

ORIGINAL ARTICLE

Processing-dependent and -independent pathways for recognition of iodinated contrast media by specific human T cells

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Summary

Background One to three percent of patients exposed to intravenously injected iodinated contrast media (CM) develop delayed hypersensitivity reactions. Positive patch test reactions, immunohistological findings, and CM-specific proliferation of T cells *in vitro* suggest a pathogenetic role for T cells. We have previously demonstrated that CM-specific T cell clones (TCCs) show a broad range of cross-reactivity to different CM. However, the mechanism of specific CM recognition by T cell receptors (TCRs) has not been analysed so far.**Objective** To determine how T cells specifically recognize CM.**Methods** CM-specific TCCs were generated from human blood of three CM-allergic patients and a specific TCR was transfected into a mouse T cell hybridoma. Functional analysis such as proliferation assays, IL-2 secretion assays, and calcium influx experiments were performed using irradiated, glutaraldehyde-fixed, CM-pre-incubated, human leucocyte antigen (HLA)-DR-matched or -mismatched antigen-presenting cells (APCs), and HLA-blocking antibodies. **Results** We identified two mechanisms of T cell stimulation: some TCCs and the transfectant reacted to CM independent of uptake by APCs because proliferation/IL-2 secretion occurred in the presence of glutaraldehyde-fixed APCs, and intracellular calcium increased within seconds after drug addition. Other TCCs required functional APCs, compatible with uptake and presentation of CM on MHC-class II molecules, as implied by three findings: (1) glutaraldehyde fixation of APCs abrogated presentation; (2) CM could not be washed away from CM-pre-incubated APCs; and (3) the optimal pulsing time was 10–20 h. Because allogeneic, MHC-matched, CM-pulsed APCs could induce proliferative responses as well, the ability of CM uptake and presentation is not unique to APCs from patients with CM-induced delayed hypersensitivity.**Conclusion** Our data suggest that CM may be stimulatory for T cells either by direct binding to the MHC–TCR complex or by binding after uptake and processing by APCs. This questions the assumed inert nature of CM.**Keywords** allergy, antigen presentation/processing, cell activation, cell proliferation, human, T cell receptors, T cells

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Introduction

Iodinated contrast media (CM) are among the most frequently used pharmaceuticals for intravascular injections, with about 80 million administrations per year worldwide. Consisting of highly concentrated solutions of tri-iodinated phenyl derivatives, they are administered in

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volumes of 50–150 mL or more. They are rapidly distributed in the body and almost completely recovered unmetabolized in the urine within 24 h [1, 2].

Injections of CM may cause adverse reactions, many of which resemble immune-mediated side-effects, which can be divided into immediate (<1 h) and delayed ($\geq$ 1 h) hypersensitivity reactions [3]. By reducing the osmolality of the injected solution, the frequency of immediate adverse reactions after CM administration has been reduced significantly [4]. However, all CM (ionic and non-ionic, monomers and dimers) can elicit hypersensitivity reactions in susceptible individuals.

Whereas CM-induced immediate hypersensitivity reactions often present with pruritus, urticaria, angioedema, erythema, dyspnoea, nausea/vomiting, hypotension, and collapse [3, 5, 6], most of the delayed hypersensitivity reactions are macular or maculopapular rashes, delayed-appearing urticarial rashes and rarely, more serious, bullous exanthema [2, 3, 6–8]. Their incidence appears to be in the range of 1–3% when monitored over a period of 7 days after CM exposure [6–11]. Because CM are used for diagnostic purposes only, the level of tolerance for side-effects is very low. To further minimize them, a better understanding of the mechanisms of side-effects is necessary.

Recently, there has been increasing evidence that T cells mediate many of the delayed skin symptoms after CM exposure [2, 12, 13]. Supporting this hypothesis are the clinical symptoms, the high frequency of recurring reactions in the same subject, and in IL-2-treated patients, the positive results from skin and provocation tests of affected subjects, and the histopathology of skin eruptions and positive skin test sites [2, 14]. Moreover, we recently detected CM-reactive T cells in sensitized patients by *in vitro* lymphocyte transformation tests (LTTs) [12]. CM-specific T cell clones (TCCs) isolated from human blood of patients showed a broad range of cross-reactivity to different CM [13].

Two mechanisms may explain T cell stimulations by low-molecular-weight compounds such as drugs: first, the hapten concept, where a chemically reactive compound covalently binds a protein or peptide and elicits an immune response to the neo-epitope. This includes the prohaptent hypothesis, according to which an inert compound, unable to covalently bind to peptides or proteins is metabolized, leading to a chemically reactive compound, the hapten [15–20]. This would necessitate that the CM is taken up by APCs, metabolized, and presented to T cells as part of peptides on MHC molecules. Conversely, and because CM are chemically inert compounds, they may stimulate T cells by direct interaction with the T cell receptor (TCR) from the outside, initiating signals within T cells, which are strengthened by an additional MHC interaction (pharmacological interaction of drugs with immune receptors, the p–i concept) [20–24]. A cornerstone observation in support of the p–i concept was the finding that

‘non-hapten’ drugs such as sulphamethoxazole, lidocaine, carbamazepine, and others are recognized by drug-specific T cells even after fixation of the APCs, indicating that intracellular uptake of the drug is not needed for stimulation [21, 25, 26]. Further, no T cell stimulation was observed if the APCs were pre-incubated with these drugs and subsequently washed before addition to the TCCs. This drug-specific but metabolism and processing-independent stimulation was also observed when drug-specific TCRs were transfected into mouse T cell hybridomas [27, 28].

Here, we addressed the question of how CM-specific T cells recognize chemically inert CM. We demonstrate that CM can trigger a T cell-mediated reaction by interacting with some TCRs and MHCs, directly supporting the p–i hypothesis. Other T cells, however, required prior uptake and processing of CM by APCs. Our findings are clinically relevant because a small part of the usually very large and rapidly excreted amount of CM may be taken up by tissue or blood cells, remain in the body longer than expected, and may give rise to delayed immune reactions.

Patients, materials and methods

Patients

Patient 1 (F, 71 years) had a generalized rash 18 h after an angiography with iohexol (Omnipaque®). Patient 2 (M, 60 years) developed a generalized maculopapular exanthema 72 h after iomeprol (Iomeron®) exposure. He had experienced a similar rash after exposure to an unknown CM the year before. Both patients have been described previously [13]. Patient 3 (M, 51 years) developed a generalized exanthema and high fever after the second and third dose of CM within 15 days (ioversol, Optiray® and ioxithalamate-meglumine, Telebrix®, Guerbet, Zürich, Switzerland).

Human leucocyte antigen typing of patients and other donors

The human leucocyte antigen (HLA) genotype was determined by PCR using HLA Sequence Specific Primers Kits for HLA A*, B*, and DRB1* (2000-020; Protrans, Hockenheim, Germany). The HLA genotype of patient 1 was A*25/32, B*35/51, DRB1*01/08. The HLA genotype of patient 2 was A*24/26, B*49/51, DRB1*04/07. The HLA genotype of patient 3 was A*02/03, B*07/18, DRB1*11/15. To determine the HLA-DR restriction of TCCs from patient 2, the following Epstein–Barr virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCLs) were used as allogeneic APCs in proliferation assays: HLA of EBV A: A*24/x, B*38/44; DRB1*01/04 (match with patient 2: HLA-A*24 and DRB1*04); EBV B: A*02/11, B*08/44; DRB1*03/04 (match: HLA-DRB1*04); EBV C: A*24/32, B*14/51, DRB1*07/12 (match: HLA-A*24 and DRB1*07); EBV D: A*02/68, B14/50, DRB1*07/13 (match: HLA-DRB1*07), EBV E: A*03/30, B*18/35, DRB1*01/08 (no HLA match).

