

Original article

Amoxicillin conjugates to HLA class I molecules and interferes with signalling through the ILT2/LIR-1/CD85j inhibitory receptor

Background: Drugs behave as haptens and are recognized by specific T-cell receptors in the context of major histocompatibility complex (MHC) molecules in allergic subjects. Natural killer cell receptors (NKR) are MHC class I-specific receptors that modulate the threshold of activation of immunocompetent cells. Amongst them, ILT2/LIR-1/CD85j is an inhibitory NKR widely distributed in several cell lineages and with a broad spectrum of recognition of human leucocyte antigen (HLA) class I ligands.

Methods: We have evaluated, at the biochemical and cellular level, the ability of amoxicillin (AX) conjugate to HLA class I molecules and to interfere with the inhibitory signal delivered by the HLA class I receptor ILT2/LIR-1/CD85j.

Results: We have detected AX bound to cell membrane proteins and in particular to HLA class I molecules. Preincubation with AX rendered target cells susceptible to NK cell-mediated lysis. In conjugation experiments, target cell-bound AX hampered tyrosine phosphorylation of ILT2/LIR-1/CD85j upon ligand recognition and the subsequent recruitment of SHP-1 phosphatase.

Conclusion: Conjugation of AX to HLA class I molecules may mask HLA recognition by inhibitory receptors and attenuate the negative signal delivered by SHP-1 phosphatase, thus lowering the threshold of activation of effector cells.

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Immune reactions against xenobiotics (drugs, metals and other chemical agents) can lead to hypersensitivity and/or autoimmunity (1). Amongst them, a relevant group is composed of allergic reactions to drugs (2). Drug allergies are classified into immediate, and nonimmediate or delayed reactions. While it is clearly established that immediate reactions are immunoglobulin (Ig)E-mediated, the mechanisms involved in delayed reactions have not been completely elucidated, although T cells seem to be involved (3). The development of drug-specific immediate and nonimmediate immune responses is the result of drugs or drug-derived metabolites behaving as haptens and being recognized by specific T-cell receptors (TCR) in the context of human leucocyte antigen (HLA) molecules. As with other chemical haptens, the ability of any drug to induce an immune response depends on its reactivity with proteins that will be processed by antigen-presenting cells. Drugs-like β -lactam antibiotics easily form covalent bonds with free amino groups (4). The molecular targets of such haptenation processes may be soluble or cell-bound proteins, including major histocompatibility complex (MHC) molecules.

Molecules encoded by MHC class I genes are recognized by three distinct groups of cell surface immunoreceptors: the TCR, the CD8 and the natural killer cell receptors (NKR). In humans, NKRs are grouped in

three protein families: CD94/NKG2 heterodimers, killer cell immunoglobulin-like receptors (KIRs) and immunoglobulin-like transcripts (ILTs) also called leucocyte immunoglobulin-like receptors (LIRs; 5). CD94/NKG2 heterodimers are C-type lectins and specifically recognize the nonclassical HLA class I molecule HLA-E, KIRs belong to the immunoglobulin superfamily and recognize alleles of classical HLA class I molecules with different specificities. ILTs/LIRs/CD85 antigens also belong to the immunoglobulin superfamily. Two members of this family, ILT2/LIR-1/CD85j and ILT4/LIR-2/CD85d, have been shown to recognize classical and nonclassical HLA class I molecules with broad specificity (6–8). Inhibitory and activating receptors with similar structural features have been described within the three families. While the function of activating receptors is not well established, it is known that inhibitory receptors regulate NK cell activity and modulate the threshold of activation of T cells favouring effector functions against transformed or virus-infected cells that downregulate HLA class I expression on the cell membrane (9, 10). The molecular basis for the delivery of the inhibitory signal is common to all of them: tyrosine residues within immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic regions become phosphorylated upon HLA ligation and recruit SHP-1 phosphatase which is

responsible for the abrogation or attenuation of activating signals.

