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J Immunol 2014; 192:2984-2993; Prepublished online 3 March 2014; doi: 10.4049/jimmunol.1302306
http://www.jimmunol.org/content/192/7/2984

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/03/01/jimmunol.1302306.DCSupplemental

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Oxypurinol Directly and Immediately Activates the Drug-Specific T Cells via the Preferential Use of HLA-B*58:01

James Yun,*† Maria J. Marcaida,‡ Klara K. Eriksson,* Heidi Jamin,* Stefano Fontana,§ Werner J. Pichler,* and Daniel Yerly*

Allopurinol (ALP) hypersensitivity is a major cause of severe cutaneous adverse reactions and is strongly associated with the HLA-B*58:01 allele. However, it can occur in the absence of this allele with identical clinical manifestations. The immune mechanism of ALP-induced severe cutaneous adverse reactions is poorly understood, and the T cell–reactivity pattern in patients with or without the HLA-B*58:01 allele is not known. To understand the interactions among the drug, HLA, and TCR, we generated T cell lines that react to ALP or its metabolite oxypurinol (OXP) from HLA-B*58:01+ and HLA-B*58:01− donors and assessed their reactivity. ALP/OXP-specific T cells reacted immediately to the addition of the drugs and bypassed intracellular Ag processing, which is consistent with the “pharmacological interaction with immune receptors” (p-i) concept. This direct activation occurred regardless of HLA-B*58:01 status. Although most OXP-specific T cells from HLA-B*58:01+ donors were restricted by the HLA-B*58:01 molecule for drug recognition, ALP-specific T cells also were restricted to other MHC class I molecules. This can be explained by in silico docking data that suggest that OXP binds to the peptide-binding groove of HLA-B*58:01 with higher affinity. The ensuing T cell responses elicited by ALP or OXP were not limited to particular TCR VB repertoires. We conclude that the drug-specific T cells are activated by OXP bound to HLA-B*58:01 through the p-i mechanism. The Journal of Immunology, 2014, 192: 2984–2993.

Allopurinol (ALP) is a commonly prescribed drug to treat gout; a hypersensitivity reaction occurs in ~2% of patients. Rarely, a severe cutaneous adverse reaction (SCAR) to ALP can develop and manifest clinically as Stevens–Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) or drug rash with eosinophilia and systemic symptoms. Although the overall incidence of SCAR is very low, ALP remains a major cause of SCAR and is the most common cause of SJS/TEN in Europe and Israel (1). Recent studies (2, 3) showed that ALP-induced SCAR is strongly associated with the HLA-B*58:01 allele. All Han Chinese and Thai individuals with ALP-induced SCAR had HLA-B*58:01, and most of the Korean patients also had this allele (2, 4, 5). In all of these populations, the frequency of the HLA-B*58:01 allele is high (6.5–10%) (5). Therefore, recent recommendations suggest that HLA-B*58:01 screening should be considered before prescribing ALP to those from high-risk ancestry, such as Han Chinese or Thai (6, 7). Nonetheless, 45% of patients of European ancestry with ALP-induced SJS/TEN did not have HLA-B*58:01, suggesting that this allele is not an absolute risk factor (3). Furthermore, the positive-predictive value of HLA-B*58:01 is estimated to be only 2.7%, implying that other risk factors are also important (8).

There are three main concepts to explain how T cells can be stimulated by a drug. The hapten concept postulates that a small chemical compound cannot be immunogenic on its own because of its small size. Therefore, it first must bind covalently to an endogenous protein to form an antigenic hapten-carrier complex, which, after processing to an immunogenic hapten-modified peptide, can elicit a T cell response. The second mechanism, known as the “pharmacological interaction with immune receptors” (p-i) concept, suggests that a drug may directly and reversibly bind to TCR and/or HLA protein and not the antigenic peptide (9, 10). Therefore, the immune receptor, rather than the Ag itself, is modified by a noncovalently bound drug. It is a typical “off-target” activity of a drug, whereby the peculiar consequences of the p-i concept are caused by this off-target activity being directed to the immune receptors, leading to immune stimulation. Consequently, the classic Ag-processing pathway normally required for T cell stimulation of proteins is bypassed. Finally, immune reactivity by an altered peptide repertoire was described recently as an alternative mechanism for abacavir hypersensitivity syndrome, which is associated with the HLA-B*57:01 allele (11–13). Through this mechanism, abacavir binds specifically to HLA-B*57:01 and alters the peptide-binding groove, which results in the selection of altered self-peptides that bind abacavir–HLA-B*57:01 complexes. However, the study (14) on HLA-B*15:02 and carbamazepine-induced SJS/TEN showed that this drug binds to this HLA, as well as to other structurally related HLA-B75 family members, directly and reversibly, in a manner consistent with the p-i concept. Unlike HLA-B*57:01 and abacavir, the presence of
certain TCR repertoires is also an important risk factor (15). Furthermore, our group (16) recently showed that fluoxatocillin-specific T cells restricted to HLA-B*57:01 recognize the drug via the p-i mechanism and not as a haptenized peptide. Therefore, it is not clear whether the altered peptide repertoire can be generalized to other HLA-associated drug hypersensitivity syndromes. In particular, it is unknown how ALP or its metabolite oxypurinol (OXP) is able to stimulate T cells. Also, the role of the danger hypothesis, which complements the above three models and postulates that additional danger signals are required to stimulate T cell responses, has not been explored (17).