Antibodies and drugs

For HLA restriction experiments, the following purified mouse anti-human antibodies were used: anti-HLA-DR (L243, 1 : 20), -DP (SPV-L3, 1 : 20) and -DQ (B7-21, 1 : 20), and anti-HLA-class I (W6/32, 1 : 10). For flowcytometry, we used fluorochrome-labelled anti-human CD3, CD4, CD8 (BD/Pharmingen, Franklin Lakes, NJ, USA) and monoclonal antibodies against 24 different TCR-V β chains (Immunotech, Marseille, France) [29]. CM used for all the experiments were as follows: iohexol (Omnipaque[®], GE Healthcare, Wädenswil, Switzerland and Bayer Schering Pharma), iomeprol (Iomeron[®], Bracco Suisse S.A., Mendrisio, Switzerland), iobitridol (Xenetix[®], Guerbet), ioversol (Optiray[®], Guerbet), and iodixanol (Visipaque[®], GE Healthcare). The concentrations used were not toxic for peripheral blood mononuclear cells (PBMCs) and had elicited a proliferation in sensitized individuals, but not in healthy exposed or unexposed individuals (data not shown).

Culture medium

Culture medium consisted of RPMI-1640 (52400-25; Invitrogen, Basel, Switzerland) supplemented with 10% pooled heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland), 2 mmol/L L-glutamine (K0202; Biochrom, Berlin, Germany), 25 μ g/mL holo-transferrin (T4132; Sigma-Aldrich, Buchs, Switzerland), 100 μ g/mL streptomycin, and 100 IU/mL penicillin (4-01F00-H; Amimed, Allschwil, Switzerland).

Generation and characterization of contrast-medium-specific T cell clones

T cell lines (TCLs) were generated by stimulation of PBMCs with the chosen CM (5 mg I/mL) and 180 IU/mL human recombinant IL-2 (Proleukin[®], Roche Pharma, Reinach, Switzerland), followed by one CM-specific restimulation after 14 days. TCCs were generated using the limiting dilution technique as described earlier [29]. TCLs and TCCs were restimulated with allogeneic, irradiated PBMCs in combination with PHA (14 μ g/mL; Laboratoires Eurobio, Cortaboeuf Cedex B, France) and 300 IU/mL human recombinant IL-2. TCCs were phenotyped by flowcytometry (FACS-Canto, BD Bioscience, San Diego, CA, USA) and further, TCR-V α and -V β genes were determined by PCR typing as described previously [30].

Generation of dendritic cells

PBMCs were cultured for 2 h at 37 °C and 5% CO₂ in six-well plates at a density of 10⁷ cells per well in 3 mL of culture medium. Medium was removed, wells were gently rinsed twice, and a fresh culture medium containing

800 IU/mL GM-CSF and 500 IU/mL IL-4 was added. Every second day, half of the medium was exchanged by a fresh medium containing the same cytokine concentrations. On day 5, dendritic cells (DCs) were recovered, washed, and used as APCs as described below.

Specificity assays with T cell lines and T cell clones

5 $\times$ 10⁴ T cells (TCCs or TCLs) were incubated with either 2.5–5 $\times$ 10⁴ irradiated (45 Gy), autologous PBMCs, or alternatively with 1 $\times$ 10⁴ irradiated (60 Gy), autologous EBV-transformed B cell lines (B-LCLs) in the presence or absence of antigen. After 48 h, 0.5 μ Ci ³H-thymidine was added, and incorporated radioactivity was measured using a β -counter 14–16 h later (Inotech Filter Counting System INB 384; Inotech Biotechnologies, Basel, Switzerland). In some experiments, allogeneic EBV-transformed B-LCLs were used as APCs as indicated.

Fixing and pulsing experiments with T cell clones

To investigate direct, processing-independent presentation of CM, APCs were fixed with 0.05% glutaraldehyde before coincubations with TCCs and drugs were started. In pulsing experiments, 10⁶ APCs (EBV-transformed B-LCLs or PBMCs) were incubated $\pm$ antigens for the indicated time periods. Antigen-pulsed APCs were washed thrice, resuspended in culture medium (PBMCs in 2 mL, EBV-transformed B-LCLs in 5 mL), irradiated, and 50 μ L was added to 5 $\times$ 10⁴ TCCs. Proliferation was determined as described above. To determine the kinetic of CM uptake, presentation, and T cell reactivity, APCs (EBV-transformed B-LCLs) were incubated $\pm$ CM for 1.25, 2.5, 5, 10, and 20 h. The stimulation index (SI) was calculated as cpm in the presence of CM-pulsed APCs divided by cpm in the presence of mock-pulsed APCs. The experiments with DCs as APCs were performed as follows: 0.5–0.6 $\times$ 10⁶ *in vitro* generated DCs were pulsed $\pm$ CM for 20 h in the presence of GM-CSF and IL-4, washed four times, and 10⁴ DCs were used as APCs without irradiation. Pulsing was performed with iomeprol (eliciting CM), iohexol (cross-reactive CM), or iobitridol (non-reactive CM), followed by coincubation with the same or another CM. For all experiments (coincubations, pulsing, and fixation), incubation times of APCs with TCCs were 48 h, followed by an overnight (o/n) incubation with ³H-thymidine.

Intracellular Ca²⁺ measurements

TCCs were loaded in RPMI with 2 μ g/mL fluo-4 AM (F14201; Invitrogen, Carlsbad, CA, USA), in the presence of 2.5 mM probenecid (P8761, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), and 0.2% pluronic water (P6866, Invitrogen) for 30 min in a shaking water bath at 37 °C. After two washings with RPMI/2.5 mM

probenecid, 0.4 Mio TCCs were pipetted together with 0.4 Mio EBV-transformed B-LCLs in 180 µL RPMI/probenecid into black 96-well clear bottom plates and spun down for 1 min. Measurement was performed 20 min later on a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments Inc., Highland Park, Winooski, VT, USA; excitation: 485; emission: 528) for 3 min. After adding 20 µL of drug solution, the measurement was immediately continued for 12 min. Ionomycin (407952; Calbiochem, San Diego, CA, USA) stimulation was used as a positive control, and RPMI was the negative control. The results are shown as the normalized fluorescence (F/F_0).

Generation of TCR-transfected mouse T cell hybridoma

The TCR transfectant T5227 was generated by transfecting the Vβ- and the Vα-chain from the TCC Iom28 (Table S1) into the TCR⁻ mouse T cell hybridoma 54ζ17. Transfection and cell culture have been described previously [27, 28]. Expression of the TCR on the cell surface of the transfectant was assessed by flowcytometry, and functionality was tested by IL-2 secretion assays after anti-CD3 or drug stimulation.