In the present study, we sought to investigate whether drug/hapten modification of HLA class I molecules could affect recognition by inhibitory receptors and therefore affect the threshold of activation of effector cells. To that end, we analysed amoxicillin (AX)-modified HLA class I interactions with the ILT2/LIR-1/CD85j inhibitory receptor, which will be referred to as ILT2 in the rest of this report. ILT2 is a 110 kDa transmembrane protein which contains four ITIMs within its cytoplasmic tail that become phosphorylated and recruit SHP-1 upon receptor binding to classical and nonclassical HLA class I molecules (6, 11). It is detected on the surface of a proportion of NK cells and T lymphocytes, on all B lymphocytes, monocytes and myeloid DCs. In addition to inhibition of NK cell-mediated killing, published data support a role for ILT2 in the inhibition of CD3/TCR-mediated activation of T lymphocytes and downregulation of antigen recognition by CD8⁺ T cells (6, 12–14). We have studied, at the functional and biochemical level, ILT2 receptor activity upon interaction with AX-modified HLA class I-expressing cells. Our results suggest that AX may mask recognition by inhibitory NKRs upon modification of HLA class I molecules.

Methods

Cell lines and antibodies

The HLA class I-defective B cell line 721.221 and 221.B2705 transfectant, were a generous gift from P. Parham (Stanford University School of Medicine, Stanford, USA). The 221.G1 and 221.AEH cell lines were kindly provided by P. Le Bouteiller (Hôpital de Purpan, Toulouse, France) and D. E. Geraghty (Fred Hutchinson Cancer Research Center, N. Seattle, WA, USA), respectively. 721.221 and 221.G1 cell lines were grown in RPMI 1640 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Cambrex Bio Science). 221.B2705 and 221.AEH cells were cultured in the same medium plus 300 µg/ml of hygromycin B (Merck, Darmstadt, Germany). The ILT2 transfectant of the rat basophilic leukaemia RBL cell line RBL.ILT2 (11) was grown in RPMI 1640 plus 10% (v/v) heat-inactivated FCS in the presence of G418 (1 mg/ml; Gibco Invitrogen, Grand Island, NY, USA). The NKL cell line (15) was cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS plus 100 U/ml of hr-IL-2 (PeproTech, Rocky Hill, NJ, USA) and 1% sodium pyruvate (Cambrex Bio Science).

The monoclonal antibodies (mAbs) used were: HP-1F7 (anti-HLA class I) and HP-F1 (anti-ILT2) kindly provided by M. López-Botet (Universitat Pompeu Fabra, Barcelona, Spain), anti-amoxicillin-specific mAbs (16) kindly provided by C. Mayorga (Hospital Carlos Haya, Málaga, Spain). Fluorescein isothiocyanate (FITC)-conjugated polyclonal rabbit antimouse F(ab')₂ was purchased from DakoCytomation (Glostrup, Denmark); antiphosphotyrosine mAb 4G10 and rabbit polyclonal anti-SHP-1 were purchased from Upstate Biotechnology (Lake Placid, NY, USA), and peroxidase-conjugated goat antimouse IgG and goat antirabbit IgG were purchased from Pierce (Rockford, IL, USA).

Cell haptenation

For cell haptenation, 721.221 cells and HLA class I stable transfectants of the same cell line (2.5×10^5 cells/ml) were incubated overnight in RPMI 1640 plus 10% heat-inactivated FCS in the presence of AX at a final concentration of 1 mg/ml.

Flow cytometry

Flow cytometry analyses were performed in a FACScalibur cytofluorometer with five parameters and three colours (SSC, FSC, FL-1, FL-2 and FL-3) and analysed with CELLQUEST software (BD Bioscience, San Jose, CA, USA). Immunofluorescence staining was carried out as previously described (17). Briefly, cells were incubated with saturating concentrations of the various mAbs followed by washing and labelling with FITC-conjugated polyclonal rabbit antimouse F(ab')₂ antibodies. Two thousand events were collected and analysed per sample.

Cytotoxicity assays

The NKL cell-mediated cytotoxicity was measured in a standard 4-h ⁵¹Cr-release assay. 721.221 and 221.B2705 cell lines, preincubated or not with AX, were labelled with ⁵¹Cr (AB, Uppsala, Sweden) and used as targets. Assays were conducted in triplicate using U-bottom 96-well microtitre plates and specific lysis was calculated as $100 \times [\text{Specific release (cpm)} - \text{Spontaneous release (cpm)}] / [\text{Maximum release (cpm)} - \text{Spontaneous release (cpm)}]$. In every case spontaneous release was <20% of the maximum lysis. Target cell haptenation was confirmed by flow cytometry in each experiment.

