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HLA Haplotype Determines Hapten or p-i T Cell Reactivity to Flucloxacillin

Natascha Wuillemín,* Jacqueline Adam,* Stefano Fontana,† Stephan Krähenbühl,‡ Werner J. Pichler,* and Daniel Yerly*

Dose-induced liver injury (DILI) is a main cause of drug withdrawal. A particularly interesting example is flucloxacillin (FLUX)-DILI, which is associated with the HLA-B*57:01 allele. At present, the mechanism of FLUX-DILI is not understood, but the HLA association suggests a role for activated T cells in the pathomechanism of liver damage. To understand the interaction among FLUX, HLA molecules, and T cells, we generated FLUX-reacting T cells from FLUX-naive HLA-B*57:01+ and HLA-B*57:01− healthy donors and investigated the mechanism of T cell stimulation. We found that FLUX stimulates CD8+ T cells in two distinct manners. On one hand, FLUX was stably presented on various HLA molecules, resistant to extensive washing and dependent on proteasomal processing, suggesting a hapten mechanism. On the other hand, in HLA-B*57:01+ individuals, we observed a pharmacological interaction with immune receptors (p-i)–based T cell reactivity. FLUX was presented in a labile manner that was further characterized by independence of proteasomal processing and immediate T cell clone activation upon stimulation with FLUX in solution. This p-i–based T cell stimulation was restricted to the HLA-B*57:01 allele. We conclude that the presence of HLA-B*57:01 drives CD8+ T cell responses to the penicillin-derivative FLUX toward nonhapten mechanism.

Flucloxacillin (FLUX) is a β-lactam antibiotic that is widely used for treatment of methicillin-sensitive staphylococci that produce penicillinase. Its use has been associated with a characteristic cholestatic hepatitis that is more common in females (1) and the elderly and with prolonged treatment courses (2, 3). The onset is usually within 1–45 d after starting treatment (3). Cholestatic jaundice associated with the use of FLUX is rare but often severe (2, 4, 5). In 2009, a genome-wide association study identified the HLA-B*57:01 allele as a risk factor for FLUX-induced liver injury (FLUX-DILI). Among FLUX-DILI cases, 85% carried the risk allele, which has a frequency of 5% in white populations. Carriers of the HLA-B*57:01 allele have an 80-fold increased risk for developing liver disease on FLUX treatment. Despite the strong association with HLA-B*57:01, only 1 in every 500 to 1000 individuals with this genotype will develop DILI on treatment with FLUX. The study by Daly et al. (6) only considered patients with FLUX-DILI. However, FLUX can cause other hypersensitivity reactions like interstitial nephritis (7) or rash (1). To our knowledge, there are no studies investigating a potential HLA association with these other hypersensitivity reactions.

The poor prognosis of drug-induced liver injury (DILI) makes this type of reaction a major safety issue during drug development and marketing as well as a common cause for the withdrawal of drugs from the pharmaceutical market. DILI can occur as a result of dose-dependent, intrinsic drug toxicity or by idiosyncratic mechanisms. For the latter, two mechanisms may be involved: allergic hypersensitivity or metabolic idiosyncrasy associated with the accumulation of reactive metabolites. In the case of FLUX-DILI, the HLA-B*57:01 association reveals a probable role for activated T cells in the pathomechanism of liver damage. Indeed, FLUX-specific lymphocyte responses were detected in certain patients with liver injury (8), making an immune-mediated pathogenesis very likely. Many hepatotoxic drugs fail to be detected during clinical trials, partly because of the low incidence of liver injury. In the case of FLUX, a prospective test based on genotype for those who may develop DILI will have a very high false-positive rate because the proportion of HLA-B*57:01 carriers who will develop DILI on treatment is very low. Better understanding of the mechanisms leading to liver injury is, therefore, essential to accurately predict and possibly prevent a drug’s potential hepatotoxic effects before its release into the pharmaceutical market.