IL-2 accumulation experiments

To assess IL-2 secretion, 5×10^4 TCR transfectants were co-cultured with 5×10^4 autologous EBV-transformed B-LCLs and indicated CM concentrations. After 72 h of incubation at 37 °C, IL-2 accumulation was determined indirectly by measurement of the proliferation of murine CTLL cells, whose growth is IL-2 dependent. Hundred microlitres of supernatant from the above assays was added to 100 µL medium containing 5×10^3 CTLL cells. After 24 h, 0.5 µCi ^3H -thymidine was added and incorporated radioactivity was measured after o/n incubation [27, 28]. In all assays, radioactivity incorporated into CTLL cells was typically <1000 cpm in the absence of any IL-2, and up to 100 000 cpm in the presence of maximally 50 U/mL of IL-2. Dose-response curves for the IL-2-mediated growth of CTLL cells were performed in all assays and showed a typical sigmoidal shape.

Fixing and pulsing experiments with TCR-transfected mouse T cell hybridoma

APC were pulsed for 6 h in the presence of iomeprol, washed three times, and incubated in culture medium for the rest of the culture period (66 h). Fixation of APCs was performed as described above. IL-2 secretion was measured as described.

Results

CM-specific T cell clones show dose-dependent proliferation

All patients had positive LTTs to the eliciting (iohexol, iomeprol, and ioversol, respectively) and to several other CM [13] (data not shown). TCLs and TCCs were generated to the eliciting CM (iohexol in patient 1, iomeprol in patient 2, and ioversol in patient 3), and from patient 2 also to iodixanol, to which the LTT was also positive. The clonality of TCCs was tested by TCR Vβ-chain analysis by flowcytometry and TCR Vα- and Vβ-chain sequencing (supporting information, Table S1). The TCCs and TCLs (after cloning, with a restricted number of TCRs) of patient 1 and patient 2 were analysed for their dose-dependent proliferation. Most of the TCCs of patient 1 reacted to comparably low concentrations of iohexol (0.31–2.5 mM, Figs 1a and b), whereas the TCCs/TCLs of patient 2 required higher concentrations of CM (iomeprol, iodixanol, or iohexol; 5–10 mM, Figs 1c and d, and data not shown) to achieve optimal proliferation. The proliferation of CD4⁺TCCs/TCLs of patient 2 (Fig. 1c) was always lower than the proliferation of the CD8⁺TCC (Fig. 1d). Proliferation was always stronger when PBMCs were used instead of EBV-transformed B-LCLs (data not shown), and some TCCs proliferated only in the presence of PBMCs (e.g., TCC Iom5.15; Fig. 1c). For further experiments, 0.63 mM iohexol for TCCs of patient 1, and 12.5 mM iomeprol, iodixanol, iohexol, or ioversol for TCCs/TCLs of patient 2 and patient 3 were used, respectively. In conclusion, the TCCs/TCLs generated clearly showed a dose-dependent proliferation to the eliciting CM, indicating a specific response to CM.

Recognition of CM by specific T cell receptors is HLA restricted

Next, we tested whether specific TCCs are activated in the context of specific HLA alleles. CM-specific proliferation of TCCs Om25, Om27, and Om30 from patient 1 were inhibited by anti-HLA-DP (inhibition 74–80%, mean of two experiments for every TCC) and also by anti-HLA-DR antibodies (41–54%), respectively (Fig. 2a and data not shown). Proliferation of the TCC Iom76 from patient 2 was reduced to almost baseline levels in the presence of anti-HLA-DR (inhibition 73%), but not by anti-HLA-DP or -DQ, indicating a clear HLA-DR restriction of this clone (Fig. 2b). Proliferation of the CD8⁺ TCC Iom28 was reduced by anti-MHC-class I (inhibition 54%) but not by anti-MHC class II antibodies (0–21%, Fig. 2c), indicating that all CM-specific TCCs recognized the drugs when presented by HLA molecules.

In patient 2, we found a restriction to HLA-DRB1*07 in all tested iomeprol-specific CD4⁺ TCCs when using various allogeneic HLA-haplotype-matched or -mismatched

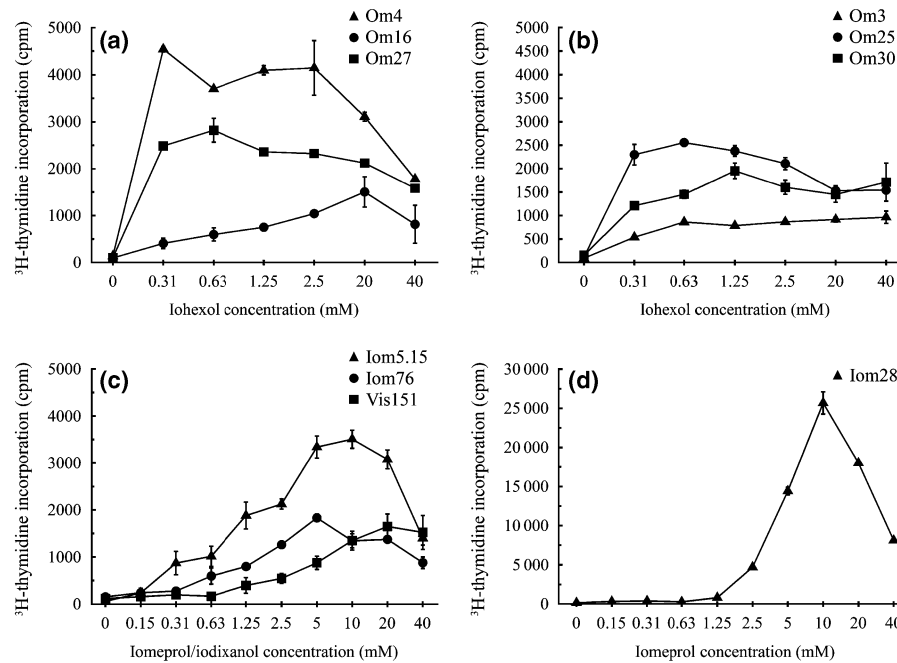


Fig. 1. Dose-dependent proliferation of selected iohexol- or iomeprol-specific T cell clones/lines (TCCs/TCLs). Proliferative responses of iohexol-specific $\text{CD}4^+$ TCCs of patient 1 [(a) TCCs Om4, Om16, and Om27; (b) TCCs Om3, Om25, and Om30], iomeprol- (TCCs Iom5.15 and Iom76) and iodixanol-specific (TCC Vis151) $\text{CD}4^+$ TCCs/TCLs (c), and the iomeprol-specific $\text{CD}8^+$ TCC (d, TCC Iom28) of patient 2. Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCLs) were used as antigen-presenting cells (APCs) for all TCCs, except for Iom5.15 and Vis151, where peripheral blood mononuclear cells (PBMCs) were used. One out of one to two experiments per clone is shown as the mean cpm of triplicate cultures $\pm$ SEM.

