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Allopurinol hypersensitivity is primarily mediated by dose dependent oxypurinol-specific T cell response

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T cell responses in allopurinol hypersensitivity

Authors:

James Yun MBBS^{1,2}, Johan Mattsson PhD³, Karin Schnyder¹, Stefano Fontana MD⁴, Carlo R. Largiadè PhD³, Werner J. Pichler MD¹, Daniel Yerly PhD¹

Affiliation:

¹ Department of Rheumatology, Clinical Immunology and Allergology,

Inselspital/University Hospital of Bern, Bern, Switzerland

² Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland

³ Institute of Clinical Chemistry, Inselspital/University Hospital of Bern, Bern, Switzerland

⁴ Regional Blood Transfusion Service of the Swiss Red Cross, Bern, Switzerland

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Corresponding Author:

Werner J. Pichler MD

Department of Rheumatology, Clinical Immunology and Allergology, Inselspital/University

Hospital of Bern, Bern, Switzerland 3010

Ph: +41 3 1632 2264

Fax: +41 3 1633 2747

Email: werner.pichler@insel.ch

Abstract

Background: Allopurinol is a main cause of severe cutaneous adverse reactions (SCAR).

How allopurinol induces hypersensitivity is unknown. Predisposing factors are the presence of the HLA-B*58:01 allele, renal failure and possibly the dose taken.

Objective: Using an *in vitro* model, we sought to decipher the relationship between allopurinol metabolism, HLA-B*58:01 phenotype and drug concentrations in stimulating drug-specific T cells.

Methods: Lymphocyte transformation test (LTT) results of patients who had developed allopurinol hypersensitivity were analysed. We generated allopurinol or oxypurinol-specific T cell lines (ALP/OXP-TCLs) from allopurinol naïve HLA-B*58:01⁺ and HLA-B*58:01⁻ individuals using various drug concentrations. Their reactivity patterns were analysed by FACS and ⁵¹Cr release assay.

Results: Allopurinol allergic patients are primarily sensitised to oxypurinol in a dose dependent manner. TCL induction data show that both the presence of HLA-B*58:01 allele

and high concentration of drug are important for the generation of drug-specific T cells. The predominance of oxypurinol-specific lymphocyte response in allopurinol allergic patients can be explained by the rapid conversion of allopurinol to oxypurinol *in vivo* rather than to its intrinsic immunogenicity. OXP-TCLs do not recognise allopurinol and vice versa. Finally, functional avidity of ALP/OXP-TCL is dependent on both the induction dose and HLA-B*58:01 status.

Conclusions: This study establishes the important synergistic role of drug concentration and HLA-B*58:01 allele in the allopurinol or oxypurinol-specific T cell responses. In contrast to the prevailing concept that type B reactions are dose independent, T cell responses in allopurinol hypersensitivity are, in fact, highly dose dependent.

Clinical Relevance: Despite the prevailing dogma that Type B adverse drug reactions are dose independent, allopurinol hypersensitivity is primarily driven by oxypurinol-specific T cell response in a dose dependent manner, particular in the presence of HLA-B*58:01 allele.

Introduction

Allopurinol is a commonly prescribed drug used to lower uric acid levels in gout. Hypersensitivity reactions to allopurinol occur in approximately 2% of patients [1,2]. While severe allopurinol hypersensitivity is relatively uncommon, the drug is a major cause of severe cutaneous adverse reactions (SCAR) and is the most common cause of Stevens-Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) in Europe and Israel [3]. Recent studies have shown its strong association with HLA-B*58:01 allele [4,5]. However, in European cohorts, 45% of patients with allopurinol-induced SJS/TEN did not have this allele, suggesting that this allele is not absolutely necessary [5]. Furthermore, positive

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predictive value of HLA-B*58:01 is estimated to be 2.7%, implying that other risk factors are important [6]. Nonetheless, the recently updated American College of Rheumatology guidelines recommend that HLA-B*58:01 testing should be considered in selected patient subpopulations at higher risk for allopurinol hypersensitivity such as those with Han Chinese or Thai ancestry [7].

In addition to HLA-B*58:01 allele, high dose regimen, renal failure and concomitant diuretic therapy has been associated with allopurinol hypersensitivity [3,8]. Subsequently, a widely accepted guideline for allopurinol dose regimen based on creatinine clearance has been proposed although recent studies have questioned the clinical utility of this guideline in preventing hypersensitivity reactions [8-11]. Since the classical teaching states that type B reactions are dose independent and unpredictable, a dose adjustment regimen to prevent allopurinol hypersensitivity is rather counterintuitive [12]. On the other hand, a recent study of abacavir-specific T cells from HLA-B*57:01⁺ individuals suggests that functional avidity plays an important role in T cell reactivity, implying that drug concentrations matter [13]. In addition, abacavir alters the available peptide repertoire in a dose dependent manner [14,15]. Finally, piperacillin binds to albumin in a dose dependent manner [16]. All these studies, in addition to the success of drug desensitisation protocols, point to the possibility that in drug hypersensitivity reactions, dose is an important determining factor.

Allopurinol is rapidly metabolised by xanthine oxidase (XO) to oxypurinol and has a plasma half life of 1-2 hours. Oxypurinol is then excreted via the kidney with a half life of approximately 15 hours. Previous studies have suggested that oxypurinol may play a role in immune-mediated allopurinol hypersensitivity [17-19]. However, these studies did not take into account the recently described association with HLA-B*58:01 allele and no further

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studies were done to delineate immune mechanisms involved in allopurinol-specific T cell response. Therefore, we analysed the influence of HLA-B*58:01 allele and/or drug concentration on T cell reactivity to allopurinol or oxypurinol.