Nowadays, two main concepts are used for the explanation of T cell stimulation by a drug. First, the hapten theory postulates that small chemical compounds bind covalently to endogenous proteins to form hapten-carrier complexes that are antigenic and induce T cell responses. A typical example is penicillin G and its derivatives, which have been shown to covalently bind to lysine residues of serum proteins (9–11). Second, the pharmacological interaction with immune receptors (p-i) concept implies direct and reversible interactions of the drug between TCR and/or HLA molecules (12, 13). In the last decade, strong associations between drug hypersensitivity and defined HLA alleles have been discovered, like the ones of abacavir and carbamazepine with HLA-B*57:01 and HLA-
B*15:02, respectively. In this context, noncovalent interactions of drugs with HLA molecules have been investigated in several studies. Carbamazepine binds to the surface of HLA-B*15:02 molecules (14), whereas abacavir was shown to interact within the peptide-binding groove of HLA-B*57:01 molecules (15), modifying the property for peptide binding (16–18). This study aims at elucidating the mechanism of FLUX presentation and T cell stimulation by FLUX.

Because FLUX belongs to the penicillin family, it is thought to form haptened peptides. In this study, we show that FLUX can indeed behave as a hapten, as it can be stably bound to proteins and presented after proteasomal processing. In HLA-B*57:01 individuals, however, an additional mechanism of FLUX presentation takes place, leading to the expansion of CD8+ T cells, which recognize FLUX according to the p-i concept in an HLA-B*57:01–restricted manner.

Materials and Methods

Healthy donors

Nine FLUX-naive HLA-B*57:01+ healthy donors (HD) were selected from Bern’s blood donation center according to their HLA-B*57:01 status and enrolled in the study (shown in italics: HD576, HD586, HD587, HD602, HD603, HD617, HD618, HD630, and HD631). Furthermore, eight FLUX-naive HLA-B*57:01 HD were enrolled in the study (HD386, HD468, HD604, HD616, HD632, HD636, HD639, and HD655). The presence of HLA-B*57:01 was excluded by staining with anti-HLA class I B17 Ab (USBiological, Swampscott, MA) and/or HLA class I typing (Table I). All HD gave written informed consent prior to being enrolled in the study, and the study was approved by the local ethical committee.

Primary induction, T cell line, and T cell clone generation

PBMC 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, Bern, Switzerland), 2 mM L-glutamine (Biochorm, Berlin, Germany), 25 µg/ml transferrin (Biotest, Dreieich, Switzerland), 50 U/ml penicillin, and 50 µg/ml streptomycin (Bioconcept, Allschwill, Switzerland). PBMC (4 × 10^6 cells in 2 ml culture medium [CM]) were cultured with various FLUX (Floxelapen; Actavis, Regensdorf, Switzerland) concentrations (1, 10, 100, and 500 µg/ml). Cells were supplemented with 50 IU/ml IL-2 (Roche, Basel, Switzerland) every other day starting from day 5 of T cell culture to maintain Ag-specific proliferation. FLUX-reacting T cells were expanded by three to four in vitro restimulations with autologous PBMC and FLUX every 14 d. FLUX-reacting T cell clones (FLUX-TCC) were generated by limiting dilution as described previously (19), and specificity of TCC was maintained as described previously (20, 21). TCC were considered to be FLUX-reacting if cytotoxicity in the culture exceeded 30% and/or when the difference in the number of spot-forming cells between wells with and without FLUX exceeded 50 (22). TCC were further expanded as previously described (23). Clones were selected for functional studies based on Ag specificity and the availability of cells. TCC that were resistant to further expansion were not used in functional assays.

T cell stimulation with FLUX

T cells were either stimulated with FLUX-pulsed APC (APCFLUX) or with freshly added FLUX (500 µg/ml) in the presence of unpulsed APC (APC + FLUX). The latter is subsequently referred to as FLUX in solution. For FLUX-pulsing, APC were incubated 14 h in CM containing FLUX (500 µg/ml) at 37°C, followed by three washing steps to completely remove unbound FLUX. Unless otherwise stated, autologous EBV-B lymphoblastoid cell lines (BLCL) were used as APC.