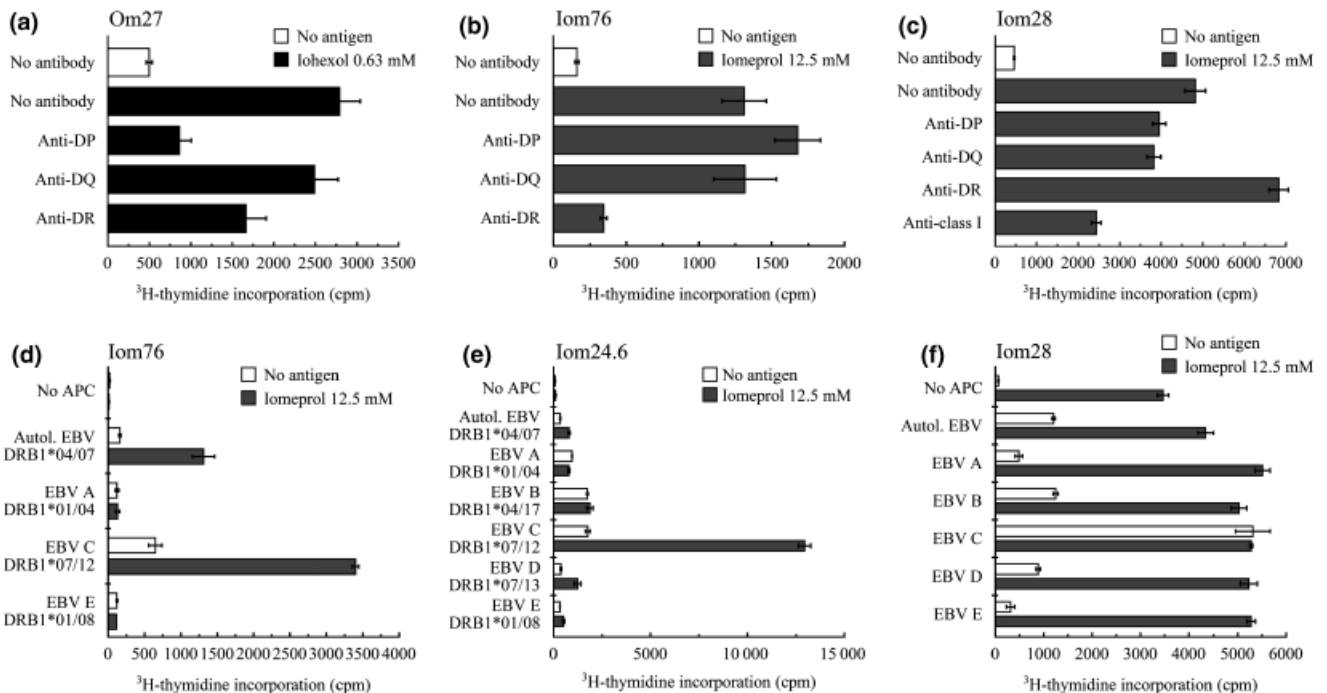


Fig. 2. Human leucocyte antigen (HLA) restriction of contrast media (CM)-specific T cell clones (TCCs) in proliferation assays. CM-specific proliferation of TCCs was measured in the presence of autologous, Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCLs) and anti-HLA-DP, -DQ, -DR, or anti-MHC-I blocking antibodies [(a) TCC Om27, patient 1; (b) TCC Iom76; (c) TCC Iom28 patient 2]. (d-f) Proliferation assays with TCC Iom76, Iom24.6, and Iom28 and different allogeneic, HLA-haplotype-matched or -mismatched APCs. EBV, EBV-transformed B-LCLs. One of two to four experiments per clone and the mean cpm of triplicate cultures $\pm$ SEM are shown.

EBV-transformed B-LCLs as APCs (TCCs Iom76, Fig. 2d; Iom24.6, Fig. 2e; EBV A, B, C, D, and E; HLAs are described in the Materials and methods). No stimulation was induced in these TCCs in the absence of APCs, indicating that HLA-DR expressed on T cells was not sufficient to present the drug to the TCR. In some experiments, allogeneic, HLA-DRB1*07-matched APCs induced a stronger response than autologous APCs (EBV C; Figs 2d and e), supporting the HLA-class II-restricted CM reactivity of the CD4⁺ TCCs, and demonstrating that the patient's APC are not unique in their ability to present CM.

The CD8⁺ TCC of patient 2 Iom28 proliferated in the presence of the eliciting CM (iomeprol) even in the absence of added APCs, which may be because of self-presentation (Fig. 2f) [31]. This clone also showed an enhanced responsiveness to allogeneic EBV-transformed B-LCLs, and in the presence of EBV C even without CM, possibly due to some alloreactivity [31]. Together, these data indicate that HLA is required for CM presentation to CM-specific TCCs, that TCCs are HLA haplotype-restricted, and that some TCCs may show alloreactivity, similar to other drug-specific T cells [31].

CM-specific T cell activation relies on direct presentation and processing of CM by APCs

We further investigated whether presentation of CM to specific TCCs depends on drug uptake and processing by APCs. CM uptake was inhibited by fixing APCs with glutaraldehyde before cocubation with TCCs and CM [21, 25]. Unexpectedly, only two of the tested CM-specific TCCs proliferated after addition of CM to fixed APCs, reaching 20–30% of the proliferative response in comparison with cocubation with CM and non-fixed APCs (TCCs Om25, Fig. 3a; Iom28, Fig. 3b). This may be explained by a processing-independent, direct interaction of the CM with either the MHC or the TCR [23, 24].

For comparison, the reactivity of the protein antigen tetanus toxoid (TT) was analysed with a TT-specific TCC from patient 2 (TCC TT34, Fig. 3c). Because TT must be taken up and processed before its peptides are presented, no proliferation was observed when the TT-specific TCC was cocubated with fixed APCs and TT. However, this TT-specific TCC reacted if cocubated with APCs and TT, or if APCs were pre-incubated with TT for 20 h, followed by three washes of the APCs to remove free TT (subsequently referred to as 'pulsing') and incubation of pulsed APCs and TCC in the absence of the drug. A pre-incubation time of 2 h was too short to allow TCC reactivity (Fig. 3c).

Some CM-specific TCCs/TCLs reacted similarly compared with the TT-specific TCC, namely not or only very moderately to CM presented on fixed APCs (TCCs Om27, Iom76, Vis4, Vis78, and Opt35, Figs 3d–h, and data not shown for Iom4.6, Vis151). This indicates that these TCCs/

TCLs may only react to CM if functional APCs are present. If APCs were pulsed with CM for 2 h, followed by an incubation with the TCCs/TCLs in the absence of the CM, only a few of the tested CM-specific TCCs/TCLs showed an increased proliferation compared with the mock-pulsed APCs (Figs 3e and f), whereas others did not react at all (Figs 3d, g and h). Extending pre-incubation of viable APCs with CM to o/n increased the proliferation of all six TCCs/TCLs that were previously not able to show a marked proliferative response upon presentation of CM on fixed APCs (Figs 3d–h, and data not shown for Iom4.6, Vis151). We assume that this time period allowed antigen (CM) uptake, metabolism, and processing. In fact, proliferation was often increased after o/n pulsing compared with cocubation of CM with non-fixed APCs and TCC/TCL (Figs 3e–g). Importantly, similar results were also obtained with MHC-matched but allogeneic, EBV-transformed B-LCL as APCs (data not shown), which suggests that CM processing is not restricted to patients with CM allergy.

Together, these experiments with CM-specific TCCs/TCLs generated from three different patients indicate that these T cells respond by two mechanisms to CM: first, to intact CM directly (Figs 3a and b), or second, to CM taken up and processed by APCs (Figs 3d–h). All four different CM used were able to stimulate T cells by this processing-dependent mechanism, suggesting that uptake and presentation by APCs is not restricted to a particular CM, and is possibly the more common mechanism.