Methods

Samples from patients and healthy donors

Lymphocyte transformation tests (LTT) are performed as a routine diagnostic test for allopurinol hypersensitivity in Inselspital, Bern, as described elsewhere [20]. The results of LTT performed for suspected allopurinol hypersensitivity between February 2004 and June 2012 were analysed. For T cell line (TCL) generation, seven HLA-B*58:01⁺ allopurinol naïve individuals selected from Bern's blood donation centre were enrolled in the study. Additionally, four allopurinol allergic patients with positive LTT and seven HLA-B*58:01⁻ allopurinol naïve healthy individuals were recruited. The study was approved by the ethics committee of the Canton of Bern and all participants gave informed consent.

TCL generation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation and were cultured in RPMI-1640 (Gibco, Basel, Switzerland) supplemented with 10% heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland), 2mM L-Glutamine (Biochrom, Berlin, Germany), 25µg/ml transferrin (Biotest, Dreieich, Germany), 50U/ml penicillin and 50µg/ml streptomycin (Bioconcept, Allschwil, Switzerland). Allopurinol or oxypurinol-specific TCLs (ALP/OXP-TCLs) were generated by incubating lymphocytes (4-6 x 10⁶ cells/2ml in 24 well plate) with 1, 10 or 100µg/ml of allopurinol or oxypurinol (Sigma-Aldrich, Buchs, Switzerland). 50 IU/ml of IL2 (Roche, Basel,

Switzerland) were added every 2-3 days from day 4-5 onward. Every 10-14 days, TCLs were checked for specificity on FACS and were expanded by restimulating with the drug and irradiated autologous PBMC.

FACS analysis

10-14 days after the induction or restimulation, the cells were incubated with autologous PBMC in the presence or absence of the inducing drug for 6 hours. In experiments assessing cross-reactivity, 100µg/ml of xanthine or hypoxanthine (Sigma-Aldrich, Buchs, Switzerland) was also used as antigen. The specificity of TCL was assessed by CD107a upregulation as a marker of degranulation and cytotoxicity [21]. Anti-CD107a-PE antibody (BioLegend, London, UK), 6µg/ml monensin and 10µg/ml brefeldin A (Sigma-Aldrich, Buchs, Switzerland) were added at 1.5 hours. Surface staining was performed with anti-CD3-PerCP-Cy5.5 antibody, anti-CD4-PE-Cy7 antibody and anti-CD8-APC-Cy7 antibody (BioLegend, London, UK). Intracellular staining with anti-IFN γ -APC antibody (BioLegend, London, UK) was performed using BD Cytofix/Cytoperm kit (BD Biosciences, Basel, Switzerland). FACS analysis was performed on FACSCantoTM cytometer using FACSDiva software (BD Biosciences, Basel, Switzerland).

Cytotoxicity assay

To assess the functionality of TCLs, standard 4 hour ⁵¹Cr release assay was performed as described elsewhere [22]. In this assay, the amount of leaked ⁵¹Cr from target cells correlates with T cell mediated cytotoxicity. Briefly, autologous EBV-transformed B-lymphocyte cell lines (EBV-BLCLs) were incubated with the drug overnight. These were then labelled with ⁵¹Cr for 1 hour and were washed extensively. Cytotoxic activity was determined after 4 hour

incubation with or without the drug at varying effector/target ratios. Percent cytotoxicity was determined by:

$100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})]$.

Maximum release was determined by lysis of target cells with 0.5% Triton X-100.

Xanthine dehydrogenase (XDH) and aldehyde oxidase 1 (AOX1) PCR

RNA was extracted from PBMC, EBV-BLCL and Huh7 cells (hepatocarcinoma cell line) with RNeasy Mini Kit (Qiagen, Basel, Switzerland). cDNA was generated by using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo-dT as a primer. Standard PCR reactions were performed using the following primers; β actin forward primer (positive control): 5'-CCCTGAAGTACCCCATCGAG, β actin reverse primer (positive control): 5'-CGATGGAGGGGCCGACTCG, XDH forward primer: 5'-GGCATATCATTGGTGCTGTG, XDH reverse primer: 5'-CCTTTTGGAAACAGCAATGGT, AOX1 forward primer: 5'-AACCTTCCATCCAACACAGC and AOX1 reverse primer: 5'-TTCTTATGATCCCCCGTCAG.

Allopurinol and Oxypurinol level determination via LC-MS/MS

Cell medium concentrations of allopurinol and oxypurinol were determined using a UHPLC-MS/MS method. Sample preparation consisted of simple protein precipitation. Acetonitrile (900 μ l) containing the Internal standard 1-methyxanthine-2,4,5,6,13C₄,1,3,9,15N₃ was added to 100 μ l of cell medium. After centrifugation the supernatant was diluted 1:100 with water for sample analysis.

Chromatographic separation of the aqueous samples was performed on a Acquity UPLC system (Waters Corp. Milford, USA) equipped with a Acquity UPLC HSS T3 C18 Column, 100Å, 1.8 µm, 1 mm X 100 mm (Waters Corp. Milford, USA) using Eluent A: Water with 0.1% formic acid and Eluent B: Acetonitrile with 0.1% formic acid as mobile phase. The runtime was 5.5min with a column temperature of 40°C and a flow rate of 0.2ml/min. A gradient was applied as follows: 0-1min 100% A; 1- 2.5min from 100 to 5% A; 2.5- 4min 5% A; 4-4.1min from 5 to 100% A.