Reactivity testing upon FLUX stimulation

On day 14 of every restimulation round, reactivity of T cell lines (TCL) was examined by flow cytometry. T cells were coincubated for 6 h with autologous APC; FLUX in solution, or FLUX-pulsed autologous APC, respectively. After 3 h of incubation, brefeldin A (10 µg/ml), monensin (6 µg/ml; Sigma Chemical, Buchs, Switzerland), and anti–CD107a-PE Ab (BioLegend, San Diego, CA) were added. Surface staining was performed with anti–CD3-PerCP-Cy5.5, anti–CD4–PE–Cy7, and anti–CD8–allophycocyanin–Cy7 Abs (BioLegend). Intracellular staining with anti–IFN-γ–allophycocyanin (BioLegend) was performed according to the BD Cytometric Bead Array (BD Biosciences, Basel, Switzerland). Flow cytometry analysis was carried out on a FACScanCanto I cytometer using FACSDiva software (BD Biosciences).

Calcium influx assay

Calcium influx measurements were performed as described elsewhere (22, 23). Briefly, TCL 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 10^4 cells/well. Measurement was performed on a Synergy 4 instrument (BioTek, Highland Park, VT) with an excitation band of 485/20 nm, and fluorescence was measured at 528/20 nm. Baseline signal (Fb) was recorded for 5 min before the addition of Ags. Subsequently, fluorescence was measured for 70 min. TCL in the presence of CM served as negative control, and stimulation with PHA (2 µg/ml; Brunschwig Chemie, Basel, Switzerland) served as positive control. The results are shown as normalized fluorescence (F/Fb).

Proteasome inhibition

To block proteasome activity, autologous PBMC were preincubated in CM containing the indicated concentrations of bortezomib (Velcade; Janssen-Cilag, Basa, Switzerland) for 12 h at 37°C. Afterwards, 500 µg/ml FLUX was added for 14 h to pulse PBMC. Proteasome inhibition assays were performed with either FLUX-pulsed PBMC (APCFLUX) or FLUX in solution in the presence of autologous PBMC (APCFLUX) as stimulatory agent, as described in the T cell stimulation with FLUX section. PBMC were then coincubated with FLUX-reacting TCL (FLUX-TCL) at a 1:2 ratio in the presence of the indicated concentrations of bortezomib. We used autologous PBMC as APC for proteasome inhibition assays because bortezomib shows enhanced toxicity toward EBV-BLCL (24). If proteasome inhibition assays were performed with FLUX in solution, FLUX-TCL had previously been incubated for 12 h with bortezomib at the indicated concentrations to rule out self-presentation of FLUX. Reactivation of TCL was monitored by flow cytometry after a 6-h stimulation assay and staining for CD107a.

HLA restriction analysis by cytotoxicity assay

HLA restriction of TCL and TCC was monitored by analyzing cytotoxicity toward autologous EBV-BLCL, allogeneic EBV-BLCL with overlaps in the HLA haplotype, and the human lymphoid cell line 721.221 (25) expressing a single HLA class I molecule of interest (HLA-B*57:01, HLA-B*58:01, HLA-B*51:01, or HLA-A*02:01). 721.221 transfectants were generated according to Adam et al. (22). 51Cr-release cytotoxicity assays were performed as described previously (26). Briefly, target cells were labeled with 50 µCi sodium chromate solution (PerkinElmer, Schwerzenbach, Switzerland) for 60 min at 37°C. E/T ratio was 10:1. Specific lysis was calculated as: 100 × ([maximal release] − [spontaneous release])/[maximal release − spontaneous release].

Statistical analysis

Statistical analyses were performed using GraphPad Prism4 (GraphPad Software, San Diego, CA). Results are expressed as mean ± SD. Comparisons were drawn using unpaired t test or Mann–Whitney U test. Each experiment was at least repeated twice. The p values < 0.05 were regarded as statistically significant with *p < 0.05, **p < 0.01, and ***p < 0.001 (95% confidence interval).

