They were found to secrete a similar panel of cytokines (results not shown).

The number of clones generated from hypersensitive patients, their CD phenotype, and the SMX-NO–specific proliferative response are summarized in Table I. No significant difference in the secretion of IFN-γ, IL-5, or IL-13 was observed when clones from hypersensitive patients and the in vitro priming assay were compared (Fig. 4).

Because hypersensitive patient clones and clones isolated from in vitro priming secreted similar Th1 and Th2 cytokines, a panel of 17 clones (from multiple donors) with a strong growth pattern was selected to study IL-17 and IL-22 secretion. Approximately 50% of clones were found to secrete IL-22 following exposure to SMX-NO. In contrast, IL-17 secretion was only detected with one clone (Fig. 5A). IL-22–secreting clones were isolated from hypersensitive patient PBMC and following in vitro priming (Fig. 5B). Importantly, the isolation of SMX-NO–responsive, IL-22–secreting clones from the priming assay was not dependent on the presence of PD-L1 block.

**FIGURE 4.** SMX-NO–specific activation of T cell clones isolated from hypersensitive patients and following in vitro priming. Clones were incubated with APCs ± SMX-NO, and (A) proliferative responses and (B) IFN-γ, IL-5, and IL-13 were measured using [3H]thymidine incorporation and ELISPOT, respectively (*p < 0.05 when SMX-NO and control wells were compared).
of Th22-polarizing cytokines, and clones were not maintained under Th22-polarizing conditions.

ELISPOT was also used to study secretion of the cytolytic molecules perforin, granzyme B, and FasL. Interestingly, the clones were found to secrete either FasL or granzyme B but not perforin. The IL-22high clones belonged exclusively to the FasL-producing subset (Fig. 5A).

To confirm Ag specificity, SMX-NO clones were cultured with flucloxacillin or carbamazepine prior to analysis of proliferation. SMX-NO–responsive clones were not activated with either drug (Supplemental Fig. 3).

IL-22highFasLhigh clones express skin-homing receptors and migrate in response to CCL17 and CCL27

A panel of seven clones showing the different cytokine signatures (two clones secreting FasLhigh IL-22high granzyme Blow; two clones secreting FasLhigh IL-22low granzyme Bhigh; three clones secreting FasLlow IL-22high granzyme Bhigh) was then selected to explore which clones express CCR4, CCR10, and CLA and hence have the ability to migrate toward skin. All FasLhigh clones expressed high levels of CCR4, CCR10, and CLA (Fig. 6A) and migrated in the presence of CCL17 and CCL27 (Fig. 6B).

PD-L1 signaling does not regulate the functionality of Ag-specific T cells

Although PD-1 is most commonly described as a marker of cell exhaustion, it has also been reported that PD-1high cells are highly cytotoxic and/or proliferative (22, 23). Thus, our SMX-NO–specific clones were used to measure PD-1 expression on individual clones, explore the relationship between PD-1 expression and effector function, and analyze whether PD-L1 block alters the levels or profile of cytokines secreted following Ag stimulation.

Flow cytometric analysis of PD-1 on 40 clones revealed a 4-fold difference in expression (Fig. 7A). PD-1 was stably expressed on the surface of clones; Fig. 7B shows PD-1 staining on two representative clones maintained in culture for 10 d (±SMX-NO stimulation). PD-1 expression did not correlate with the strength of the drug-specific proliferative response or secretion of IFN-γ, IL-5, IL-13, IL-17, IL-22, perforin, granzyme B, or FasL (r² < 0.2 for all parameters tested; results not shown; 17 clones depicted in Fig. 5A were used for the comparisons). Despite this, PD-L1 block resulted in a modest increase in IFN-γ, IL-13, and granzyme B secretion when clones were stimulated with SMX-NO (Fig. 7C).