We (18) showed recently that the HLA-B*57:01 allele is an important factor in generating ALP- or OXP-specific T cell responses from healthy drug-naive donors and that these responses are highly dose dependent. Furthermore, these drug-specific T cells universally lack cross-reactivity and have the ability to discriminate between structurally related ALP and OXP (Fig. 5). However, there has not been a detailed study assessing the reactivity pattern of ALP/OXP-specific T cells. In particular, it is not known whether the hapten concept, the p-i concept, or an altered peptide repertoire is responsible for the reactivity or whether HLA-B*57:01 is involved directly in the drug-specific T cell response. Therefore, we sought to elucidate the interactions among T cells, HLA molecules, and the drugs that are responsible for ALP hypersensitivity.

Our findings initially suggested yet another T cell reactivity pattern in HLA-associated drug hypersensitivity. Unlike HLA-B*57:01 and fluoxatocillin (16), all ALP/OXP-specific T cells were activated via the p-i mechanism, regardless of their HLA-B*57:01 status. Unlike HLA-B*57:01 and abacavir (19), the drug binding was labile, and unlike HLA-B*15:02 and carbamazepine (15), there was no shared TCR usage within reactive T cells. Moreover, OXP-specific T cells from HLA-B*58:01* individuals were selectively restricted to this molecule, whereas ALP-specific T cells also were restricted to other unrelated HLA molecules for the drug recognition. Based on in silico data, OXP binds to the peptide-binding groove of HLA-B*58:01 with higher affinity, in a manner similar to that previously described for other HLA-associated drug-hypersensitivity syndromes (11). Therefore, the drug-specific T cell responses are not simply due to where the drug binds in HLA molecules, they also are a result of how strongly the drug binds to HLA molecules and how that affects the dynamics of the ternary complex formation and recognition by the TCR.

Materials and Methods

Samples from patients and healthy donors

Seven HLA-B*58:01* healthy ALP-naive donors, selected from Bern’s blood donation center, were enrolled in the study. All of them were of European ancestry; their other HLA class I alleles are shown in Supplemental Table I. Additionally, three ALP-allergic HLA-B*58:01* patients and eight HLA-B*58:01* healthy ALP-naive donors were recruited. Absence of the HLA-B*58:01 allele was determined in some cases by staining with anti-HLA class I B17 Ab (US Biological, Swampscott, MA) (16, 20). The study was approved by the ethics committee of the Canton of Bern, and all participants gave informed consent.

T cell line and T cell clone generation

PBMCs were isolated by Ficoll density gradient centrifugation and cultured in RPMI 1640 (Life Technologies, Basel, Switzerland) supplemented with 10% heat-inactivated human AB serum (Swiss Red Cross), 2 mM l-glutamine (Biochrom, Berlin, Germany), 25 μg/ml transferrin (Bietst, Dreieich, Germany), 50 U/ml penicillin, and 50 μg/ml streptomycin (Bioconcept, Allschwil, Switzerland). ALP/OXP T cell lines (TCLs) were generated by incubating lymphocytes (4–6 × 10^6 cells/2 ml in 24-well plates) with 100 μg/ml ALP or OXP (Sigma-Aldrich, Buchs, Switzerland) because this dose was shown to be effective in stimulating ALP/OXP-specific T cell responses from drug-naive donors (18). For control experiments, peptide-specific TCLs and T cell clones (TCCs) were generated using an HLA-B*58:01*–restricted peptide, IaW9 (IALYLOQNWW) (21). Cells were supplemented with 50 IU/ml IL-2 (Roche, Basel, Switzerland) every 2–3 d from day 4+5 onward to maintain Ag-specific proliferation. Every 10–14 d, TCLs were expanded by restimulating with the drug and irradiated autologous PBMCs. ALP/OXP-TCCs were generated by limiting dilution, as described elsewhere (22). The specificity of TCCs was assessed by increased CD107a expression on FACS, IFN-γ ELISPOT assay, or (3H)-release assay, as previously described (15, 18, 23).

T cell stimulation with ALP/OXP

ALP/OXP-TCLs were stimulated with various pulsing conditions on day 10–14 after restimulation. Autologous PBMCs or EBV-transformed B lymphocyte cell lines (EBV-BLCL) were used as APCs. To generate drug-pulsed APCs, they were incubated with either 100 μg/ml ALP or OXP for 16 h at 37°C, followed by three washing steps to remove the unbound drugs. TCLs were then incubated with these either in the presence or the absence of the freshly added drugs for 6 h. In some cases, TCLs were incubated directly with the drug, either without APCs or with unpulsed APCs. Anti-CD107a–PE Ab (BioLegend, San Diego, CA) and 6 μg/ml monensin (Sigma-Aldrich) were added at 1.5 h. Surface staining was performed with anti-CD3–PerCP-Cy5.5, anti-CD4–PE–Cy7, or anti-CD8–allophycocyanin–Cy7 Abs (BioLegend). For TCR Vβ phenotype analysis, a panel of Abs specific for different TCR Vβ (Beckman Coulter Immunotech, Marseille, France) was used in combination with anti-CD170a–allophycocyanin Ab (BioLegend). FACS analysis was performed on a FACSCan cytometer using FCSDivia software (both from BD Biosciences). Lymphocyte-gated CD3^+CD4^+ and CD3^+CD8^+ T cells were analyzed separately for CD107a expression.