Results

In vitro CD8+ T cell responses to FLUX in HLA-B*57:01 FLUX-naive individuals

To define the optimal FLUX concentration at which T cell reactivity can be induced, PBMC from 4 HLA-B*57:01 HD were incubated with various concentrations of FLUX (1, 10, 100, and 500 µg/ml). Induction of T cell reactivity to FLUX was successful after two to four rounds of in vitro restimulation with FLUX and autologous PBMC. T cell reactivity was observed in four out of four HLA-B*57:01 individuals with the highest FLUX concentration and in one out of four HLA-B*57:01 HD with 100 µg/ml FLUX. Lower FLUX concentrations (1 and 10 µg/ml) did not result in any detectable T cell reactivity (Fig. 1A).
by flow cytometry after a 6-h stimulation assay. Reactivity of CD8+ T cells from FLUX concentrations (1, 10, 100, and 500 μg/ml) and restimulated every 14 d. At the end of each restimulation round, T cell reactivity to FLUX was monitored by flow cytometry after a 6-h stimulation assay. Reactivity of CD8+ T cells from HD617 is shown based on CD107a expression after stimulation with FLUX in solution. (B) Cells were gated as lymphocytes positive for CD3 expression. Flow cytometry plots show IFN-γ production (top panel) and degranulation (CD107a, bottom panel) of CD3+ T cells in the absence (left panel) or presence (right panel) of FLUX in solution (500 μg/ml) after the third restimulation. Shown is one representative TCL. (C) The CD4+/CD8+ ratio in the CD3+ T cell population was analyzed at the end of each restimulation round.

T cell reactivity to FLUX was consistently, but not exclusively, observed in the CD8+ T cell subset. FLUX-reacting T cells responded by IFN-γ secretion and CD107a upregulation upon drug stimulation (Fig. 1B) and were cytotoxic against autologous APC. Only part of the CD8+CD107a+ T cells secreted IFN-γ upon FLUX stimulation, indicating that CD107a is a more sensitive marker for the detection of FLUX-reacting T cells. T cell cultures were successfully enriched for FLUX-reacting T cells, which was reflected in a relative expansion of CD8+ T cells compared with CD4+ T cells with increasing number of restimulation rounds (Fig. 1C).

**FIGURE 1.** FLUX-reacting T cells are mainly CD8+ and can be expanded over time. (A) FLUX-TCL from HLA-B*57:01+ HD were induced with various FLUX concentrations (1, 10, 100, and 500 μg/ml) and restimulated every 14 d. At the end of each restimulation round, T cell reactivity to FLUX was monitored by flow cytometry after a 6-h stimulation assay. Reactivity of CD8+ T cells from HD617 is shown based on CD107a expression after stimulation with FLUX in solution. (B) Cells were gated as lymphocytes positive for CD3 expression. Flow cytometry plots show IFN-γ production (top panel) and degranulation (CD107a, bottom panel) of CD3+ T cells in the absence (left panel) or presence (right panel) of FLUX in solution (500 μg/ml) after the third restimulation. Shown is one representative TCL. (C) The CD4+/CD8+ ratio in the CD3+ T cell population was analyzed at the end of each restimulation round.

**FLUX-reacting T cells can be generated in HD with various HLA haplotypes**

FLUX-T cell responses were induced with 500 μg/ml of FLUX in five other HLA-B*57:01+ HD, because this concentration was shown to be the most effective for TCL induction. Altogether, FLUX reactivity could be detected in nine out of nine HLA-B*57:01+ individuals (Fig. 2A, Table I). Daly et al. (6) identified the HLA-B*57:01 allele as a risk factor for FLUX-DILI. Because there exist other non-HLA-associated FLUX hypersensitivity reactions like interstitial nephritis (7) or rash (1), we also tried to generate FLUX-TCL from HLA-B*57:01+ individuals. Generation of FLUX-reacting T cells was possible in seven out of eight HLA-B*57:01+ HD. Stimulation by FLUX in solution, however, was not observed in the CD8+ T cell subset. CD4+ FLUX-reacting T cells reacted by CD107a upregulation and IFN-γ secretion upon FLUX stimulation, whereas we did not observe increase in IL-4, IL-5, or IL-13 (Supplemental Fig. 1A). HLA-blocking experiments revealed HLA class II restriction of the CD4+ FLUX-reacting T cells (Supplemental Fig. 1B). Interestingly, we did not observe a relative expansion of CD8+ T cells compared with CD4+ T cells in HLA-B*57:01+ TCL with an increasing number of restimulation rounds (Supplemental Fig. 1C), as was the case in the HLA-B*57:01+ FLUX-TCL. This goes hand in hand with the fact that CD4+ FLUX-TCC were more frequent in HLA-B*57:01+ individuals (Supplemental Table I).