Cell conjugation, immunoprecipitation and Western blotting

RBL.ILT2 cells (2×10^6) were incubated for 2 min at 37°C with 10^6 of 221.AEH cells preincubated or not with AX in RPMI 1640 plus 10% FCS (haptenation of HLA-E-expressing cells was confirmed by flow cytometry). Further, cell conjugates were centrifuged at 200 g for 3 min and lysed for 45 min in 250 µl of cold lysis buffer containing 1% (v/v) Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM Na₃VO₄ and 1 mM NaF. HLA class I molecules or ILT2 receptors were immunoprecipitated from lysates with specific mAbs bound to protein A-sepharose (AB). Immunocomplexes were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking nonspecific binding, membranes were incubated with specific mAbs and immunoreactivity was detected using an enhanced chemoluminescence kit (AB). Densitometry of specific bands was performed with 1D-MANAGER software (TDI, Madrid, Spain).

Pervanadate treatment

The RBL.ILT2 cell line (10^6 cells) was incubated in 1 ml of RPMI 1640 containing freshly prepared sodium pervanadate (0.1 mM Na₃VO₄ and 10 mM H₂O₂) for 10 min at 37°C. Upon treatment, cell lysates were prepared and ILT2 protein was immunoprecipitated as described above.

Statistical analysis

When applicable, statistical significance was assessed by Mann–Whitney *U*-test and accepted at *P* < 0.05.

Results

Amoxicillin binds to cell membrane proteins

In order to explore the effect of HLA haptenation by drugs in NKR–ligand interactions, we first analysed whether AX could bind spontaneously to cell membrane proteins under standard cell culture conditions. We cultured the HLA class I-defective B cell line 721.221 and 221.B2705, 221.G1, and 221.AEH transfectants of the same cell line-expressing HLA-B*2705, HLA-G1 and HLA-E, respectively, in the presence or absence of AX. Amoxicillin-specific mAbs were used to detect the membrane-bound antibiotic by indirect immunofluorescence and flow cytometry analysis. As illustrated in Fig. 1, AX was able to bind to the extracellular region of cell membrane proteins of the various cell lines in the cell culture media and conditions tested.

Amoxicillin conjugates to MHC class I molecules

Once we had demonstrated that AX binds to cell membrane proteins, we investigated whether it was able to bind to MHC class I molecules. For this purpose, we used HLA-G1 and HLA-B*2705-expressing cell lines (221.G1 and 221.B2705, respectively), as both of them express high levels of cell surface HLA class I. For control cells we used the parental cell line 721.221 that is devoid of cell surface expression of these molecules. Upon an overnight incubation in the presence or absence of AX, the cells were collected and HLA class I molecules were immunoprecipitated from cell lysates with a specific mAb. As illustrated in Fig. 2, Western blot analysis of immunoprecipitated proteins with a mixture of anti-AX mAbs shows that bands of about 45 kDa (the expected molecular weight for HLA class I heavy chains) develop exclusively in lanes corresponding to HLA class I transfectants preincubated with the drug (lane 4 in the