Immediate increase of intracellular calcium concentration in a CD8⁺ TCC exposed to CM

The two pathways of CM presentation were confirmed by measurement of intracellular calcium concentrations ([Ca²⁺]_i). The processing-dependent TCC Iom76 and the processing-independent TCC Iom28 were analysed after stimulation with iomeprol and iobitridol, a non-stimulatory CM. The TCC Iom76 did not show an increased fluorescence after stimulation with iomeprol (Fig. 4a). In contrast, a very rapid increase of [Ca²⁺]_i was measured in the TCC Iom28 after stimulation with iomeprol, but not after stimulation with the non-reactive CM iobitridol (Fig. 4b). The rapid response of the TCC Iom28 confirms the direct, processing-independent stimulation of this TCR. In contrast, the failure of Iom76 to react rapidly is consistent with the dependence on CM processing for this TCR.

T cell proliferation correlates with the duration of CM uptake and processing by APCs

The similar response of some CM-specific TCCs and the TT-specific TCC (Fig. 3) suggested that CM may be taken up and presented on MHC molecules after intracellular processing. Hence, time-dependent pulsing experiments were performed both with a TT-specific CD4⁺ TCC (TT8,

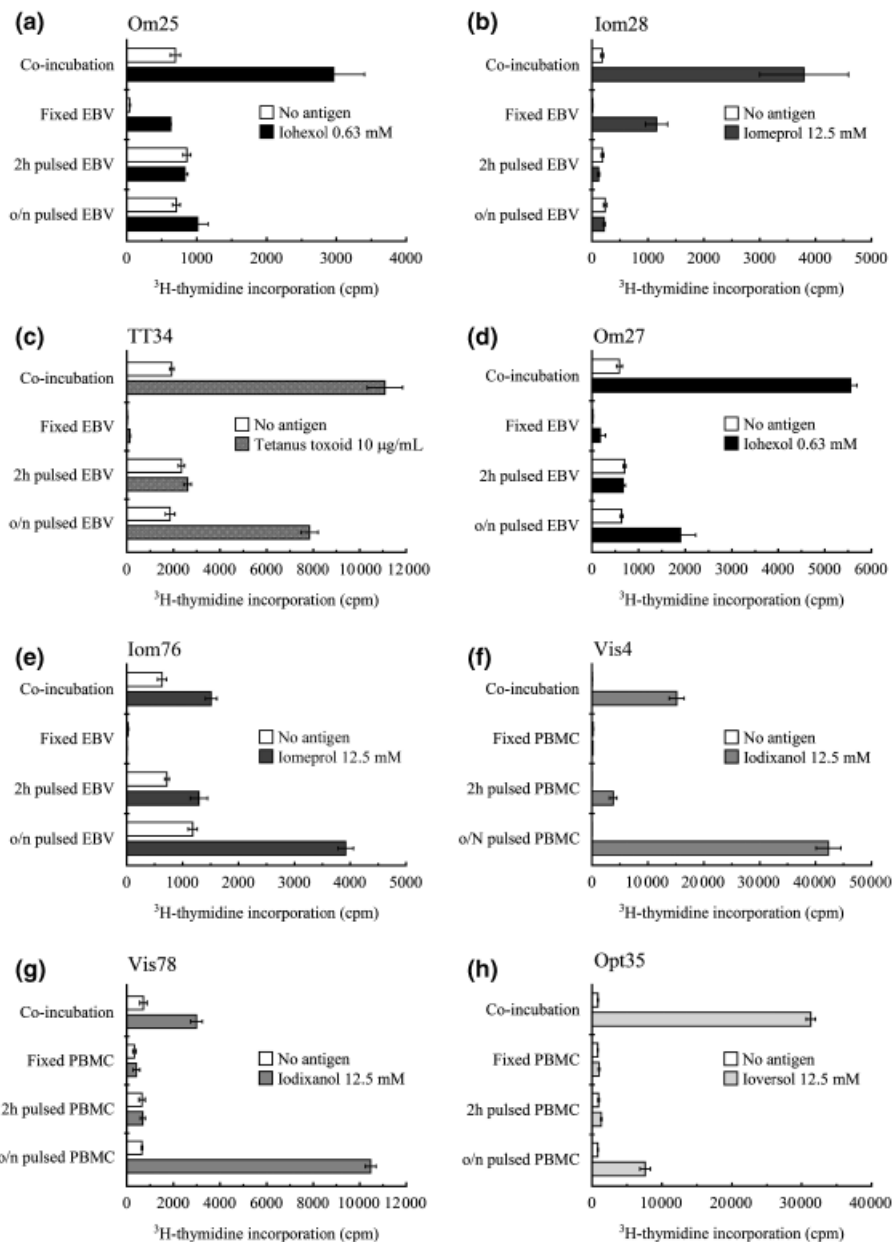


Fig. 3. Contrast media (CM) processing by antigen-presenting cells (APCs) is needed for stimulation of specific T cell clones (TCC) proliferation. Proliferative responses of TCCs were determined with non-fixed (1 and 2, bar from top to bottom in each graph) or glutaraldehyde-fixed APCs (3 and 4, bar) in the absence or presence of the antigen (coincubation). Further, TCCs were incubated with CM- or TT-pulsed APCs (5–8, bar). Pulsing time: 2 or 18 h. APCs used: Epstein–Barr virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCLs) or PBMCs. Different TCCs/TCLs from three patients are shown: (a and b) TCC that reacted with fixed APCs: (a) iohexol-specific TCC Om25 of patient 1; (b) iomeprol-specific TCC Iom28 of patient 2; (c–h) TCCs/TCLs that reacted with antigen-pulsed APCs: (c) TT-specific TCC TT34 of patient 2; (d) iohexol-specific TCC Om27 of patient 1; (e) iomeprol-specific TCC Iom76 of patient 2, (f and g) iodixanol-specific TCLs Vis4 and Vis78 of patient 2; (h) ioversol-specific TCC Opt35 of patient 3. EBV, EBV-transformed B-LCLs; o/n, overnight. One of one to four experiments per clone and mean cpm of triplicate cultures $\pm$ SEM are shown.

Fig. 5a) and the CM-specific CD4⁺ TCC Iom76 (Fig. 5b) from patient 2. In agreement with previous investigations using TT protein antigen [32], a pulsing time of more than 10 h was needed for optimal stimulation of the TT-specific TCC, compatible with an uptake and processing of the antigen. Very similar processing kinetics were observed with the CD4⁺ TCC Iom76 when stimulated with either

iomeprol or iohexol (a CM that induces cross-reactivity), with the strongest response after 10–20 h of pre-incubation. We also measured TCR down-regulation as an early readout for a specific interaction of the TCR with MHC–peptide complexes: here, an 18-h pre-incubation of APCs with CM resulted in stronger TCR down-regulation than a 2-h pre-incubation (data not shown) [32].