**The HLA-B*57:01 allele determines the reactivity pattern of FLUX-reacting T cells**

To investigate the reactivity pattern of FLUX-TCL/TCC, several stimulation conditions and their impact on T cell activation were analyzed by flow cytometry. FLUX-reacting T cells were either stimulated with FLUX in solution (APC+FLUX) or APC-FLUX as described in the Materials and Methods section. Of note, stimulation by APC-FLUX would require a covalent association of FLUX with HLA or a very strong noncovalent interaction, which would resist washing steps. Stimulation by FLUX in solution, however, would allow labile binding of drug to HLA. Yet, it does not exclude formation of stably presented hapten, because the duration of the assay is 6 h, allowing drug uptake and processing to take place.

If FLUX-TCL were stimulated with FLUX in solution, we observed T cell reactivity in all TCL (Fig. 3A, 3C). FLUX-pulsed APC only activated a minority (23.5%; 4 out of 17) of HLA-B*57:01+ TCL (Fig. 3A, 3B). The magnitude of the FLUX-T cell response in terms of CD107a upregulation and IFN-γ secretion after the third restimulation was not significantly different in HLA-B*57:01+ and HLA-B*57:01- HD (Fig. 2C, 2D). As mentioned, T cell reactivity was not exclusively observed in the CD8+ T cell subset. CD4+ FLUX-reacting T cells reacted by CD107a upregulation and IFN-γ secretion upon FLUX stimulation, whereas we did not observe increase in IL-4, IL-5, or IL-13 (Supplemental Fig. 1A). HLA-blocking experiments revealed HLA class II restriction of the CD4+ FLUX-reacting T cells (Supplemental Fig. 1B). Interestingly, we did not observe a relative expansion of CD8+ T cells compared with CD4+ T cells in HLA-B*57:01+ TCL with an increasing number of restimulation rounds (Supplemental Fig. 1C), as was the case in the HLA-B*57:01+ FLUX-TCL. This goes hand in hand with the fact that CD4+ FLUX-TCC were more frequent in HLA-B*57:01+ individuals (Supplemental Table I).
Immediate activation of HLA-B*57:01+ TCC by FLUX in solution

So far, we only examined T cell response to FLUX 6 h after drug encounter. To characterize the kinetics of T cell activation early after FLUX stimulation, we generated TCC, so that calcium influx measurements were possible. We generated a total of 86 FLUX-TCC from 2 HLA-B*57:01+ HD (HD617 and HD630) and 50 FLUX-TCC from 2 HLA-B*57:01− HD (HD639 and HD655) by limiting dilution. The FLUX-reacting TCC were identified as described in Materials and Methods.

Activation of HLA-B*57:01+ and HLA-B*57:01− FLUX-TCC upon stimulation with FLUX in solution (APC+FLUX) or APCFLUX was monitored by calcium influx measurements. After 5 min of baseline measurements, FLUX-pulsed APC were added to FLUX-TCC. None of the tested HLA-B*57:01+ TCC reacted to FLUX-pulsed APC. The activation of HLA-B*57:01+ FLUX-TCC

Table I. HLA class I typing of HLA-B*57:01+ and HLA-B*57:01− individuals

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<th>B1</th>
<th>B2</th>
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B17 status has been determined by staining with an HLA class I Ag B17 Ab. n.d., HLA allele was not determined.
required the addition of FLUX in solution, and TCC reactivity was detected even in the absence of autologous APC. HLA-B*57:01+ FLUX-TCC displayed an immediate activation after the addition of FLUX in solution, and the maximum activation level was reached within 1–5 min (Fig. 4A). This fast activation kinetic was consistently observed among all analyzed (20 out of 20) HLA-B*57:01+ CD8+ TCC (Fig. 4B), supporting the data observed in TCL. Conversely, in HLA-B*57:01− FLUX-TCC, the addition of APCFLUX resulted in an immediate calcium influx, reaching its maximum within 2 min (Fig. 4C), whereas the majority did not react upon stimulation with FLUX in solution during the duration of the assay (Fig. 4D). These results imply, that most HLA-B*57:01− FLUX-TCC cannot be activated immediately by the addition of FLUX in solution, suggesting the require-
ment of a time-dependent activation step to render immunogenic FLUX.