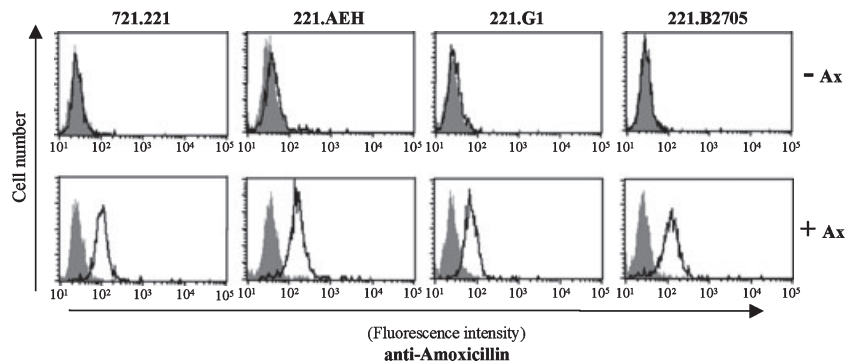


Figure 1. Amoxicillin binds to cell membrane proteins. The major histocompatibility complex class I-negative parental cell line 721.221 and HLA-E, HLA-G1 and HLA-B2705 transfectants of this cell line (221.AEH, 221.G1 and 221.B2705, respectively) were cultured overnight in RPMI 1640 plus 10% heat-inactivated fetal calf serum in the presence (lower panels) or absence (upper panels) of 1 mg/ml of amoxicillin. After treatment, cells were collected, incubated with saturating concentrations of a mix of specific anti-amoxicillin monoclonal antibodies (white histograms) or control immunoglobulin G (grey histograms), washed, stained with a secondary FITC-conjugated rabbit antimouse F(ab')₂, and analysed by flow cytometry.

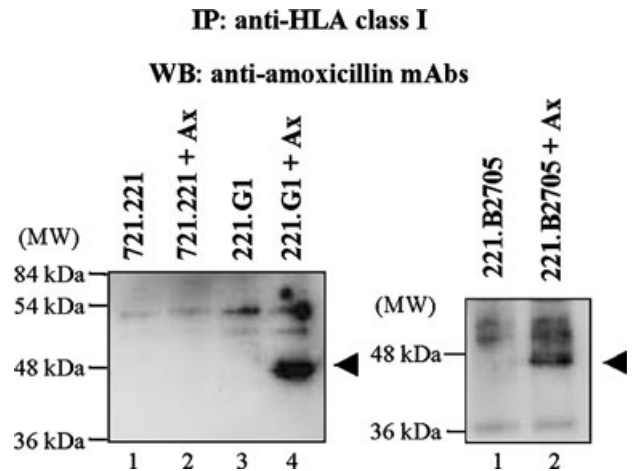


Figure 2. Amoxicillin binds to major histocompatibility complex class I molecules. 721.221, 221.G1 and 221.B2705 cell lines were incubated overnight in RPMI 1640 plus 10% heat-inactivated fetal calf serum in the presence or absence of 1 mg/ml of amoxicillin. After this time cells were collected, lysed and immunoprecipitated with a specific antihuman leucocyte antigen (HLA) class I monoclonal antibody (mAb; HP-1F7). Immunoprecipitates were transferred to a PVDF membrane, which was incubated with a mix of mAbs anti-amoxicillin. Arrows indicate 45 kDa specific bands corresponding to haptenized HLA class I molecules.

left panel, and lane 2 in the right panel). This result indicates that AX is able to form covalent bonds with the heavy chain of HLA-B*2705 and HLA-G1 when it spontaneously conjugates to cell surface proteins.

Target cell preincubation with amoxicillin increases cytotoxicity mediated by the cell line NKL against 221.B2705 cells

The blockage of cytotoxicity through recognition of HLA molecules by inhibitory NKRs has been widely docu-

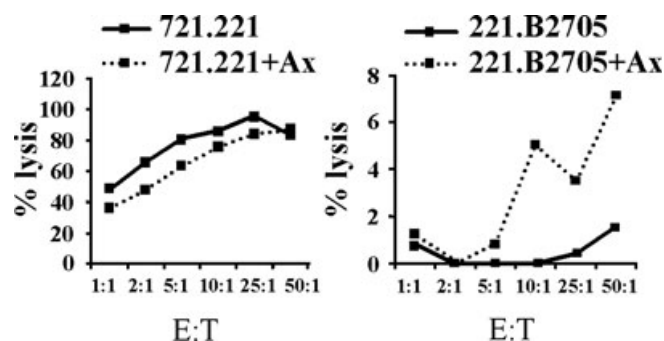


Figure 3. Target cell preincubation with amoxicillin increases cytotoxicity mediated by NKL cell line against 221.B2705 cells. Cytotoxicity mediated by human NKL cell line was measured in a 4-h ^{51}Cr -release assay against class I-deficient 721.221 cells and the transfectant 221.B2705 preincubated (discontinuous line) or not (continuous line) with 1 mg/ml of amoxicillin. Assays were conducted in triplicates. In every case spontaneous release was <20% of the maximum lysis. Results are statistically significant at effector : target (E : T) ratios 10 : 1, 25 : 1 and 50 : 1 for the killing of amoxicillin-preincubated 221.B2705 cells (221.B2705 + AX; $P < 0.05$ by Mann-Whitney U -test).