Proteasome inhibition

To assess the requirement for proteasome, ALP/OXP-TCLs were pre-incubated with various concentrations of bortezomib (Velcade; Janssen-Cilag, Baar, Switzerland) for 16 h. Afterward, 100 μg/ml ALP or OXP was added to the TCLs for an additional 6 h before FACS analysis was performed. APCs were not used for proteasome inhibition because all TCLs were able to self-present the drug in the absence of APCs (Fig. 1B).

Calcium-influx assay

Calcium-influx assays were performed as previously described (19, 24). Briefly, TCLs were incubated with 2 μg/ml Fluo-4 AM (Invitrogen, Carlsbad, CA) and plated in half-area, clear-bottom, 96-well plates (VWR International, Dietikon, Switzerland) at 2 × 10^5 cells/well. A Synergy 4 microplate reader (BioTek, Winooski, VT) was used with an excitation band of 485/20 nm, and fluorescence was measured at 528/20 nm. Baseline signal (F0) was recorded for 5 min before the addition of the drugs. A total of 2 μg/ml PHA (Brunschwig, Basel, Switzerland) served as a positive control, and the culture medium served as a negative control. Fluorescence was measured for 1 hour. The results are shown as normalized fluorescence (F/F0).

HLA-restriction analysis

HLA restriction of TCLs and TCCs was assessed by FACS. First, self-presentation of the drugs by T cells was minimized by reducing the stimulating drug concentrations. This was possible because ALP/OXP-TCLs are highly dose dependent (18). Then, ALP/OXP-specific responses could be restored by adding an excess of APCs, such as EBV-BLCLs, with higher MHC density in the presence of the drug. The ideal drug doses and cell concentrations were chosen carefully to minimize the self-presentation of the drugs by T cells while achieving adequate presentation of the drugs by APCs for T cell reactivity. To investigate whether HLA-B*58:01 is necessary for ALP/OXP-specific T cell responses, the MHC class I–deficient human lymphoid cell line 721.221 expressing a single HLA class I molecule of interest (HLA-A^*02:01, HLA-B^*51:01, HLA-B^*57:01 or HLA-B^*58:01) was added as APCs (25). These 721.221 transfectants were generated according to Adam et al. (19). In addition, allogeneic EBV-BLCLs with overlapping HLA haplotypes were used to determine other HLA usage.

In silico docking

For in silico docking, ALP and OXP molecular structures were obtained from the ZINC database (ID 13298313 and 13542567, respectively) (26). The structures of 57 HLA molecules that are commonly found in the human population were obtained from the protein data bank when available or modeled from closely related HLA molecules (highest sequence identity) using Modeller 9.12 (Supplemental Table I) (27, 28). AutoDock Tools 1.5.6 was used to assign hydrogen and Gasteiger charges, and docking of the drugs to HLA molecules was performed with AutoDock Vina (29). All docking runs were performed at least twice in the absence of peptide (unless otherwise indicated), and the docking grid of minimum peptide.
dimensions $30 \times 30 \times 30 \text{ Å}$ was used to encompass the entire peptide-binding groove. Additionally, two TCRs from TCCs that recognize ALP or OXP were modeled. Donor 604 ALP-TCC A1 TCR was modeled using PDB 3PL6 chain C and 3UTP chain E for $\alpha$- and $\beta$-chains, and Donor 616 OXP-TCC C7 TCR was modeled using PDB 2XN9 chain A and PDB 2CDE chain B (Supplemental Table III). These models were used for ALP/OXP docking using grids that included either the whole receptor or the region responsible for peptide–MHC (pMHC) recognition.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5.02 (GraphPad, San Diego, CA). Comparisons were performed using the unpaired $t$ test or the nonparametric Mann–Whitney $U$ test. The $p$ values $< 0.05$ were regarded as statistically significant.

**Results**

**Labile binding of the drugs is responsible for ALP/OXP-specific CD8$^+$ T cell response**

As our recent study (18) showed, ALP/OXP-TCLs could be generated from HLA-B$^*$58:01$^+$ ALP-naive healthy donors or HLA-B$^*$58:01$^-$ ALP-allergic donors by incubating PBMCs with either ALP or OXP. FACS analysis showed that ALP/OXP-TCLs specifically increased the expression of degranulation marker CD107a in response to the drug, as previously reported (Fig. 1A) (18). From these ALP/OXP-TCLs, we were able to generate CD8$^+$ ALP/OXP-TCCs from both HLA-B$^*$58:01$^+$ and HLA-B$^*$58:01$^-$ donors.

To understand how ALP or OXP stimulates T cells, we explored whether T cells could be activated by APCs preincubated with the drug, as is the case for abacavir-specific T cells and hapten-specific T cells (16, 19, 30, 31). First, APCs were incubated with either the drug or the culture medium overnight. The next day, these APCs were washed extensively before they were added to ALP/OXP-TCLs, with or without the drug. No ALP/OXP-TCLs reacted to the drug-pulsed APCs if the drugs were not freshly added (Fig. 1B). In contrast, ALP/OXP-TCLs were activated by the freshly added drug whether the APCs were pulsed or not (Fig. 1B). In fact, they were activated by the drug even when the APCs were absent (Fig. 1B). This pattern was observed for both HLA-B$^*$58:01$^+$ ($n = 4$) and HLA-B$^*$58:01$^-$ ($n = 5$) TCLs. This self-presentation of the drugs by the T cells was also preserved in all ALP/OXP-TCCs, regardless of their HLA-B$^*$58:01 status (data not shown). Overall, these findings suggest that the labile binding of ALP and OXP is responsible for T cell reactivity.