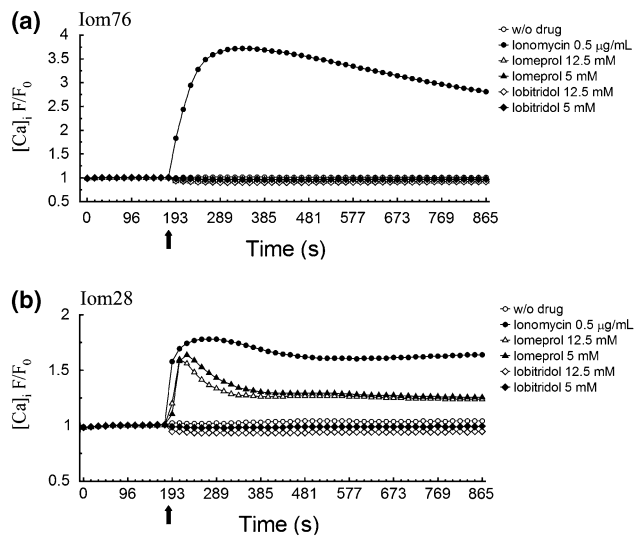


Fig. 4. Increased intracellular Ca^{2+} concentrations after T cell clones (TCC) stimulation with contrast media (CM). TCC Iom76 (a) and Iom28 (b) were stimulated with the specific drug (iomeprol), with a non-reactive CM (iobitridol), with a positive control (ionomycin), or with culture medium only. Arrows indicate the addition of the stimuli. One of three similar experiments is shown.

Cross-reactive drugs are presented in the same way to the same TCR and by different APCs

The cross-reactivity of several CM-specific TCCs was previously described by our group [13]. Here, we wondered whether drugs that elicit cross-reactivity are presented in the same way as the original drug. We could demonstrate that the iomeprol-specific TCC Iom76 was stimulated by iomeprol-pulsed APCs (EBV-transformed B-LCLs; Figs 3e and 5b), but also by iohehexol-pulsed APCs (data not shown and Fig. 5b) and that fixation of the APCs abrogated reactivity to both drugs. The same reactivity pattern was observed by the iodixanol-specific TCC Vis78 (Fig. 3g) when tested with its related and also stimulatory derivative iohehexol (data not shown). Both experiments indicated that different CM use the same way of CM presentation.

Further, we were interested in the influence of APC pulsing with culprit, cross-reactivity-inducing or non-stimulatory CM. In these experiments, we used *in vitro* generated DCs as APCs. As DCs are the primary APCs of the immune system, it was important to demonstrate that they were also able to present CM to TCCs. Moreover, in contrast to EBVs or PBMCs, DCs do not need to be irradiated for proliferation experiments, which somehow may have influenced the CM presentation by APCs. The experiment was performed with the TCC Iom76. DCs were generated *in vitro* from PBMCs of patient 2, pulsed with two stimulatory (iomeprol or iohehexol) and one non-stimulatory CM (iobitridol), or with culture medium for 20 h. Pulsed DCs were incubated with the TCC Iom76

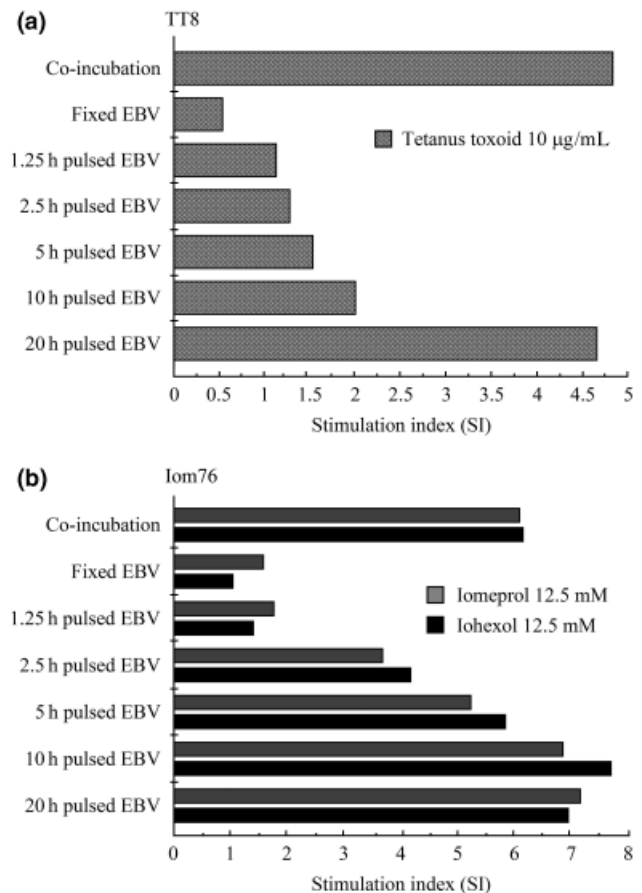


Fig. 5. Kinetics of contrast media (CM) processing by antigen-presenting cells (APCs) for the stimulation of T cell clones (TCCs). APCs (EBV-transformed B-LCLs) were prepulsed for the indicated time with TT, iomeprol, or iohehexol, washed, and co-cultured with TCCs (a, TT8; b, Iom76) for 48 h. The stimulation index was calculated from triplicate culture as cpm in the presence of CM-pulsed APCs divided by cpm in the presence of mock-pulsed APCs (background proliferation for TT8: 83–347 cpm; Iom76: 225–772 cpm). EBV, Epstein-Barr virus; EBV, EBV-transformed B-LCLs, B-LCLs; B-lymphoblastoid cell lines. For TCC Iom76, one of two similar experiments is shown.

either in culture medium or with iomeprol, iohehexol, or iobitridol. DCs pulsed with iomeprol or iohehexol induced a strong reaction, whereas iobitridol failed to induce any proliferation (Fig. 6). In iomeprol- or iohehexol-pulsed DCs, a co-incubation with iomeprol or iohehexol further increased the reactivity, whereas a co-incubation with iobitridol had no enhancing or inhibitory effect (Fig. 6, second and third panel).

Together, these data demonstrate that CM presentation is similar among all stimulatory variants and that non-stimulatory CM does not alter the specific recognition. Additionally, these experiments indicate that CM recognition is independent of the type of APC and the stimulatory efficacy of DCs may even be superior to irradiated EBV-transformed B-LCLs.

Specificity of the TCC Iom28 is demonstrated by transfection of the CM-specific TCR in a mouse T cell hybridoma

The TCC Iom28 did not show any cross-reactivity [13] and recognized the drug on fixed APCs. To demonstrate that the specificity of this TCC relies on a specific interaction of the CM with the TCR, and to further analyse the role of self-presentation, we transfected the TCR V α - and V β -chain of the TCC Iom28 into a mouse T cell hybridoma (T5227). The response of the transfectant T5227 to iomeprol was dose-dependent (Fig. 7a) and – similar to the original TCC – it exclusively recognized iomeprol but no

other CM (Fig. 7b). In contrast to the TCC, the transfectant T5227 required the presence of APCs to be stimulated (Fig. 7c), most likely because mouse T cell hybridoma do not express any human MHC molecules that are required for self-stimulation. These findings support the idea that the original TCC reacted because of self-presentation of CM on self-MHCs. Further, T5227 recognized the CM on glutaraldehyde-fixed APCs, whereas pulsing of the APCs did not increase reactivity over the background. Lastly, it was necessary that iomeprol was present during co-incubation. Taken together, this TCR-transfected mouse T cell hybridoma proves the specific recognition of CM by a particular TCR, and the role of MHC in this processing-independent T cell activation.