Detection was made on a Xevo TQ-S triple quadrupole system (Waters Corp. Milford, USA) operated in Positive Electrospray Ionisation (ESI) mode. Multiple Reaction Monitoring (MRM) was used for quantification, monitoring the following transitions: 153.0>135.9 for Oxypurinol, 136.9>109.9 for Allopurinol and 174.0>115.1 for 1-Mehtylxanthine (IS). The calibration range of the method was 0.1 – 120µg/ml for both analytes with a limit of quantification of 0.1µg/ml.

Statistical analysis

Statistical analysis was performed using non-parametric Kruskal-Wallis test, followed by Wilcoxon signed-rank test for patients' LTT results and Mann-Whitney *U*-test for others. Dose response curves and EC₅₀ were determined using Prism 5.02 (GraphPad Software Inc., CA, USA).

Results

LTT results of allopurinol allergic patients

There were 26 patients with a suggestive history of allopurinol hypersensitivity who had LTT performed with allopurinol and oxypurinol. Twelve of 26 (46%) patients had positive

LTT to oxypurinol but only one patient (4%) had low positive LTT to allopurinol (Table 1). These results showed dose dependent proliferation for oxypurinol (Table 1 and Figure 1). Given these findings, we hypothesised that oxypurinol is possibly more immunogenic and/or that patients were primarily sensitised to oxypurinol.

ALP/OXP-TCL induction

As HLA-B*58:01 allele is highly associated with allopurinol-induced SCAR, we sought to generate ALP/OXP-TCLs from allopurinol-naïve individuals with or without HLA-B*58:01 allele. Given the dose dependent proliferation on LTT, we hypothesised that drug concentrations may play a role in inducing allopurinol or oxypurinol-specific T cells. Therefore, 1, 10 or 100µg/ml of either allopurinol or oxypurinol were used for induction and restimulation as described above.

Restimulation and specificity assays were performed up to four rounds. ALP/OXP-TCLs as determined by CD107a upregulation in CD8 T cells could be demonstrated (Figure 2A).

Unlike CD107a upregulation in CD8 T cells, intracellular IFNγ expression in CD8 or CD4 T cells were either transient or sporadic (data not shown). To make sure that CD107a upregulation was functionally relevant, cytotoxicity assay was performed using ⁵¹Cr release assay. As expected, ALP/OXP-TCLs that upregulated CD107a in response to the drugs were cytotoxic (Figure 2B and 2C).

In all HLA-B*58:01⁺ donors (7/7), ALP/OXP-TCLs could be generated using 100µg/ml. Furthermore, with this dose, ALP/OXP-TCLs could be generated repeatedly (data not shown). But ALP/OXP-TCLs were generated in only 1/6 HLA-B*58:01⁺ donors using 10µg/ml and 1/7 HLA-B*58:01⁻ donors using 100µg/ml. With the lowest dose of 1µg/ml,

ALP/OXP-TCL could not be generated at all from healthy donors. Taken together, HLA-B*58:01 molecule and high drug concentration work synergistically to induce ALP/OXP-TCLs (Figure 3).

Altogether, four ALP-TCLs and five OXP-TCLs could be generated, suggesting that oxypurinol is not more immunogenic than allopurinol *per se*.

Allopurinol Metabolism

XO is converted from XDH and is primarily responsible for the conversion of allopurinol to oxypurinol. In the absence of XO, AOX1 can also metabolise allopurinol to oxypurinol [23]. However, it is not known whether these enzymes are present in PBMC or not. Therefore, mRNA expression of XDH and AOX1 were checked in PBMC and EBV-BLCL, and were found to be absent (Figure 4A). To ensure that the culture medium did not contain metabolising enzyme, allopurinol or oxypurinol was incubated with PBMC and their subsequent levels were checked by LC-MS/MS as described above. Indeed, allopurinol and oxypurinol concentrations did not decrease over time (Figure 4B). Importantly, oxypurinol was not detected in the culture medium incubated with allopurinol. This indicates that when allopurinol is added to the culture medium for either TCL induction or for LTT, it remains as allopurinol and is not metabolised to oxypurinol.

Cross-reactivity

Allopurinol and oxypurinol only differ by one oxygen atom (Figure 5). Given extensive T cell cross-reactivity that has been documented for T cell immune responses to beta-lactams, sulfonamides, quinolones, radiocontrast media and anti-epileptics, we explored whether allopurinol-specific T cells can recognise oxypurinol or not, and vice versa.[24-29] To study

the differences in HLA-B*58:01⁺ and HLA-B*58:01⁻ individuals, we additionally generated ALP/OXP-TCL from HLA-typed allopurinol allergic individuals.

Surprisingly, all ALP-TCLs were unable to recognise oxypurinol and, likewise, none of OXP-TCLs reacted to allopurinol (Figure 6A). This inability to recognise the alternative drug occurred irrespective of HLA-B*58:01 status. T cell clones tend to display more fine specificity than TCLs as TCLs contain many different T cell receptors (TCRs) with higher probability of TCRs that can cross-react [24]. However, even though there were some restricted TCR usages based on V β staining, many ALP/OXP-TCLs consisted of more than one TCR (data not shown). Therefore, this extremely fine specificity is a universal feature of allopurinol or oxypurinol-specific T cells. This lack of cross-reactivity also explains why PBMCs from allopurinol allergic patients respond strongly to oxypurinol on LTT but poorly to allopurinol.