**Ag processing is not required to activate ALP/OXP-specific T cells**

Hapten-specific T cells normally require APCs to process haptenated proteins before they are loaded as haptenized peptides onto MHC molecules on the surface (16, 32). Bortezomib is a proteasome inhibitor that can block the Ag-processing pathway of intracellular peptides (19). It also was shown to interfere with the activation of flucloxacinil-specific T cells that depend on hapten formation (16). Although ALP/OXP-TCLs do not need APC for activation, this does not exclude the possibility of intracellular processing of the drugs by the T cells. Therefore, ALP/OXP-TCLs were preincubated with varying concentrations of bortezomib for 16 h to block this Ag-processing pathway and then the drug was added to the TCLs incubated for an additional 6 h. ALP/OXP-TCLs were still able to recognize ALP/OXP, even in the presence of bortezomib (Fig. 1C). Once again, this pattern was observed for both HLA-B$^*$58:01$^+$ ($n = 3$) and B$^*$58:01$^-$ ($n = 4$) TCLs. This implies that the classical Ag-processing pathway of the drug using proteasome is not required for T cell activation.

**ALP or OXP is able to activate T cells immediately by binding directly on the surface**

The p-i concept suggests that a drug is able to stimulate T cells by binding directly and immediately to either MHC molecules or TCR on the surface (10). Given the above findings, we sought to establish further whether the p-i mechanism is involved in the T cell recognition of the drug. First, CD107a expression kinetics of ALP/OXP-TCLs were assessed by incubating the TCLs with the

**FIGURE 1.** CD8$^+$ T cell reactivity to ALP/OXP. (A) Representative FACS plot of an ALP/OXP-TCL from Donor 638 (HLA-B$^*$58:01$^+$) after stimulation with either culture medium or ALP (100 $\mu$g/ml). Cells were gated as lymphocytes positive for CD3 and CD8 expression. FACS plots show CD107a of CD8$^+$ T cells in the absence or the presence of the drug. (B) CD8$^+$ T cell reactivity in the presence of various APC conditions. APCs were incubated with the drug overnight (drug-pulsed APCs) and then washed extensively before they were added to ALP/OXP-TCLs, with or without the drug. The gray lines/triangles represent HLA-B$^*$58:01$^+$ ALP/OXP-TCLs, and the black lines/circles represent HLA-B$^*$58:01$^+$ ALP/OXP-TCLs ($n = 9$). (C) Reactivity of ALP/OXP-TCLs in the presence of varying concentrations of bortezomib, a proteasome inhibitor ($n = 7$). $^{**}p < 0.01$, Wilcoxon signed-rank test.
drug for various lengths of time. Typically, peak CD107a expression was seen at 2–4 h, and the initial CD107a increase was seen at 30 min (Fig. 2A). This pattern was similar to the kinetics of the peptide-specific CD8⁺ T cell response that recognized preformed pMHC (data not shown). This implies that ALP/OXP-TCLs are able to recognize the drug within 30 min of its exposure, about as rapidly as peptide-specific T cells that can recognize preformed pMHC.

The reactivity of ALP/OXP-TCCs was assessed using a Ca²⁺-influx assay to further analyze the kinetics. Remarkably, ALP/OXP-TCCs could be activated within seconds of the drug exposure, regardless of HLA-B*58:01 status (Fig. 2B, left and middle panels). Although this Ca²⁺-influx pattern was similar to that of PHA stimulation, this immediate reactivity was not due to nonspecific T cell activation by the drugs, because peptide-specific TCCs did not show such Ca²⁺ influx in response to ALP or OXP (Fig. 2B, right panel). In addition, the reactivity of ALP/OXP-specific T cells was shown to be highly drug specific because there was no cross-reactivity between these two drugs (18). Altogether, these findings imply that ALP or OXP is able to stimulate the drug-specific T cells immediately by binding directly on the surface.

**OXP-specific T cells from HLA-B*58:01⁺ donors are selectively restricted to HLA-B*58:01 for the drug recognition**

Because the HLA-B*58:01 allele is highly associated with ALP-induced SCAR, we also hypothesized that HLA-B*58:01 plays a role in this interaction (2–5). Our recent data also revealed that the presence of HLA-B*58:01 is an important immunogenic factor in generating the ALP/OXP-specific T cell response (18). To investigate whether this molecule is directly involved, we used APCs expressing different HLA molecules to stimulate T cells in the presence of the drugs, as described in Materials and Methods. This experiment revealed different patterns for the ALP/OXP-specific T cell response. First, all OXP-TCLs (7/7) from HLA-B*58:01⁺ donors were restricted exclusively to HLA-B*58:01 for the drug recognition (Fig. 3A, left panel, Table I). In comparison, some HLA-B*58:01⁺ ALP-TCLs (3/6) or their TCCs were not restricted to this molecule for the drug recognition (Fig. 3A, middle panel, Table I). As expected, ALP/OXP-TCCs from HLA-B*58:01⁻ donors were not restricted to HLA-B*58:01 for their activation (Fig. 3A, right panel, Table I). Interestingly, at the clonal level, not all OXP-TCCs from HLA-B*58:01⁺ donors were restricted to HLA-B*58:01, as was the case for Donor 653 OXP-TCC C3 (Table I). This implies that, within an OXP-TCL, there may be occasional drug-specific T cells that are restricted to other HLA molecules. Nonetheless, these data suggest that HLA-B*58:01⁺ OXP-specific T cells are preferentially restricted to this molecule more than ALP-specific T cells are for their activation.