Stable presentation of FLUX requires proteasomal processing

The stable presentation of FLUX and its time dependence in HLA-B*57:01+ individuals suggest a hapten mechanism of FLUX-presentation. Due to their antigenic characteristics, haptns have to be processed by the processing and presentation machinery to be suitably presented. To verify these characteristics, we investigated the role of the proteasome in FLUX presentation and performed inhibition assays with the reversible proteasome inhibitor bortezomib. To this end, PBMC that were preincubated with bortezomib were pulsed with FLUX and used as APC in a 6-h stimulation assay with FLUX-TCL. The activation of FLUX-TCL with FLUX-pulsed PBMC (APCFLUX) was dependent on proteasomal processing in all analyzed HLA-B*57:01+ TCL, which is reflected by a decreased CD107a response with increasing bortezomib concentrations (Fig. 5A). This held true for the atypical HLA-B*57:01+ TCL from HD587, which was reacting to stimulation with APCFLUX (Fig. 5B). Because the majority of HLA-B*57:01+ FLUX-TCL reacted to FLUX in solution, we also performed proteasome inhibition assays with FLUX in solution (APC+FLUX). If HLA-B*57:01+ FLUX-TCL were stimulated with FLUX in solution, T cell reactivity was not inhibited by bortezomib, implying independence of proteasomal processing (Fig. 5C) and confirming an alternate presentation pathway.

Noncovalent binding of FLUX is restricted to the HLA-B*57:01 molecule

According to the previous experiments, T cells were able to react to FLUX presented either in a labile or stable manner. Because reactivity toward labily presented FLUX was mainly observed in the HLA-B*57:01+ HD pool, we investigated whether it was restricted to the HLA-B*57:01 molecule. Therefore, 51Cr-release assays with single HLA class I allele-expressing target cells (721.221) and/or allogeneic EBV-BLCL with overlap in the HLA haplotype were performed. Restriction experiments with four HLA-B*57:01+ FLUX-TCC showed that reactivity to labily presented FLUX was restricted to the HLA-B*57:01 allele, because only targets expressing the HLA-B*57:01 molecule were efficiently killed in the presence of FLUX (Fig. 6A). Restriction experiments were also performed with the atypical HLA-B*57:01+ TCL reacting to FLUX-pulsed APC (HD587). This TCL was not able to kill 721.221 target cells expressing HLA-B*57:01, and therefore, restriction to this allele could be excluded. The TCL from HD587 only killed autologous EBV-BLCL and allogeneic EBV-BLCL from HD631 previously pulsed with FLUX (Fig. 6B), implying HLA-A*01 or HLA-C*06 as the presenting allele. As FLUX-pulsed target cells from HD535 (HLA-A*01+) were not killed, HLA-C*06 could finally be identified as the FLUX-presenting HLA molecule. In summary, p-i-like immune responses were restricted to the HLA-B*57:01 allele, whereas hapten-like immune responses occurred in individuals with various HLA haplotypes, suggesting no need for the presence of a certain HLA allele.

Discussion

We report the generation of FLUX-TCL/TCC in HLA-B*57:01+ and HLA-B*57:01- FLUX-naive HD. The reacting T cells were mainly CD8+, cytotoxic, and IFN-γ secreting. We show that FLUX, a penicillin derivative, is able to stimulate T cells not only according to the hapten but also according to the p-i concept. Analysis of T cell reactivity pattern and HLA restriction revealed that the presence of the HLA-B*57:01 molecule is crucial for stimulation according to the p-i concept. As HLA-B*57:01+ individuals are more prone to suffer from FLUX-DILI (6), one might speculate that the HLA-B*57:01-restricted FLUX presentation might be primarily responsible for the induced liver damage.