Hypoxanthine and xanthine are naturally occurring purine derivatives and share structural similarity to allopurinol and oxypurinol, respectively (Figure 5). Therefore, we further investigated whether ALP/OXP-TCLs can cross-react with these molecules. Most ALP/OXP-TCLs (9/10) did not recognise xanthine and hypoxanthine (Figure 6B). However, one OXP-TCL from a HLA-B*58:01⁺ donor (donor 616) was able to recognise xanthine but not hypoxanthine (Figure 6B and C). This xanthine recognition was functionally relevant as xanthine specific cytotoxicity could be demonstrated on ⁵¹Cr release assay (data not shown). Remarkably, this TCL still did not recognise allopurinol, once again highlighting the fine specificity of oxypurinol-specific T cells (Figure 6C).

Functional T cell avidity

As high drug dose increases the likelihood of developing ALP/OXP-TCL, we sought to establish whether HLA status has any influence on T cell reactivity. When EC₅₀ of TCL generated using 100µg/ml or 10µg/ml of the drugs were assessed, they were found to have lower and higher avidity, respectively (Figure 7A). This implies that higher concentration of drug *in vitro* results in preferential expansion of low avidity T cells and vice versa. When the effect of HLA-B*58:01 status on the functional avidity of TCL were analysed, TCL from HLA-B*58:01⁺ donors had higher functional avidity compared to those from HLA-B*58:01⁻ individuals (Figure 7A).

Regardless of HLA status or drug concentration used to induce TCL, ALP/OXP-TCLs have steep dose-response curves with the loss of reactivity at 1/10th and 1/100th of the induction dose used for HLA-B*58:01⁻ and HLA-B*58:01⁺ individuals, respectively (Figure 7B). This implies that allopurinol or oxypurinol-specific T cells are highly dose dependent, not only for their induction but also for activation, particularly if these T cells are generated from HLA-B*58:01⁻ donors. Conversely, in individuals with HLA-B*58:01 allele, ALP/OXP-TCLs are able to recognise lower concentration of drugs, particularly if the TCLs were induced with the lower dose.

Discussion

Even though allopurinol hypersensitivity has been described more than 40 years ago, there has not been a study that characterised allopurinol or oxypurinol-specific T cells in detail. In contrast to the previously held assumption that allopurinol-specific T cells cross-react with oxypurinol, our study shows that allopurinol hypersensitivity is primarily mediated by T cells that react against oxypurinol in a dose dependent manner [19]. In fact, oxypurinol-

specific T cells do not recognise allopurinol and vice versa, due to the remarkable ability of these drug-specific T cells to recognise one oxygen difference. Putting our data together, allopurinol allergic patients are likely to develop oxypurinol-specific T cell response, not because oxypurinol is more immunogenic, but because the allopurinol molecule is rapidly metabolised to oxypurinol *in vivo* and drug concentration is a critical factor in immunogenicity. This effect is particularly pronounced in those with HLA-B*58:01 allele, resulting in higher sensitising potential with the generation of higher avidity T cells. This implies that beyond epidemiological association, the HLA-B*58:01 molecule is likely to play an important additional role in pathogenesis. Therefore, our study shows for the first time that immunogenicity of allopurinol, or rather, oxypurinol, is likely due to its dose dependent interaction with T cells, particularly in the presence of HLA-B*58:01 molecule.

The lack of cross-reactivity between allopurinol and oxypurinol is particularly fascinating as extensive T cell cross-reactivity has been documented for other drug classes [24,25,28]. Carbamazepine-specific T cells can recognise structurally related drugs as carbamazepine analogues with preserved 5-carboxamide moiety are able to specifically bind HLA-B*15:02 molecule [29]. In contrast, even a slight modification of abacavir abolishes T cell reactivity as it is no longer able to bind HLA-B*57:01 molecule [30]. These imply that for both abacavir and carbamazepine, drug binding to HLA molecule is a key determining factor for cross-reactivity.

However, the lack of cross-reactivity in ALP/OXP-TCL cannot be related to the differences in ability of allopurinol or oxypurinol to bind to HLA-B*58:01 molecule as the lack of cross-reactivity was also consistently observed in HLA-B*58:01⁻ individuals. This implies that unlike carbamazepine, allopurinol and oxypurinol-specific T cells are able to discern minute

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differences between these two drugs. It is not surprising that febuxostat, another structurally unrelated XO inhibitor, was well tolerated in 12 out of 13 individuals with severe allopurinol hypersensitivity [31].

Oxypurinol has been suggested as an alternative drug for allopurinol intolerant patients [32]. However, up to 40% of allopurinol intolerant patients treated with oxypurinol developed allergic reactions [33]. These reactions were usually similar to what the patient developed for allopurinol, occurring mostly within the first week and in some cases, after the first dose. In light of our data, these findings are expected as allopurinol allergic individuals actually develop immune responses primarily against oxypurinol. Therefore, in those with immune-mediated allopurinol hypersensitivity, the use of oxypurinol is likely to be unsuccessful and hazardous.

While allopurinol can inhibit T cell activation at high doses, this is unlikely to be the explanation for the patients' negative LTT results to allopurinol since it can still induce and stimulate ALP-TCLs in our study [34]. Instead, inability of oxypurinol-specific T cells to recognise allopurinol is likely the explanation for the negative LTT results. Therefore, *in vitro* diagnostic tests for allopurinol hypersensitivity must include oxypurinol to avoid a high false negative rate. This is supported by our findings and by the previous LTT reports that showed oxypurinol responses were stronger and predominant [17,18]. Our study further illustrates how *in vitro* diagnostic tests can yield false negative results for a particular drug when PBMC lack metabolising enzymes and a metabolite, not the drug, is the main antigen. This limitation must be recognised and ideally overcome by including drug metabolites in the test panel.