**ALP/OXP-specific T cells that are not restricted to HLA-B*58:01 molecules are restricted to other MHC class I molecules for the drug recognition**

To determine whether another HLA molecule is used for the drug recognition when HLA-B*58:01 is not used, ALP/OXP-TCLs or TCCs were stimulated with the drug in the presence of HLA-matched allogeneic EBV-BLCLs (Fig. 3B, Supplemental Table I). ALP-TCCs from Donor 604 (HLA-B*58:01⁺) reacted exclusively with EBV-BLCLs from Donors 615 and 616, but not with others, implying that HLA-A*33:03, but not other 21 MHC class I molecules that were also present on EBV-BLCLs, were used by TCR.
Likewise, HLA-B*58:01+ ALP-TCLs from Donor 616 and Donor 638 were restricted to HLA-C*07:02 and HLA-A*01:01 or HLA-A*02:01, respectively (Table I). Similarly, an OXP-TCC from Donor 651 (HLA-B*58:01) was restricted to HLA-C*04:01 molecules exclusively but not to other 19 MHC class I molecules that were also expressed on EBV-BLCLs (Fig. 3B right panel, Table I, Supplemental Table I). Therefore, when HLA-B*58:01 is not used for the drug recognition, another HLA molecule is used instead. Donors 604, 615, and 616 share HLA-A*33:03, which is absent in all other donors (left panel). Donor 603, 631, 651, and 653 share HLA-C*04:01, which is absent in all other donors (right panel). HLA types of various donors are shown in Supplemental Table I.

ALP/OXP-specific T cells do not use limited TCR repertoires

Ko et al. (15) recently showed that the presence of both HLA-B*15:02 and certain TCR repertoires is important for a carbamazepine-specific T cell response. In contrast, abacavir stimulation results in polyclonal expansion of the drug-specific T cells (11, 30). To assess the use of the TCR repertoire in the ALP/OXP-specific response, TCLs were stimulated with the drug, and the drug-specific population was analyzed by gating on the CD3+CD8+CD107a+ population. Only a few TCR Vβ were used in each ALP/OXP-TCL from both HLA-B*58:01+ and HLA-B*58:01− donors (Supplemental Table IV). All five TCCs generated from Donor 604 ALP-TCL had identical TRAV and TRBV, implying they are of the same clonality (data not shown, Table I). In contrast, a cross-sectional analysis of ALP/OXP-TCLs (n = 20) did not reveal any public TCR (Supplemental Table IV). When ALP/OXP-TCLs were generated from the same HLA-B*58:01+ drug-naïve donors (Donors 616, 638, and 650) at different time points, drug-specific T cells with different TCR Vβ were expanded, implying that a particular TCR repertoire within an individual for the drug-specific response is not preserved (Supplemental Table IV).

OXP binds to the F pocket of HLA-B*58:01

In silico docking has been used with accuracy to predict the binding of abacavir and carbamazepine to HLA-B*57:01 and HLA-B*15:02, respectively (11). We first performed ALP and OXP docking on 57 HLA molecules commonly found in the human population; the best docking score was identified for OXP with HLA-B*58:01 (−27.3 kcal/mol) (Table II). In contrast, ALP binds to HLA-B*58:01 with a lower affinity (−26.4 kcal/mol) (Table II). This is remarkably consistent with the epidemiological data that showed the association of ALP hypersensitivity with the HLA-B*58:01 allele and our in vitro data that revealed that HLA-B*58:01 is used more selectively for OXP-specific T cells than for ALP-specific T cells (Table I) (2, 3).

Analysis of the docking results showed that both OXP and ALP are placed within the F pocket of the peptide-binding groove of HLA-B*58:01. Fig. 4 shows the OXP docking solution with the best score, where OXP makes van der Waals interactions with the surrounding residues of the F pocket and a hydrogen bond with the oxygen atom at position 6 in the pyrimidine ring, the hydrogen bond with Arg97 is also disturbed, leading to a lower affinity (Fig. 5). Docking of ALP and OXP to HLA-B*58:01 in the presence of different peptides did not reveal any alternative binding sites for the drugs (data not shown). In addition, docking of ALP and OXP to two different TCRs from
ALP/OXP-TCCs also did not reveal a likely binding site (data not shown). Together, these data suggest that ALP and OXP bind to the F pocket of the peptide-binding groove and that OXP binds to B*58:01 with higher affinity.

**Discussion**

We (18) showed recently that T cells specific for the metabolite OXP are mainly responsible for ALP hypersensitivity, and the HLA-B*58:01 allele plays an important role in generating ALP/OXP-specific T cell responses. In this study, we now show that ALP and OXP activate the drug-specific T cells directly and immediately in a manner consistent with the p-i mechanism. Interestingly, the p-i mechanism is involved for the parent drug and its metabolite in both HLA-B*58:01 + and HLA-B*58:01 − individuals. However, OXP-specific T cells were more selectively restricted to HLA-B*58:01 than were ALP-specific T cells for the drug recognition. Our in silico data could explain this by showing that the extra oxygen in the OXP molecule strengthens the interaction with the F pocket of HLA-B*58:01.