The generation of FLUX-reacting T cells required iterative in vitro restimulations for 4–8 wk. This induction time is similar to that of carbamazepine, another drug leading to HLA-restricted CD8+ T cell responses (27). Compared to the induction of abacavir-reacting T cells, which is also associated with HLA-B*57:01, the generation of FLUX-TCL is slower and requires
higher molar drug concentration (1 mM). Abacavir-reacting T cells were already detected 14 d after induction with 30 μM abacavir (28). Possible explanations may lie in a lower precursor frequency of FLUX-reacting T cells or lower stability of the FLUX-HLA-B*57:01 complex. Indeed, Hamdahl et al. (29) demonstrated that the stability of the peptide within the HLA molecule was crucial for the generation of an optimal T cell response. Perhaps this observation is also true for drugs binding to HLA complexes. In fact, the affinity of noncovalently bound abacavir to HLA-B*57:01 is potentially higher than that of FLUX, because multiple binding sites in a protein imply that after processing, a novel concept to explain T cell stimulation by drugs. In the presence of abacavir, the conformation of the peptide-binding cleft is modified, so that the peptide anchoring site displays other steric and electrostatic properties. In this case, peptides with a short aliphatic residue at the C terminus became favored over peptides with a tryptophan or phenylalanine usually seen at the C terminus of HLA-B*57:01-binding peptides (16–18). Crystal structures have shown that abacavir bound the F-pocket of HLA-B*57:01 only by noncovalent interactions (17). In this sense, this novel concept fulfills the p-i concept. Nevertheless, Chessman et al. (28) showed that the presentation of abacavir was TAP and tapasin dependent, a property typically observed for haptens. In summary, FLUX differs from abacavir on at least two points. First, FLUX did not modify the affinity of HLA-B*57:01-binding peptides for the HLA molecule (16), and second, as discussed above, abacavir cannot be washed away from HLA-B*57:01+ abacavir-pulsed APC. To define the T cell reactivity patterns upon FLUX stimulation, we used three characteristics to dissect the hapten and p-i concept: 1) stability or lability of FLUX binding to APC; 2) involvement of proteasomal processing or not; and 3) FLUX-TCC activation kinetics upon stimulation with FLUX in solution. In contrast to labily or noncovalently bound drugs, haptens cannot be removed from APC by extensive washing steps (30, 31). Referring to this, TCL that reacted to FLUX-pulsed APC were considered to recognize hapten–peptide complexes (23). The hapten hypothesis was additionally supported by the involvement of the proteasome and a slow activation kinetic of FLUX-TCC upon stimulation with FLUX in solution, implying the need of processing of FLUX–protein complexes prior to presentation of FLUX–peptides. FLUX that was removed from APC by washing was assumed to be rather labily bound to HLA, and therefore, we concluded that a noncovalent binding took place. T cell reactivity to labily presented FLUX was further characterized by its independence of proteasomal processing and the immediate activation kinetic of FLUX-TCC, which was too fast to allow FLUX uptake, processing, and presentation to take place. There is a possibility for an immediate TCC activation due to a direct and rapid binding of FLUX as a hapten onto peptides embedded in the HLA. However, such a chemical reactivity would be stable and not affected by extensive washing. In contrast, we did not observe HLA-B*57:01+ TCL being activated by FLUX-pulsed APC, indicating a labile binding.

Moreover, we believe that drug hypersensitivity to a hapten–peptide complex is unlikely to be restricted to a single HLA allele, because multiple binding sites in a protein imply that after processing, a number of potential drug-bound peptides are available for loading onto different types of HLA molecules (32). In agreement with this, our data revealed the presence of a hapten-like immune response to FLUX in TCL from individuals with various HLA haplotypes. Interestingly, only 35% of HLA-B*57:01+ FLUX-TCC reacted to FLUX presented as a hapten. The majority of HLA-B*57:01+ T cell reactivity could be explained by the p-i concept. The conformation of the HLA-B*57:01 molecule seems to be optimal for a labile interaction among HLA, TCR, and drug, because p-i immune response to FLUX was restricted to this allele. Although FLUX-haptenized peptides must also be formed and presented in the HLA-B*57:01+ HD as efficiently as in the HLA-B*57:01+ individuals, we rarely detected additional hapten responses in HLA-B*57:01+ TCL. If detected, they were related to FLUX presentation by other HLA molecules. Most probably, both mechanisms occur together, but T cell stimulatory signals upon recognition of labile FLUX-HLA-B*57:01 complexes appear to be more powerful than signals upon FLUX–
hapten recognition, leading to increased expansion of T cells recognizing FLUX according to the p-i concept. Thus, in HLA-B*57:01+ individuals, the FLUX–HLA-B*57:01-directed response is immunodominant, suggesting that noncovalently bound drugs might be more immunogenic for the TCR repertoire than a haptenized peptide.