Surprisingly we were able to find one of 10 ALP/OXP-TCLs that cross-reacted with xanthine. This shows that endogenous small molecules such as purine derivatives can, albeit uncommonly, activate T cells. It is possible that *in vivo* deletion of T cells that react with such 'autoantigen' occurs to prevent harmful immune responses. However, in a manner analogous to autoimmunity triggered by infection, oxypurinol-specific T cells may rarely cross-react with xanthine as was shown in our study. Further studies need to be performed to pursue the idea that endogenous, non-peptide structures can act as an autoantigen, be it directly, by inducing a new immune response, or indirectly, by cross-reactivity of T cells, induced by an immunogenic drug.

It has been shown that naïve T cells can differentiate into high- or low-avidity cytotoxic T cells depending on the peptide concentrations used for stimulation [35]. We found this also to be the case for small molecules; TCLs stimulated with high dose of drug resulted in lower avidity CD8 T cells and vice versa. Interestingly, we also found that HLA-B*58:01⁺ ALP/OXP-TCLs had higher avidity. Whether this is due to the ability of allopurinol or oxypurinol to bind to HLA-B*58:01 molecule resulting in increased number of drug-MHC complexes available for T cells or not, needs to be explored further.

Our data show that allopurinol or oxypurinol-specific T cells are extremely susceptible to the effect of drug concentrations and dose is crucial for both immunogenicity and reactivity. These *in vitro* data are consistent with the epidemiological study that showed the allopurinol dose was higher in patients with SJS/TEN and another study that identified higher starting dose as a risk factor in allopurinol hypersensitivity [3,36]. Study of life-threatening allopurinol toxicity showed that most patients had renal insufficiency before the initiation of the drug and developed worsening renal function as a part of hypersensitivity reaction [8].

Since oxypurinol clearance is related to renal function, oxypurinol concentration is much higher in those with renal failure. A recent study of patients with chronic renal insufficiency in Korean populations showed that allopurinol-induced SCAR occurred in 18% of patients carrying HLA-B58 allele compared to none in those without it [9]. Interestingly, this proportion is also higher than the estimated positive predictive value of 2.7% in those with HLA-B*58:01 allele [6]. All these epidemiological findings are consistent with our data which highlight the importance of HLA status and drug concentration for T cell reactivity. It appears that in allopurinol-induced SCAR, the widely held dogma that Type B reactions are dose independent needs to be questioned.

The fact that drug desensitisation is widely used in clinical practice further supports the view that Type B reactions are dose dependent. The mechanism behind drug desensitisation is largely unknown but the dose response curve of ALP/OXP-TCLs (Figure 7B) provide a clue as to the mechanism behind allopurinol desensitisation, which is successful in up to 78% of mild cases [2]. Since the dose response curve is notably steep with loss of reactivity at 1/100th of the induction dose regardless of HLA-B*58:01 status, slow titration of doses may render T cells anergic. However, whether success rate for allopurinol desensitisation also depends on HLA-B*58:01 status is not known.

In light of dose dependent activation of drug-specific T cells, the potential role of oxypurinol level monitoring and active interventions to remove oxypurinol in patients with SCAR warrant further investigations. Probenicid has been shown to increase renal excretion of oxypurinol [37]. In patients with allopurinol-induced SCAR and preserved renal function, this may be a safe adjunct to therapy. The half life of oxypurinol in anuric patients is estimated to be approximately 10 days, the use of dialysis or hemofiltration may have

potential benefits, particular when a standard 4 hour hemodialysis can reduce oxypurinol level by 39% [8]. Clinical trials will need to be performed to evaluate their actual usefulness. Our study is limited by its *in vitro* design, particularly with prolonged culture conditions in the presence of IL2. These conditions are likely to render T cells more responsive to antigen stimulations. Nonetheless, the association noted in the epidemiological studies with HLA-B*58:01 allele and drug concentrations can be replicated in our study, signifying the involvement of these factors in immunopathogenesis. It may also be questioned whether such supra-physiological dose (100µg/ml) of allopurinol or oxypurinol is relevant *in vivo*. While the normal plasma concentration of oxypurinol is 5-10µg/ml, studies involving anuric patients showed that a single dose of allopurinol can result in up to 50µg/ml of oxypurinol *in vivo*, suggesting that the dose used in our conditions is not beyond the realm of possibility [8]. Finally, translating *in vitro* data to *in vivo* situation can be complicated but in the absence of animal models in HLA associated drug hypersensitivity, we believe that our study offers some valuable insights.

Type B reactions are becoming less ‘unpredictable’ as HLA associations are being discovered. Our study shows that HLA status is important for drug-specific T cell response. Further studies will need to be performed to assess how allopurinol or oxypurinol interacts with this HLA molecule. Finally, due to the paucity of data on the role of dose in immune-mediated drug hypersensitivity, it is not clear whether our dose dependent conclusion can be generalised to other drug hypersensitivity reactions. Further studies with other drugs must also be undertaken to determine whether this is the case and, indeed, one of the defining features of Type B reactions, namely dose independence, should be revised.

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Conflict of Interest

The authors declare no conflict of interests. This work is supported by the Swiss Center for Applied Human Toxicology (SCAHT) and Swiss National Science Foundation (SNF Grant Nr. 310030-129828-1).

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Figure legend 1

Lymphocyte transformation test (LTT) results of allopurinol allergic patients with positive LTT (n = 12). Patients' PBMC are tested against varying concentrations (1, 10 and 100µg/ml) of allopurinol and oxypurinol. Bars represent median with interquartile range. * p<0.05, ** p<0.01, *** p<0.001

Figure legend 2

(A) Each ALP/OXP-TCLs were tested for specificity via CD107a upregulation on FACS. Representative FACS plots of an OXP-TCL from Donor 616 (HLA-B*58:01⁺) gated on CD3⁺CD8⁺ cells after stimulation with either culture medium or oxypurinol were shown.