The hapten and prohapten mechanisms have been well described for T cell responses to penicillins and sulfamethoxazole (33–36). However, several features are inconsistent with the hapten mechanism for ALP/OXP-specific T cell responses. First, ALP/OXP-specific T cells require the drug to be present in solution; when the drug-pulsed APCs were washed, the reactivity was completely abolished (Fig. 1B). Because covalently bound hapten is resistant to the washing steps, this implies that the labile drug binding is responsible for T cell activation. Second, ALP/OXP-specific T cell responses were proteasome independent, whereas hapten-dependent flucloxacinil-specific T cells required drug presentation via a proteasome-dependent Ag-processing pathway (Fig. 1C) (16). Finally, the drugs activated ALP/OXP-TCCs immediately, whereas the processing-dependent hapten mechanism exhibited slower kinetics (Fig. 2B) (16, 24, 36). Therefore, ALP/OXP-specific T cells depend on labile, immediate, and direct binding of the drug to TCR or HLA rather than by hapten formation. This finding is particularly interesting because, contrary to an assumption that drug metabolites induce immune response via hapten formation, the metabolite OXP used the p-i mechanism exclusively for T cell activation.

An altered peptide repertoire due to the drug binding to HLA was proposed recently as an alternative mechanism for T cell reactivity in drug hypersensitivity (11–13). Crystallographic structures revealed that, although abacavir binds noncovalently to HLA-B*57:01, it is embedded underneath the altered peptides and is able to form stable peptide–abacavir–HLA-B*57:01 complexes (11). Subsequently, these stable complexes are able to activate abacavir-specific TCCs, even after extensive washing steps (19). These studies also suggest that peptides, and not the drug itself, are directly recognized by the reactive T cells. However, the immediate reactivity of ALP/OXP-TCCs and some abacavir-specific TCCs cannot be explained by an altered peptide repertoire, because this process cannot occur immediately (Fig. 2B) (19). Moreover, it is unlikely that an altered peptide repertoire due to ALP or OXP on HLA-B*58:01 is responsible for T cell reactivity, because washing steps abolish T cell activation (Fig. 1B). Therefore, a noncovalent interaction of the drug with the HLA molecule (p-i HLA) that alters the presented peptide repertoire due to ALP or OXP on HLA-B*58:01 is responsible for T cell activation.
which T cell reactivity in HLA-B*57:01+ individuals was driven by the hapten mechanism (16). Second, ALP and OXP do not bind to HLA-B*58:01 stably, because washing steps removed T cell reactivity. This is in direct contrast to abacavir and HLA-B*57:01; all abacavir-specific T cells were activated by abacavir-pulsed APCs, even when the soluble drug was absent (19).

In HLA-B*58:01+ individuals, not all ALP/OXP-specific T cells were restricted to HLA-B*58:01 for the drug recognition. In fact, although OXP-specific T cells were preferentially restricted to HLA-B*58:01, ALP-specific T cells were less restricted to this allele. This is in contrast to HLA-B*57:01+ abacavir-specific T cells and HLA-B*15:02* carbamazepine-specific T cells, in which these HLA molecules were used exclusively (14, 15, 19, 37). Nonetheless, the fact that ALP/OXP-specific T cells can be restricted to other HLA alleles is consistent with the epidemiological data that showed that the HLA-B*58:01 allele is not required for ALP-induced SJS/TEN (3). Our in silico data revealed that ALP binds less strongly to HLA-B*58:01 than does OXP, and OXP can bind to other HLA alleles with diverse docking scores, suggesting that the drug affinity for the HLA molecules varies, depending on the alleles and the drug and even its metabolite. This is likely an explanation for HLA-B*58:01 being used more preferentially for OXP-TCLs than for ALP-TCLs. However, because ALP hypersensitivity is driven primarily by the OXP-specific T cell response, it is not clear whether the less selective binding of ALP is relevant in vivo (18). Finally, unlike carbamazepine-specific T cells, in which limited TCR repertoires were used, no restricted TCR repertoire was used by ALP/OXP-specific T cells (15).