mented (5). Amongst these NKRs, ILT2 has been shown to inhibit lysis of target cells expressing classical and nonclassical HLA class I molecules (6). To investigate whether the binding of AX to HLA class I can interfere with the receptor–ligand interactions and therefore with the inhibitory signal, cytotoxic assays were performed in which we used a human NK cell line-expressing ILT2 (NKL) as effector cells. As target cells we used 721.221 and 221.B2705 lymphoblastoid cell lines, as it has been reported that NKL-mediated lysis of the class I deletion mutant 721.221 is inhibited by transfection of HLA-B*2705, and that this inhibition is accounted for by the ILT2 receptor (6). Cytotoxicity assays were conducted at different ratios against the two target cell lines cultured overnight in the presence or absence of AX. As illustrated in Fig. 3, while cytotoxicity mediated by the NKL cell line against 721.221 cells was not substantially affected by the presence of the drug, the protection conferred by the expression of HLA-B*2705 was partially lost by preincubation of target cells with AX. The statistical analysis of the results showed a significant increase of the cytolytic activity of NKL against the 221.B2705 transfectant preincubated with AX at 10 : 1, 25 : 1 and 50 : 1 (effector : target) ratios.

Target cell preincubation with amoxicillin reduces the recruitment of SHP-1 phosphatase to the ILT2/LIR-1/CD85j receptor after the interaction with HLA class I

The recruitment of SHP-1 phosphatase to phosphorylated tyrosines in the cytoplasmic tail of ILT2 is responsible for the inhibitory signal upon ligand recognition (6, 11, 13). We studied whether haptentation of HLA class I molecules by drugs could affect the molecular events

leading to inhibition of activating signals by ILT2. To perform these experiments, we used the RBL.ILT2 cell line as it expresses high levels of cell surface ILT2 molecules and does not express any other HLA class I inhibitory NKRs. It has been previously reported that interactions between ILT2+ cells (RBL.ILT2) and HLA-E+ cells (221.AEH) were sufficient to induce receptor tyrosine phosphorylation and SHP-1 recruitment (11). We conducted the same cell conjugation experiments in which the 221.AEH transfectants were preincubated or not with 1 mg/ml of AX. As a positive control, tyrosine phosphorylation was induced by pharmacological treatment of RBL.ILT2 cells with pervanadate. Upon cell conjugation or pervanadate treatment, ILT2 was immunoprecipitated from cell lysates with a specific mAb (HP-F1). Immunocomplexes were analysed by Western blot with anti-SHP-1, antiphosphotyrosine and anti-ILT2-specific antibodies. Conjugation of RBL.ILT2 with HLA-E-expressing cells induced tyrosine phosphorylation of ILT2 (Fig. 4A, middle panel), and recruitment of SHP-1 phosphatase as expected (Fig. 4A, upper panel). However, the degree of phosphorylation of ILT2 was lower and, interestingly, the recruitment of SHP-1 phosphatase to ILT2 was markedly reduced when ligand-expressing cells had been haptenized with AX (see Fig. 4A, lanes 1 and 2). Densitometric analysis of specific bands revealed that there was a 50% reduction in tyrosine phosphorylation and a 75% reduction in SHP-1 coprecipitation relative to the total amount of ILT2 when HLA-E-expressing cells had been pretreated with AX (see Fig. 4B).