Discussion

Delayed hypersensitivity reactions to CM are, like other drug-induced reactions, elicited by drug-specific T cells. Here, we have focused on how CM stimulate T cells, and have found two different pathways of CM recognition, both of which require MHC molecules for optimal stimulation. The first one is compatible with the p-i concept [23, 24, 33], involving direct binding of the CM to the TCR-MHC complex; it requires MHC molecules, but is independent of functional APCs, and CM are easily removed by washing of the APCs. The second pathway has all the features of classical antigen presentation: it relies on functional APCs, requires an extended time interval until optimal presentation is achieved, involves stable presentation of the CM on MHC-II molecules, and is best compatible with the pro-hapten hypothesis [17–19, 33].

CM are thought to be chemically inert and to be rapidly excreted from the body in their unmetabolized state via the urine [1, 2], which suggests that CM stimulate T cells

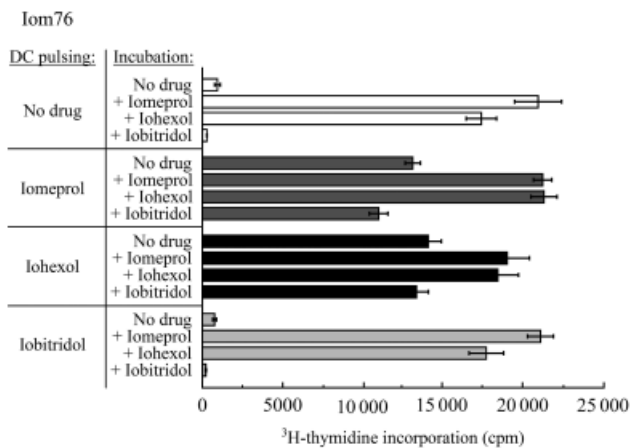


Fig. 6. *In vitro* generated monocyte-derived dendritic cells (DC) may serve as antigen-presenting cells (APC) for various contrast media (CM). DCs were pre-pulsed overnight with culture medium, iomeprol (specific), iohexol (cross-reactive), or iobitridol (non-reactive). After washing, DCs were co-incubated with the TCC Iom76 in the absence or presence of drugs for 48 h. Proliferation was determined by ³H-thymidine incorporation. The mean cpm of triplicate cultures with SEM is given.

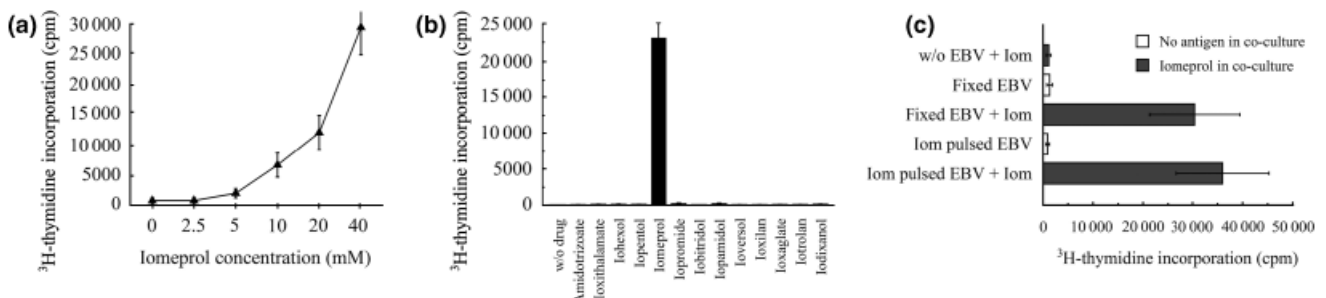


Fig. 7. Specific recognition of iomeprol by the TCR transfectant (T5227) is mono-specific, dose- and antigen-presenting cells (APC)-dependent, and processing-independent. IL-2 secretion by the transfectant T5227 upon stimulation with contrast media (CM) was measured by ³H-thymidine incorporation of CTLL cells. The dose-dependent response to iomeprol (a) and the mono-specific reaction to iomeprol but not to 11 other CM (b, drug concentration 40 mM) is shown. (c), IL-2 secretion of the transfectant T5227 was determined in the presence of iomeprol but the absence of APCs (1, bar from top to bottom), with glutaraldehyde fixed APCs [EBV-transformed B-LCLs, in the absence (2, bar) and presence (3, bar) of iomeprol], and with iomeprol-pulsed (6 h) APCs [in the absence (4, bar) or presence (5, bar) of iomeprol in co-culture]. The total incubation time was 72 h. Autologous EBV-transformed B-LCLs were used as APCs. EBV, EBV-transformed B-LCLs, B-LCLs; B-lymphoblastoid cell lines; iom, iomeprol; EBV, Epstein-Barr virus. The averages $\pm$ SEM of three experiments performed in triplicates are shown.

by directly interacting with the TCR and/or the MHC molecule, similar to T cell stimulations elicited by sulphamethoxazole, lidocaine, carbamazepine, and other drugs [23–25, 34]. Our results for some CD4⁺ TCCs as well as the CD8⁺ TCC and its TCR transfectant are compatible with this hypothesis, because these TCCs responded to CM within seconds as revealed by an increase of $[Ca^{2+}]_i$ and proliferation in the presence of fixed APCs, which indicates that uptake and processing of CM by APCs is not required. Moreover, most of these TCCs did not react if the APCs were pulsed with the CM, which indicates a labile, reversible interaction of CM with APCs. On the other hand, MHC molecules were clearly needed for full stimulation, as revealed by the failure of TCR-transfected hybridoma cells to react without APCs. This transfection also demonstrates the specificity of a particular TCR for this direct CM stimulation, as e.g., hybridoma cells transfected with sulphamethoxazole- or quinolone-specific TCR did not react to CM (data not shown). Thus, three main characteristics of the p-i concept, namely direct interaction of an immune receptor with a drug, binding from the outside, and ability of binding, could be demonstrated for some CM-specific CD4⁺ or CD8⁺ TCCs from different patients.

Nevertheless, the majority of the CD4⁺ TCCs reacted to CM in the same way as T cells respond to protein antigens such as TT. Fixation of APCs with glutaraldehyde, which abrogates antigen (CM or TT) uptake and subsequent processing, abolished T cell proliferation. Further, these TCCs reacted after antigen pulsing of APCs, which indicates stable binding of the CM to the APC surface. As the CM reactivity was HLA-DR allele dependent and as anti-MHC class II antibodies reduced reactivity, presentation occurs probably via MHC class II molecules (HLA-DP and -DR). Further support for CM uptake and processing came from studies investigating the time of APC pulsing required for T cell reactivity: the mandatory 10–24 h pre-incubation time is more compatible with intracellular metabolism and processing of CM than with direct and reversible binding of CM to the TCR or the MHC molecule.

Hence, there seem to be two different ways of CM recognition by T cells, which can occur simultaneously in the same individual: first, CM stimulating as an inert, parent compound that directly interacts with certain TCRs and requires MHC for full activation, and second, a putatively reactive, and therefore hapten-like CM derivative that is stably presented by MHC class II molecules. While CM are regarded as non-reactive molecules, they might still be pro-haptens that are transformed inside the APCs into a more reactive compound, which binds to an intracellular protein, is processed, and then presented by MHC class II molecules. The transformation into a compound that allows stable presentation on MHC molecules could apparently be performed by all the cells that were used as APCs in our studies, namely monocytes/macrophages within PBMC, EBV-transformed B-LCLs, and DCs.