Discussion

In the current study, we have focused on regulation of drug Ag-specific T cell priming through the PD-1/PD-L1 pathway, the way in which PD-1 signaling influences effector T cell responses, and the functionality of drug-responsive clones generated through in vitro priming and isolated from hypersensitive patient PBMC. The inhibitory function of PD-1 relies on the presence of an immunoreceptor tyrosine-based switch motif. On activation, the switch becomes phosphorylated and subsequently recruits the
protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase 2. This causes the inhibition of downstream pathways through the dephosphorylation of proteins such as CD3 and ZAP70 (16, 25), preventing further T cell stimulation. To assess the effect of PD-ligand blockade and in particular whether this could be used as an immunogenic boost to enhance drug-specific stimulation of naive T cells, we used an in vitro T cell priming assay and the model drug haptens SMX-NO and flucloxacillin. In agreement with our previous study, an 8-d culture period was sufficient to activate naive CD3+ T cells, and SMX-NO–specific responses were readily detectable following Ag recall using readouts for proliferation and IFN-γ or granzyme B secretion. CFSE staining revealed that naive CD4+ and CD8+ T cells were activated during priming. The dividing cells were CD45RO+, indicating a change in phenotype from naive to memory. PD-1 expression was induced on dividing T cells during priming and following Ag recall. An increase in the magnitude of the drug-specific proliferative response and levels of IFN-γ/granzyme B secretion was seen when naive T cells were exposed to PD-L1 block. In contrast, PD-L2 block had no effect and even hindered the increased activation of T cells produced from PD-L1 blockade in two of three donors. Previous studies have shown that PD-1/PD-L2 signaling inhibits TCR-mediated triggering of proliferation and cytokine release. Thus, it is not clear why PD-L2 block did not enhance the priming of naive T cells against SMX-NO. One potential explanation is that B7.1 (CD80), a CD28 costimulatory ligand, is known to interact with PD-L1, but not PD-L2 (26); this, however, requires further investigation. Interestingly, CFSE staining suggested the CD4+ cells might be more sensitive to the effects of PD-L1 block than CD8+ cells.

Next, we sought to establish whether PD-L1/PD-1 signaling negatively regulates the priming of T cells against other drug-derived
FIGURE 7. PD-1 expression on CD4\(^+\) clones and SMX-NO–specific cytokine secretion with and without PD-L1 block. (A) PD-1 expression on SMX-NO–specific CD4\(^+\) clones. (B) PD-1 expression on dividing and nondividing CD4\(^+\) clones. Clones were cultured with or without SMX-NO, and PD-1 expression was measured. (C) Proliferative responses and cytokine secretion from 10 SMX-NO–specific clones with and without PD-L1 block. ELISPOT images show differences in cytokine secretion from two representative clones ± PD-L1 block.
Ags. To do this, we focused on the β-lactam antibiotic flucloxacillin. In contrast to SMX-NO (27–29), which forms antigenic determinants through the irreversible modification of cysteine, the β-lactam ring of flucloxacillin is targeted by nucleophilic lysine residues (6, 30). We have recently characterized drug-responsive CD4+ and CD8+ T cells that express the gut-homing receptors CDR4 and CCR9 from patients with liver injury, but not tolerant controls (6). The HLA-B*57:01 genotype is a major determinant of flucloxacillin-induced liver injury, and, through priming of naive T cells from blood donors carrying the HLA risk allele, it has been possible to link the genetic association to the disease pathogenesis (6, 12, 31). Importantly, priming naive T cells against flucloxacillin generally leads to weak and inconsistent results. Confirmation of priming is often only obtained when flucloxacillin-responsive T cells are cloned. In this study, we clearly show enhanced priming of flucloxacillin-specific T cells with PD-L1 block using IFN-γ and granzyme B ELISPOT as readouts.