(B) Representative results of three ALP/OXP-TCLs (filled circles) based on CD107a upregulation display specific lysis of autologous EBV-BLCL in the presence of the drug. This is not the case for the non-specific TCLs (open circles). E:T ratio is defined as effector (TCL) to target (EBV-BLCL) ratio.

(C) Correlating cytotoxicity of ALP/OXP-TCLs based on ⁵¹Cr release assay with the difference in upregulation of CD107a on CD8 T cells by the drugs from the background.

Shown is the percent specific lysis at E:T ratio of 10:1. The difference of 3% is chosen as an arbitrary cut-off (dotted line).

Figure legend 3

The maximum observed differences in upregulation of CD107a on CD8 T cells in ALP/OXP-TCL from the background. The lines represent median values. Mann-Whitney test was used. * $p < 0.05$, ** $p < 0.01$

Figure legend 4

(A) mRNA expression of Xanthine dehydrogenase (XDH) and aldehyde oxidase 1 (AOX1) was absent in PBMC and EBV-BLCL. Huh-7 (hepatocarcinoma cell line) was used as a positive control. (B) Allopurinol or oxypurinol was added to PBMC and the drug concentrations were measured after various incubation periods. Allopurinol or oxypurinol levels did not decrease and oxypurinol was not detected in culture medium of PBMC incubated with allopurinol. Bars represent mean with SEM.

Figure legend 5

Allopurinol

Oxypurinol

Hypoxanthine

Xanthine

Figure legend 6

(A) Upregulation of CD107a on CD8 T cells after stimulation with different drugs. Alternative drug is oxypurinol for ALP-TCL and vice versa. (B) Upregulation of CD107a on CD8 T cells in response to various molecules. Boxed is the result of OXP-TCL from donor 616 (HLA-B*58:01⁺). (C) CD107a upregulation of xanthine-cross-reactive OXP-TCL. Bars represent mean with SEM. ** $p < 0.01$, *** $p < 0.001$