Discussion

Drugs behave as haptens or prohaptens and, like other low-molecular weight chemicals, become immunogenic upon binding to serum of cellular proteins. Amongst them, β -lactam antibiotics are most commonly used for the treatment of bacterial infections and represent a typical example of allergy-inducing drugs in humans. These compounds spontaneously conjugate to amino acid residues through the nucleophilic attack of the β -lactam ring by free amino groups of proteins. Therefore, concerning their function as haptens, β -lactam antibiotics represent a particularly convenient tool for investigation (18). As the discovery that drugs bind covalently to proteins generating immunogenic conjugates responsible for many drug-induced hypersensitivity reactions, much effort has been dedicated to elucidating the nature of the antigenic determinants involved in their immunogenicity and allergenicity, and in TCR and IgE recognition (19–21). On the other hand, few data are available on the interaction of drugs with other components of the immune response. Few proteins to which β -lactam antibiotics conjugate have been characterized. Although these compounds can conjugate to lysine and histidine

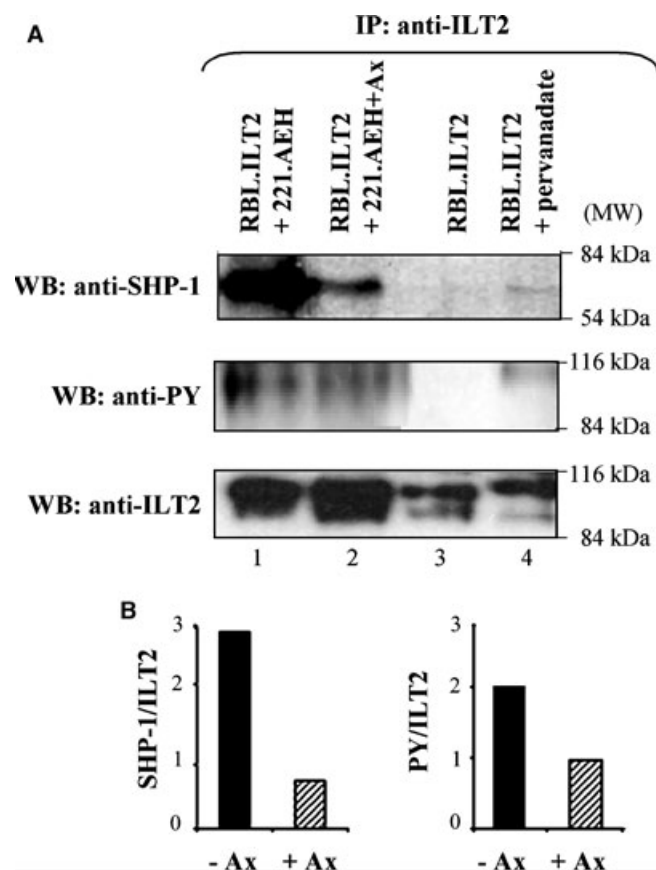


Figure 4. Target cell preincubation with the drug reduces the recruitment of SHP-1 phosphatase to ILT2/CD85j receptor after the interaction with human leucocyte antigen. (A) The RBL.ILT2 cell line was conjugated for 2 min at 37°C with the target 221.AEH preincubated or not with 1 mg/ml of amoxicillin. Lysates were immunoprecipitated with a specific anti-ILT2 monoclonal antibody (HP-F1), resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis, and transferred to a PVDF membrane, which was sequentially incubated with anti-SHP-1, antiphospho-tyrosine and anti-ILT2-specific antibodies. Lysates from RBL.ILT2 cell line, treated or not with pervanadate, were included as control samples. This gel is representative of two separate experiments with similar results. (B) Analysis of SHP-1 association with ILT2 and of ILT2 tyrosine phosphorylation (expressed in arbitrary units) as the densitometric intensity ratio of SHP-1/total ILT2 and PY-ILT2/total ILT2 in RBL.ILT2 cells conjugated to 221.AEH cells haptenated (+AX) or not (-AX) with amoxicillin (experiment shown in A).

residues, some published data suggest that conjugation is preferential for some proteins over others, and that conjugation may have functional consequences for the haptenated protein. In particular, it has been shown that benzilpenicillin differentially conjugates to cytokines and preferentially disrupts the activity of IFN- γ (4, 22, 23).