Whether PD-1 signaling regulates the activation of Ag-specific memory T cell responses has yet to be fully defined. Previous studies show that PD-1high cells can be highly cytotoxic and that PD-1 expression might be a marker of effector memory function, which seems counterintuitive (21, 22). Thus, using SMX-NO-responsive clones generated from healthy donors through priming and isolated directly from hypersensitive patient PBMC [whose T cells were primed at the time of the adverse reaction (3, 4, 13)], we assessed whether PD-1 expression correlated with the strength of the Ag-specific proliferative response and/or secretion of cytokines/cytolytic molecules. Furthermore, PD-L1/2 blocking Abs were used to assess whether PD-1 signaling regulates the activation of Ag-specific memory T cells. Detailed analysis of 40 clones revealed a 4-fold variation in PD-1 expression on T cells (2), PD-1 was stably expressed for up to 10 d after Ag stimulation, and there was no correlation between PD-1 expression and the magnitude of the drug-specific proliferative response or secretion of cytokines. Nevertheless, subtle increases in IFN-γ, IL-13, and granzyme B secretion were observed when clones were stimulated with SMX-NO in the presence of PD-1 block. PD-L2 block had no significant effect. Clearly, our data may relate in part to the manipulations involved in the long-term T cell culture; however, at present there are no ready alternatives to investigate these effects in humans. Furthermore, fully characterized animal models of drug hypersensitivity are not widely available.

Previous immunohistological studies characterizing the phenotype of T cells infiltrating inflamed skin of patients with maculopapular skin rashes describe the presence of large numbers of CD4+ T cells and lower numbers of CD8+ T cells (32, 33). Studies focusing on the SMX-specific T cell response show that CD4+ and CD8+ T cells can be activated by the drug to secrete cytokytic molecules; however, keratinocytes are specifically killed by CD4+ T cells (34). In agreement with these findings, more recent studies show that most SMX (metabolite)-specific T cells isolated from hypersensitive patients are CD4+ and secrete a mixed panel of Th1/Th2 cytokines, including IFN-γ, IL-5, and IL-13 (4, 13, 35). However, the discovery of new T cell populations (e.g., Th9, Th17, Th22) renders this classification out of date. For this reason, we conducted a detailed analysis of the cytokines released by SMX-NO–specific CD4+ T cells generated through in vitro priming and from hypersensitive patients. Following Ag recall, the SMX-NO–primed T cells from healthy donors were found to secrete IFN-γ, IL-13, and IL-22, but IL-17 secretion was not detected. CD4+ clones isolated from the priming assay also secreted IFN-γ, IL-5, and IL-13, but no IL-17. IL-22 secretion was detected from ~50% of the clones (Fig. 5). A similar pattern of cytokine secretion was seen with clones (IFN-γhigh IL-5high IL-13high IL-22low and IFN-γhigh IL-5high IL-13high IL-22high) isolated from SMX-hypersensitive patients (Fig. 5). IL-22 is a cytokine that modulates tissue responses as expression of IL-22R1 is restricted to nonhematopoietic cells. In skin, IL-22 is expressed at high levels on keratinocytes, and IL-22 has been found to enhance keratinocyte proliferation and inhibit terminal differentiation (36). Furthermore, IL-22 has been shown to mediate inflammatory responses in patients with psoriasis, and IL-22–secreting cells have been identified in patients with allergic contact dermatitis (37–39). To our knowledge, our data are, however, the first to show production of IL-22 alongside IFN-γ by Ag-specific T cells from drug-hypersensitive patients.

Given the heterogeneous secretion of IL-22 by individual CD4+ clones, the release of cytolytic molecules (perforin, granzyme B, and FasL) and expression of skin-homing chemokine receptors were also measured using ELISPOT and flow cytometry, respectively. These studies clearly show that SMX-NO–responsive CD4+ T cells release cytolytic molecules when activated through their TCR. Two subsets of drug-specific clone were identified and classified according to the production of either granzyme B or FasL. Importantly, the IL-22–secreting clones produced FasL following Ag stimulation. A preliminary analysis of skin-homing receptors on seven clones revealed they expressed high levels of CCR4, CCR10, and CLA and migrated toward CCL17 and CCL27, indicating that the receptor expression was functionally relevant. Collectively, these studies identify two pathways of killing by drug-specific T cell clones. The FasL- and IL-22–secreting clones may be crucial mediators of the immunological reaction as they are programmed to migrate toward skin.

In conclusion, our in vitro study found that PD-L1/PD-1 signaling negatively regulates the priming of drug Ag-specific T cells that secrete a heterogeneous pattern of cytokines. These data provide a foundation to explore PD-L1/PD-1 expression and activity in prospective studies of drug immunogenicity.

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Disclosures

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