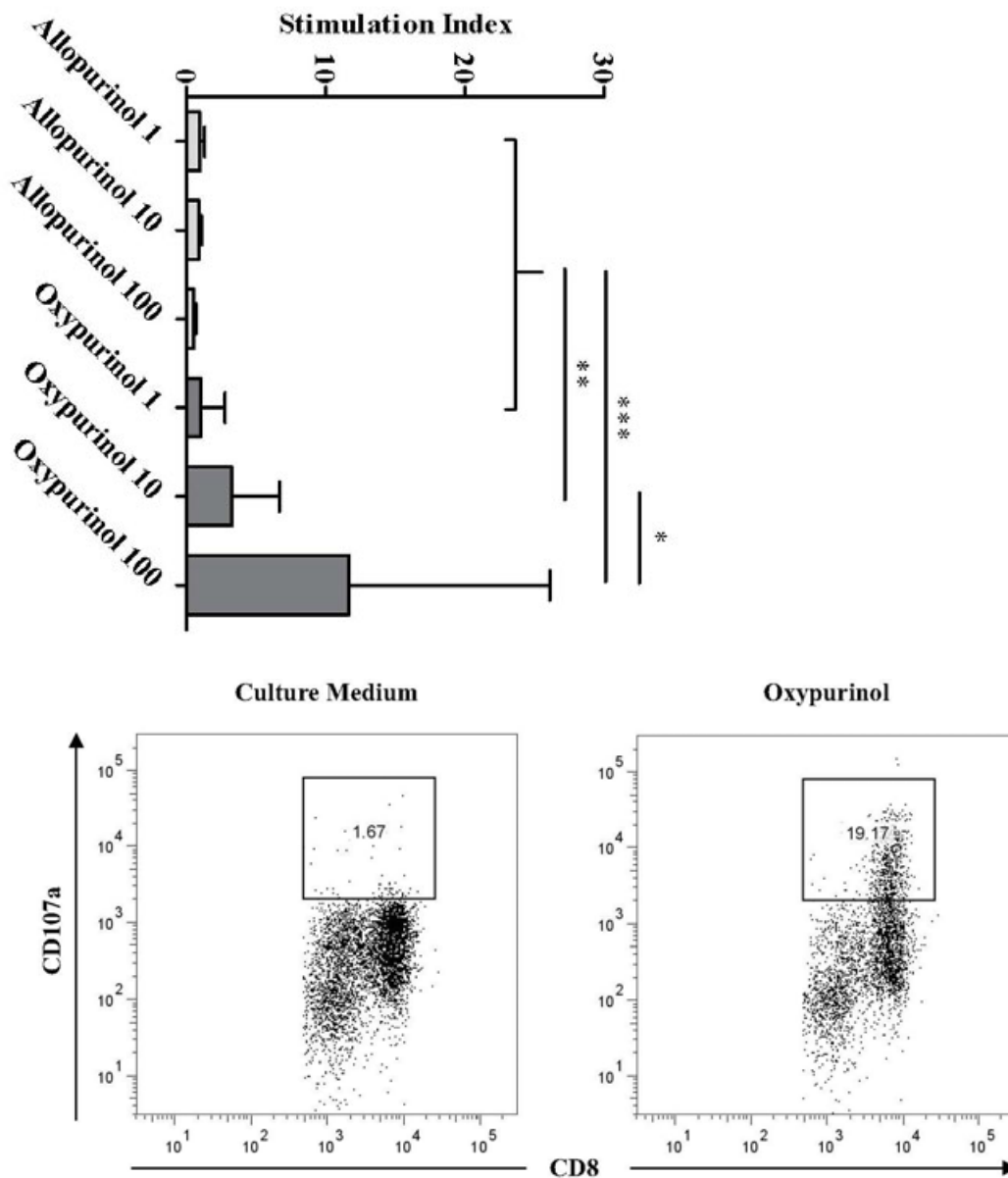
Figure legend 7

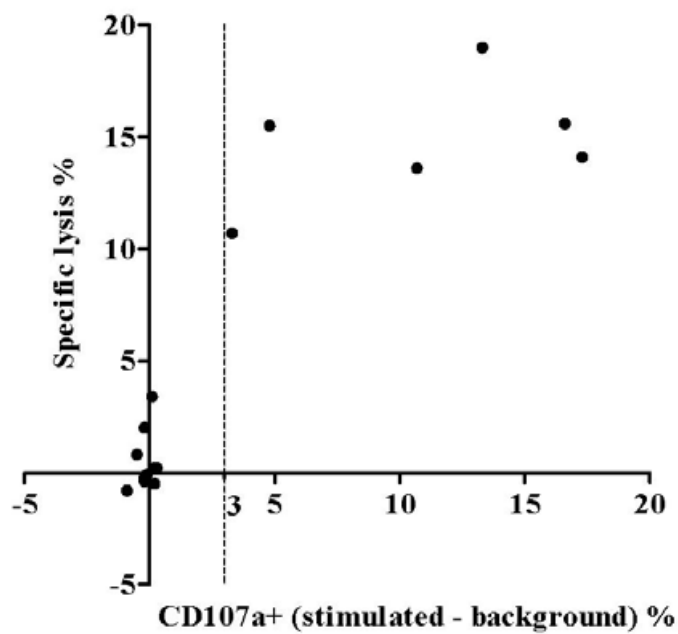
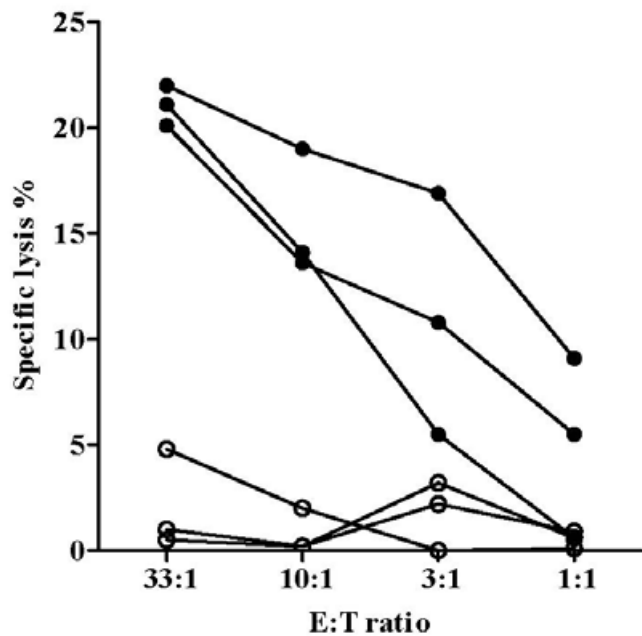
(A) EC₅₀ of ALP/OXP-TCLs as determined by CD107a upregulation on CD8 T cells. EC₅₀ is defined as the concentration of drugs required to achieve half maximum response. (B) Dose response curve of ALP/OXP-TCLs relative to the induction dose. Bars represent mean with SEM. * p<0.05, ** p<0.01

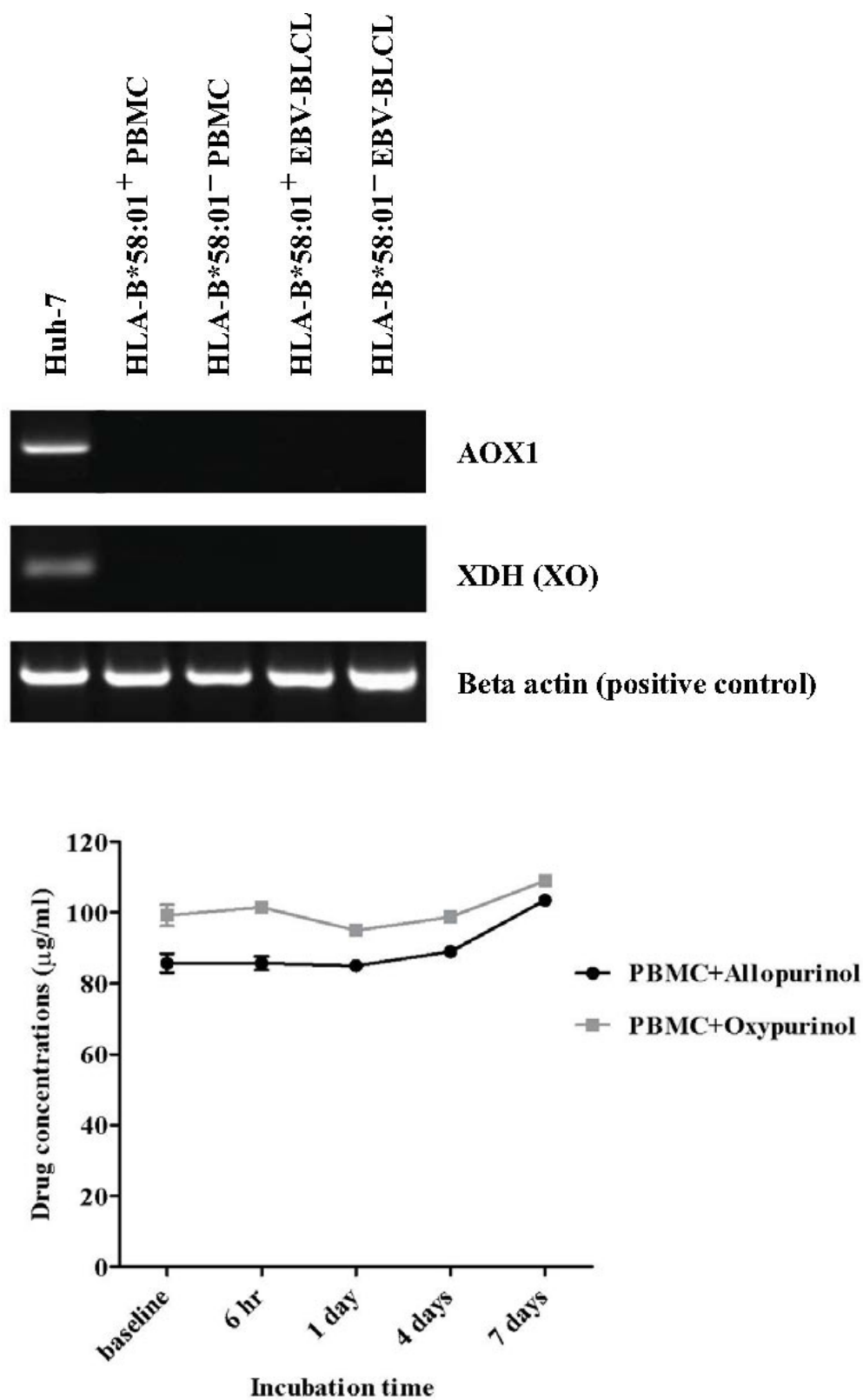
Table 1 Clinical features and LTT results of individuals with allopurinol hypersensitivity and positive LTT.