Table II. Summary results for docking of ALP or OXP to 57 MHC class I molecules

<table>
<thead>
<tr>
<th>HLA</th>
<th>Affinity for ALP (kcal/mol)</th>
<th>Affinity for OXP (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*02:01</td>
<td>-5.6</td>
<td>-5.8</td>
</tr>
<tr>
<td>A*02:06</td>
<td>-5.6</td>
<td>-5.7</td>
</tr>
<tr>
<td>A*02:07</td>
<td>-5.9</td>
<td>-6.1</td>
</tr>
<tr>
<td>A*03:01</td>
<td>-5.4</td>
<td>-5.6</td>
</tr>
<tr>
<td>A*11:01</td>
<td>-5.5</td>
<td>-6.1</td>
</tr>
<tr>
<td>A*23:01</td>
<td>-5.8</td>
<td>-6.6</td>
</tr>
<tr>
<td>A*24:02</td>
<td>-5.8</td>
<td>-6.4</td>
</tr>
<tr>
<td>A*25:01</td>
<td>-5.9</td>
<td>-6.9</td>
</tr>
<tr>
<td>A*26:01</td>
<td>-5.6</td>
<td>-6.1</td>
</tr>
<tr>
<td>A*29:02</td>
<td>-5.6</td>
<td>-6.2</td>
</tr>
<tr>
<td>A*30:01</td>
<td>-5.6</td>
<td>-6.1</td>
</tr>
<tr>
<td>A*30:02</td>
<td>-5.9</td>
<td>-6.6</td>
</tr>
<tr>
<td>A*32:01</td>
<td>-5.7</td>
<td>-6.4</td>
</tr>
<tr>
<td>A*33:03</td>
<td>-5.6</td>
<td>-6.3</td>
</tr>
<tr>
<td>A*68:02</td>
<td>-6.0</td>
<td>-6.4</td>
</tr>
<tr>
<td>A*74:01</td>
<td>-5.4</td>
<td>-5.8</td>
</tr>
<tr>
<td>B*07:02</td>
<td>-6.2</td>
<td>-6.6</td>
</tr>
<tr>
<td>B*08:01</td>
<td>-5.4</td>
<td>-5.8</td>
</tr>
<tr>
<td>B*13:02</td>
<td>-5.4</td>
<td>-6.3</td>
</tr>
<tr>
<td>B*15:01</td>
<td>-5.7</td>
<td>-6.3</td>
</tr>
<tr>
<td>B*15:03</td>
<td>-5.6</td>
<td>-6.2</td>
</tr>
<tr>
<td>B*18:01</td>
<td>-5.5</td>
<td>-6.1</td>
</tr>
<tr>
<td>B*35:01</td>
<td>-6.0</td>
<td>-6.5</td>
</tr>
<tr>
<td>B*35:03</td>
<td>-5.5</td>
<td>-5.9</td>
</tr>
<tr>
<td>B*38:01</td>
<td>-5.6</td>
<td>-6.6</td>
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<tr>
<td>B*40:01</td>
<td>-5.7</td>
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<tr>
<td>B*40:02</td>
<td>-5.5</td>
<td>-5.8</td>
</tr>
<tr>
<td>B*40:06</td>
<td>-5.5</td>
<td>-6.1</td>
</tr>
</tbody>
</table>

HLA-B*58:01 is highlighted in bold type. Both ALP and OXP bind most strongly to HLA-B*58:01 compared with other HLA molecules.

FIGURE 4. Model of OXP binding to the F pocket of HLA-B*58:01. HLA-B*58:01 is shown in surface representation to highlight the peptide-binding groove where OXP has been placed. The hydrogen bond to Arg97 is shown by the dashed line, and the drug-contacting residues are shown as sticks.

FIGURE 5. Structure of ALP and OXP.
Despite these apparent differences in T cell reactivity with regard to HLA-associated drug-hypersensitivity syndromes, our in silico data and those of other investigators (11, 14) suggest that the drug binds to the peptide-binding groove of HLA molecules. How can one explain the above differences in T cell–reactivity patterns? We propose the following model as an explanation for the p-i–HLA mechanism, based on ALP and, particularly, OXP stimulation. Docking studies (11, 14) show that OXP, abacavir, and carbamazepine bind to the peptide-binding groove of HLA molecules in a highly selective manner. However, the peptide-binding groove of stable pMHC on the cell surface is assumed to be unavailable for drug binding (Fig. 6A). Therefore, the drug is considered to bind the peptide-binding groove in the endoplasmic reticulum (ER) before an endogenous peptide is loaded. Although there is evidence for ER loading of abacavir, this alone cannot explain the immediate T cell reactivity to soluble drugs described for abacavir, flucloxacinil, and OXP, because drug loading in the ER takes time (16, 19, 37, 38). A clue to this paradox may be found in the flexibility of pMHC, as described recently for the peptides bound to HLA-B*27:05 and HLA-B*27:09 (39). According to this study, some peptides undergo partial detachment from the peptide-binding groove as the result of increased local flexibility (Fig. 6B). This means that, in some instances, drug binding can take place on the cell surface while the pMHC complex undergoes spontaneous conformational changes (Fig. 6C). These altered conformations of the presented autologous peptides may lead to the recognition of the peptide–drug–HLA complexes as “foreign” (Fig. 6D).