Because allogeneic but MHC-matched APCs could also present the CM to specific TCCs, no particular patient- or disease-related capability to process or metabolize CM was required to allow their presentation in a stable way.

This dual pathway of CM recognition is reminiscent of sulphamethoxazole recognition by T cells reported in previous studies, where some TCCs reacted with the parent compound and others with its reactive metabolite nitroso-sulphamethoxazole [35]. It is also comparable with the processing-independent recognition of paraphenylenediamine and the processing-dependent recognition of its autoxidation product Bandrowski's base by TCCs [36]. Similarly, phenindione was presented to T cells via both processing-dependent and -independent pathways [37]. Considering that CM are regarded as prime examples of chemically inert drugs, it was quite surprising to find stable presentation by APCs for this class of compounds. On the other hand, previous studies have emphasized that CM like iohexol are in fact taken up inside cells, and may accumulate there [38, 39]. Because CM are applied in very large amounts (50–150 mL), the impact of this cellular uptake may be substantial even if it is relevant for a small portion of the injected dose only.

The presumed hapten-like presentation of CM described here was observed with iomeprol, iohexol, iodixanol, and ioversol, implying that such a mechanism may be relevant for other iodinated CM as well, considering their similar structures. Moreover, it is unclear but worth considering that the cellular uptake of CM may be relevant for other CM-related side-effects as well (pruritus, nephrotoxicity, etc.) [40].

Previous reports described the predominance of CD4⁺ T cells in maculopapular reactions and a predominant CD8⁺ T cell activation in more severe skin diseases [41]. In this study analysing patients with a generalized rash or maculopapular exanthema, most of the CM-specific T cells were CD4⁺ and only a limited number of strongly reacting CD8⁺ T cells were detected. As most of the delayed hypersensitivities to CM are mild or moderate, CD4⁺ T cells may be dominant in these reactions. Whether the mode of CM recognition (pi- or hapten-like) has an influence on the severity of the disease is unknown and requires further investigation.

In conclusion, CD4⁺ and CD8⁺ T cells and a TCR transfectant recognized CM independent of antigen processing by APCs. In addition, a substantial subset of CM-specific CD4⁺ T cells recognized CM only when stably presented by MHC class II molecules. Whereas the first mechanism is compatible with the assumed inert nature of CM, the latter is only possible if CM molecules undergo an activation process, which would imply that they are not as inert as originally thought. Because many cell types may be able to take up CM, further work is needed to elucidate these processes in order to evaluate not only the

immunological but also the clinical relevance of our findings for these compounds, which are used extensively worldwide.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characterization of TCCs and TCLs (after T cell cloning) from patient 1, patient 2 and patient 3. Stimulation indexes (SIs) of the proliferation assays are given for each TCC. T cell co-receptor expression (CD4/CD8) was determined by flowcytometry. Unless noted otherwise, TCR-V α and -V β were determined by PCR as described.

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Supplemental Table 1:

Characterization of TCCs and TCLs (after T cell cloning) from patient 1, patient 2 and patient 3. Stimulation indexes (SIs) of the proliferation assays are given for each TCC. T cell co-receptor expression (CD4/CD8) was determined by flowcytometry. Unless noted otherwise, TCR-V α and -V β were determined by PCR as described.

TCCs / TCLs (name)	SI (cpm of control culture)	T cell subtype	TCR-V α	TCR-V β
TCCs patient 1 Iohexol^a-specific				
Om3	15.9 (738)	CD4+	TRAV26-1*01, TRAJ6*01	TRBV12-3 or 12-4
Om4	19.3 (574)	CD4+	n.d.	n.d.
Om7	29.1 (391)	CD4+	n.d.	TRBV12-4 ^b
Om16	2.7 (265)	CD4+	TRAV26-1, TRAJ10	TRBV6-5*01, TRBJ1-6*01
Om25	160.1 (28)	CD4+	n.d.	n.d.
Om27	159.7 (36)	CD4+	TRAV6*02, TRAJ33 *01	n.d.
Om30	47.7 (223)	CD4+	n.d.	n.d.
TCCs patient 2 Iomeprol^a-specific				
Iom28	98 (275)	CD8+	TRAV22*01, TRAJ24*02	TRBV6-6, TRBJ1-6*01
Iom4.6	262 (26)	CD4+	n.d.	TRBV30 ^b
Iom5.6	150.2 (124)	CD4+	n.d.	TRBV5-1 ^b
Iom5.15	2.5 (324)	CD4+	n.d.	Not detectable ^b
Iom24.6	22.1 (234)	CD4+	n.d.	TRBV2 ^b
Iom44.1	12.7 (68)	CD4+	n.d.	TRBV11-2 ^b
Iom50.46	22.4 (304)	CD4+	n.d.	TRBV11-2 ^b
Iom76	5.7 (142)	CD4+	TRAV1-2, TRAJ38*01	TRBV2*01, TRBJ2-1*01
TCLs patient 2 Iomeprol^a-specific (after cloning)				
Iom15.3	33.5 (183)	CD4+	TRAV19*01, TRAJ40*01	TRBV7-2 + TRBV9, TRBJ1-5 + TRBJ2-6
TCLs patient 2 Iodixanol^a-specific (after cloning)				
Vis4	4.6 (390)	CD4+ 100% CD8+ 3%	n.d.	Not detectable ^b
Vis78	7.7 (65)	CD4+	n.d.	TRBV11-2 and others ^b
Vis151	5.5 (53)	CD4+ 100% CD8+ 19%	n.d.	TRBV19 and others ^b
TCCs patient 2 TT^a-specific				
TT8	12 (332)	CD4+ 100%	n.d.	TRBV18 ^b
TT34	3 (387)	CD4+ 100%	n.d.	TRBV20 ^b
TCCs patient 3 Ioversol^a-specific				
Opt04	7.2 (234)	CD4+	TRAV13-1*01, TRAJ17*01	TRBV3-1*01, TRBJ1-2*01
Opt35	62.1 (192)	CD4+		TRBV5-1 + TRBV6-2 ^{b,e}
Opt33	54.1 (237)	CD4+	TRAV13-1*01, TRAJ49*01	TRBV3-1*01, TRBJ1-6*01 or 02
Opt40	88.7 (351)	CD4+	TRAV35*02	TRBV5-1
Opt50	34.9 (262)	CD4+		TRBV5-1 + TRBV6-2 ^{b,c}
Opt110	85.9 (205)	CD4+		TRBV5-1 + TRBV6-2 ^{b,c}

^aStimulation index (SI) in proliferation assays (first screening of the TCCs/TCLs). Drug-concentrations: Iohexol 5 mM; Iomeprol, iodixanol, and ioversol 13 mM, APCs used: PBMCs in assays with all omni-paque-, iodixanol- and ioversol-specific TCCs/TCLs, and with Iom5.6; EBV transformed B-LCLs in assays with iomeprol- and TT-specific TCCs.

^bTCR-V β determined by FACS analysis only.

^cTCCs Opt35, Opt50, and Opt110 all expressed 100% of TRBV5-1 together with various amounts of TRBV6-2. The up-regulation of TRBV6-2 might be an *in vitro* effect.

n.d.: not done.