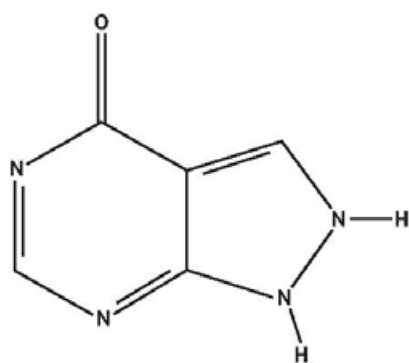
Patient	Age	Symptoms	Lymphocyte Transformation Test (LTT) SI					
			Allopurinol (µg/ml)			Oxypurinol (µg/ml)		
			1	10	100	1	10	100
399	71	MPE	0.8	0.8	0.1	0.8	6.7	11.1
651	59	DRESS/DIHS	0.9	1.1	0.1	0.9	3.7	5.4
652	28	Fixed drug eruption	1.0	1.3	0.6	1.0	6.7	12.3
658	81	DRESS/DIHS	0.9	0.9	0.7	2.4	2.6	21.7
659	49	Exanthema	1.3	1.0	2.1	5.3	76.9	54.9
660	67	Erythroderma	0.7	0.5	0.4	0.7	0.6	27.6
661	69	DRESS/DIHS	0.9	0.5	0.4	3.1	3.4	6.7
663	29	DRESS/DIHS	0.9	1.1	0.5	2.9	2.7	4.2
664	57	DRESS/DIHS	1.1	0.9	0.4	0.9	1.3	3.1
665	49	DRESS/DIHS	1.3	0.8	0.7	0.6	3.1	15.8
667	71	Exanthema	1.4	1.1	0.8	1.0	2.1	2.9
668	67	DRESS/DIHS	1.3	0.7	0.6	1.2	8.1	54.3

Highlighted in bold is SI greater than 2. PBMC from Donor 399, 651, 652 and 668 were available for TCL generation and they were HLA-B*58:01⁻. DRESS: drug rash with eosinophilia and systemic symptoms; DIHS: Drug-induced hypersensitivity syndrome; LTT: lymphocyte transformation test; MPE: maculopapular exanthema; SD: standard deviation; SI: stimulation index.

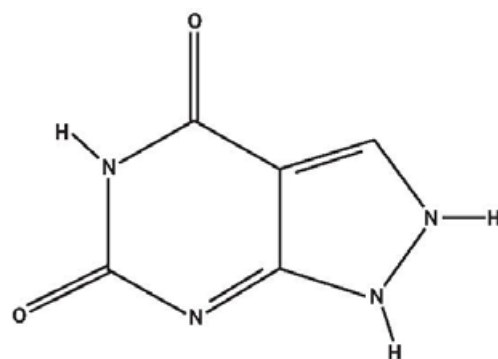




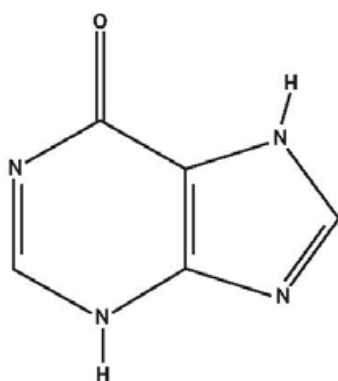




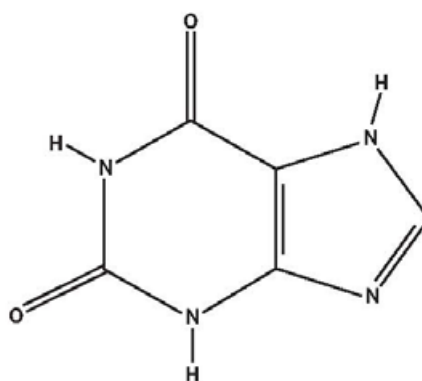
Allopurinol



Oxypurinol



Hypoxanthine



Xanthine