Our data on T cell stimulation by FLUX in HLA-B*57:01+ individuals go beyond the recent publication by Monshi et al. (33). Despite a precise description of the presentation of haptenized FLUX and the ensuing T cell stimulation, they did not address the precise role of the HLA-B*57:01 allele. Moreover, modifications of albumin by FLUX could be detected in all treated patients studied to date (10), whereas FLUX hypersensitivity reactions were only found in few patients, suggesting that the formation of a FLUX–protein complex is poorly immunogenic. The role of hapten formation in immunological, and allergic reactions may become clearer by characterization of FLUX-modified peptides eluted from HLA molecules.

This study reveals an additional mechanism of FLUX presentation, which is predominantly found in HLA-B*57:01+ individuals and thus might be relevant for the HLA-B*57:01 association. However, many points remain obscure. For instance, we were able to generate FLUX-reacting CD8+ T cells in 100% of HLA-B*57:01+ HD in vitro, whereas in vivo, only 1 in 500 responding T cells will thus be of big importance. The HLA-B*57:01 molecule is expressed on the surface of all nucleated cells and probably these cells infiltrate the liver and damage liver cells, as it has been shown for granzyme B+ and Fas ligand+ lymphocytes for liver failure associated with a sulfasalazin-induced drug reaction with eosinophilia and systemic symptoms syndrome (34). Nevertheless, it remains enigmatic why specifically cells located in the liver are harmed by FLUX-reacting CD8+ T cells, because the HLA-B*57:01 molecule is expressed on the surface of all nucleated cells and labily bound FLUX should therefore be omnipresent. Future studies focusing on liver cells as targets for FLUX-reacting T cells will thus be of big importance.

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Disclosures

The authors have no financial conflicts of interest.

References

7. Spanou, Z., M. Keller, M. Britschgi, N.在 article is not fully visible, please provide more text or clarify the request.

Supplemental Figure 1 Characterization of FLUX-reacting CD4+ T cells.

FLUX-TCL were induced with 500 ug/ml FLUX and restimulated every 14 days. At the end of each restimulation round, T cell reactivity was monitored by flow cytometry after a 6 h stimulation assay. A) Cells were gated as lymphocytes positive for CD3 expression. Flow cytometry plots show CD107a upregulation and IFNγ, IL-4, IL-5 or IL-13 production of CD4+ T cells in the absence (left) or presence (right) of FLUX in solution (500 μg/ml) after the 3rd restimulation round. Shown is one representative TCL. B) FLUX-reacting CD4+ TCC were stimulated with 500 μg/ml FLUX (APC+FLUX) in the presence of anti-human HLA-A,B,C (Biolegend) or anti-human HLA-DR,DP,DQ (BD Biosciences) blocking antibodies.
Therefore, TCC and APC were incubated 30 minutes at 37 °C in CM with 4 μg/ml of the respective antibody. Reactivation of TCC was analyzed by flow cytometry after a 6 h stimulation assay in terms of CD107a upregulation. Experiments were performed in triplicates, shown is representative experiment from 1/4 CD4+ FLUX-TCC. C) The CD4+/CD8+ ratio in the CD3+ T cell population was analyzed at the end of each restimulation round. Shown is 1 representative TCL.
**Supplemental Table I** CD4 or CD8 phenotype of FLUX-TCC from HLA-B*57:01\(^+\) and HLA-B*57:01\(^-\) individuals.

<table>
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<th>CD4 TCC</th>
<th>CD8 TCC</th>
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