We herein report that the β -lactam antibiotic AX conjugates to cell membrane proteins under standard cell culture conditions. In particular, immunoprecipitation

experiments with an anti-HLA class I mAb and Western blot analysis with anti-AX antibodies demonstrate that AX can conjugate to the heavy chain of several classical and nonclassical HLA class I molecules (HLA-B*2705 and HLA-G1) in a physiological environment. The extracellular region of the heavy chain of HLA-B*2705, HLA-G1 and HLA-E used in this study, has several lysine and histidine residues susceptible to participation in the formation of protein adducts with β -lactam antibiotics.

Inhibitory NK cell receptors (iNKRs) recognize HLA class I molecules and seem to raise the threshold of cellular activation through the initiation of an inhibitory signal cascade upon ligand interaction (24). Available data lead researchers in the field to postulate that several leucocyte lineages have adopted HLA class I recognition as a common strategy to control cell activation during an inflammatory or immune response, and that the activity of iNKRs may contribute to peripheral self-tolerance (5, 6, 10). ILT2 is a widely distributed iNKR that recognizes a broad array of MHC class I molecules in a peptide-independent manner, through the interaction with the $\alpha 3$ -domain of HLA class I molecules (25, 26). It has been reported to down-modulate myeloid cell functions, TCR-triggered activation of T lymphocytes and cytotoxicity by NK cells (6, 12, 27, 28). We took advantage of its functional activity in NK cells to analyse ILT2 recognition of AX-modified HLA. The human cytotoxic NK cell line NKL expresses ILT2, which has been reported to protect cells expressing HLA-B*2705 from lysis by NKL. As illustrated in Fig. 3, target cell haptenation with AX partially reverses this protection. This fact implies a partial loss of the inhibitory signal delivered by ILT2. As illustrated in Fig. 2, AX conjugates to HLA-B*2705; therefore it is tempting to speculate that the molecule of AX might mask HLA recognition by ILT2, hampering the inhibitory signal. It has been previously reported that ligand engagement of ILT2 induces tyrosine phosphorylation and SHP-1 recruitment, leading to the activation of phosphatase and to the delivery of the inhibitory signal (6). Therefore, to further confirm our hypothesis, we analysed the phosphorylation status of ILT2 and SHP-1 phosphatase recruitment to the receptor upon ligand binding. We found that in conditions in which AX haptenizes HLA class I proteins, ligand recognition leads to a reduced tyrosine phosphorylation of ILT2 and to a significant inhibition of phosphatase recruitment. These results further support the notion that the hapten bound to HLA class I molecules may interfere with their recognition by inhibitory receptors, and as a consequence reduce the amount of active SHP-1 phosphatase.

The results reported here suggest that post-translational modifications of HLA by β -lactams or by other drugs may have functional consequences for activity of ILT2 and other iNKRs. Reactive haptenic compounds, such as β -lactamic antibiotics, may directly form adducts with peptides within MHC molecules, or with cellular or

serum proteins that will be processed and presented by HLA. In this situation, these haptens could be 'sensed' by other inhibitory receptors whose binding site is close to the peptide groove, such as KIRs and CD94/NKG2 heterodimers (29). In this way, peptide phosphorylation has been shown to reduce the inhibitory signal delivered by KIR2DL1 (30). Although ILT2 is expressed by many cell types, KIRs and CD94/NKG2 heterodimers are expressed mainly by NK and cytotoxic T lymphocytes (CTLs; 5, 9, 10). One of the properties of chemically reactive haptens is their capacity to simultaneously generate immunogenic determinants for hapten-specific CD8+ and CD4+ T cells. However, a clear dominance of CD8+ effector T cells is observed in contact hypersensitivity and allergic contact dermatitis (31, 32). Moreover, cytotoxic mechanisms seem to be involved in various forms of T cell-mediated allergies (33). It is

tempting to hypothesize that the drug could interfere with KIR recognition in hapten-specific cytotoxic lymphocytes.

In conclusion, our results suggest that HLA haptenation by antibiotics may hamper recognition by HLA class I inhibitory receptors and mimic a loss of HLA expression on the cell surface, leading to a lowering of the activation threshold of immune cells. This modification may have implications for understanding the mechanisms by which drugs and other haptens may disrupt immune regulation in allergic subjects.

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