**TABLE III. Summary of the immune mechanisms involved in HLA-associated drug-hypersensitivity syndromes**

<table>
<thead>
<tr>
<th>HLA-B*57:01 and Abacavir</th>
<th>HLA-B*57:01 and Flucloxacinil</th>
<th>HLA-B*15:02 and Carbamazepine</th>
<th>HLA-B*58:01 and ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical manifestations</strong></td>
<td>Abacavir-hypersensitivity syndrome</td>
<td>Drug-induced liver injury</td>
<td>SJS/TEN</td>
</tr>
<tr>
<td><strong>Immune mechanism used by the associated HLA molecules</strong></td>
<td>p-i mechanism</td>
<td>p-i mechanism (preferential)</td>
<td>p-i mechanism</td>
</tr>
<tr>
<td><strong>Immune mechanism used by other HLA molecules</strong></td>
<td>Altered peptide repertoire</td>
<td>Hapten mechanism</td>
<td>Possibly altered peptide repertoire</td>
</tr>
<tr>
<td><strong>HLA molecules used for the drug recognition</strong></td>
<td>HLA-B*57:01</td>
<td>HLA-B*57:01 for p-i mechanism</td>
<td>HLA-B<em>15:02 and the related HLA-B</em>75 superfamily</td>
</tr>
<tr>
<td><strong>Site of drug binding</strong></td>
<td>Peptide-binding groove (F pocket)</td>
<td>Unknown</td>
<td>Peptide-binding groove (D pocket)</td>
</tr>
<tr>
<td><strong>Cross-reactivity with related compounds</strong></td>
<td>Unknown for p-i</td>
<td>Unknown for p-i</td>
<td>No (rarely exceptions can occur)</td>
</tr>
<tr>
<td><strong>TCR usage</strong></td>
<td>Polyclonal</td>
<td>Yes for hapten</td>
<td>Oligoclonal with limited TCR repertoire</td>
</tr>
<tr>
<td><strong>Refs.</strong></td>
<td>(11–13, 19, 44)</td>
<td>(16, 45, 46)</td>
<td>(14, 15, 47)</td>
</tr>
</tbody>
</table>

DRESS, drug rash with eosinophilia and systemic symptoms; MPE, maculopapular exanthema.

**FIGURE 6.** p-i HLA model to explain the interaction among the drug, peptide, and HLA. (A) The drug binding site on the peptide-binding groove is not normally exposed on the stable pMHC on the cell surface. (B) Some pMHCs undergo spontaneous conformational changes in dynamic equilibrium, resulting in partial peptide detachment. The drug binding site may be transiently exposed (B), resulting in drug binding (C) to the peptide-binding groove. This intermediate peptide–drug–HLA complex is unstable and may reverse to (A) or result in complete peptide detachment, with resultant dissociation of the entire HLA complex. (D) In some instances, peptide may assume a new conformation and form a new stable peptide–drug–HLA complex, which may be recognized by T cells as “foreign.”
To form such immunogenic peptide–drug–HLA complexes, several conditions must be satisfied: the peptide–HLA complex must be flexible enough to allow partial detachment of the peptide and exposure to the binding site of the drug, the drug must have sufficient affinity or concentration to allow the binding to take place, the peptide must be able to bind in a new conformation in the presence of a drug–HLA complex, and the peptide–drug–HLA complex must be recognized by T cells as “foreign.” This means that variation in the T cell–reactivity pattern occurs, not because the drug binds to HLA in a different manner but because complex interactions among peptide, drug, and HLA molecules result in variable immunogenic peptide–drug–HLA complexes on the cell surface. For example, the affinity of abacavir for HLA-B*57:01 may be high enough to resist the washing step on the surface, whereas the affinity of OXP for HLA-B*58:01 may be lower, resulting in the removal of OXP from HLA-B*58:01. Alternatively, the peptide–abacavir–HLA-B*57:01 complexes may be less flexible than the peptide–OXP–B*58:01 complexes, allowing the drug to remain in situ and resist the washing step.

If our model is correct, drug affinity to HLA is a small, albeit essential, part of the equation. Because HLA polymorphism can additionally influence peptide repertoire, peptide flexibility, pMHC stability and conformation, and thymic selection of T cell repertoires, these factors introduce a broad spectrum of variability in predicting HLA-associated drug hypersensitivity (39–43). Further studies of the molecular interactions among these various elements will be required to assess the accuracy of our model. Because some of these factors are thought to play a role in the allorecognition of pMHC by cross-reactive T cells, it also would be interesting to determine whether the alteration of pMHC by the drug can induce similar changes, with ensuing T cell alloreactivity (39).

Our study is limited by its in vitro design, particularly with prolonged culture conditions. This can affect TCR Vβ phenotype analysis of TCLs. Whether the ALP- or OXP-specific response in vivo is truly oligoclonal or not cannot be answered. Nonetheless, the cross-sectional analysis of ALP/OXP-TCLs from different individuals and from different drug-naïve donors at different time points shows that a particular TCR repertoire is not used. These findings suggest that restricted TCR usage is not likely to be responsible for ALP hypersensitivity. Furthermore, translating in vitro findings to in vivo situations is complicated, and conclusions should be made with caution. However, in the absence of animal models in HLA-associated drug-hypersensitivity syndromes, our data still offer some insights into the T cell mechanisms responsible for these reactions.

In conclusion, ALP/OXP-specific T cells are activated by direct and immediate interaction of the drugs with pMHC molecules in a manner consistent with the p-i mechanism. HLA-B*58:01 is selectively used in OXP-induced reactions in HLA-B*58:01+ donors, but other HLA molecules may be involved infrequently, as well. However, these interactions are more complex than initially anticipated from the p-i HLA model, because the drug is likely to bind to the peptide-binding groove. The affinity of the drug for the binding site is an important factor where various components of the interactions among the drug, HLA, and peptide contribute to the immunogenicity of the final peptide–drug–HLA complexes. To what extent each component contributes to the overall immunogenicity of the drugs and how these altered conformation of autologous peptides by the drugs elicit T cell responses will need to be clarified in future studies.

Acknowledgments
We thank Dr. M. Gangloff (University of Cambridge, Cambridge, U.K.) and Dr. M. Cascella (University of Bern) for advice about in silico docking data.


