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Research Article

Characterization of Healthy Donor-Derived T-Cell Responses Specific to Telaprevir Diastereomers

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ABSTRACT

Telaprevir, a protease inhibitor, was used alongside PEGylated interferon-α and ribavirin to treat hepatitis C viral infections. The triple regimen proved successful; however, the appearance of severe skin reactions alongside competition from newer drugs restricted its use. Skin reactions presented with a delayed onset indicative of a T-cell mediated reaction. Thus, the aim of this study was to investigate whether telaprevir and/or its diastereomer, which is generated in humans, activates T-cells. Telaprevir in its S-configured therapeutic form and the R-diastereomer were cultured directly with peripheral blood mononuclear cells from healthy donors prior to the generation of T-cell clones by serial dilution. Drug-specific $CD4^+$ and $CD8^+$ T-cell clones responsive to telaprevir and the R-diastereomer were generated and characterized in terms of phenotype and function. The clones proliferated with telaprevir and diastereomer concentrations of 5–20 μM and secreted IFN-γ, IL-13, and granzyme B. In contrast, the telaprevir M11 metabolite did not stimulate T-cells. The $CD8^+$ T-cell response was MHC I-restricted and dependent on the presence of soluble drug. Flow cytometric analysis showed that clones expressed chemokine receptors CCR4 (skin homing) and CXCR3 (migration to peripheral tissue) and 1 of 3 distinct TCR Vβs; TCR Vβ 2, 5.1, or 22. These data show the propensity of both R- and S-forms of telaprevir to generate skin-homing cytotoxic T-cells that may induce the adverse reactions observed in human patients.

Key words: drug hypersensitivity; T-cells; immune system.

The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

Hepatitis C (HCV) is a serious, potentially life-threatening viral infection that affects an estimated 71 million individuals worldwide (WHO, 2017). Although up to a quarter of infected patients effectively clear the virus (WHO, 2017), the vast majority develop chronic HCV infection. The associated inflammation ultimately leads to severe liver disease, including hepatocellular carcinoma, liver fibrosis, and cirrhosis, due to which HCV is the most common indication for liver transplantation in the United States (Verna and Brown, 2006). HCV has been traditionally treated with a dual regimen of PEGylated IFN-α and ribavirin which provide a sustained antiviral response (<10 IU/ml) in just 39% of patients. In contrast, an updated triple treatment regimen including telaprevir (VX-950), increases the frequency of patients that achieve viral control to 70% (Lang, 2007). Telaprevir is an NS3/4.A protease inhibitor for use against HCV genotype 1 which prevents both the cleavage of viral proteins into active polypeptides for viral assembly, and the deactivation of hepatic cellular proteins essential for mediating the interferon cascade and mounting a viral response (Jesudian *et al.*, 2012; Morikawa *et al.*, 2011; Smith *et al.*, 2011). Although administered orally as a single S-configurated diastereomer, telaprevir

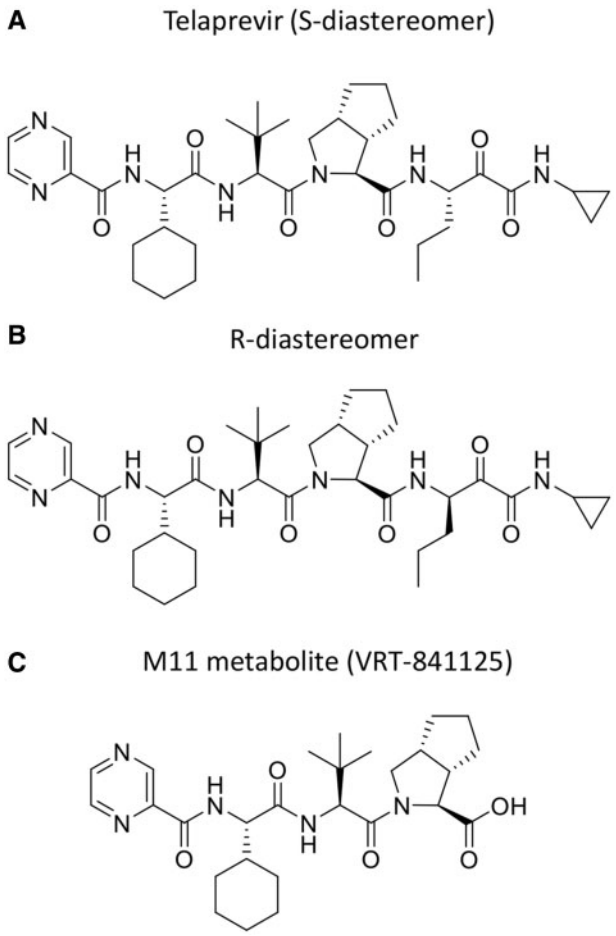


Figure 1. Chemical structures of the (A) S- (therapeutic form) and (B) R-diastereomers of telaprevir and (C) the M11 metabolite.

spontaneously forms the corresponding R-diastereomer (Figs. 1a and 1b), which is approximately 30-fold less pharmacologically active (Garg *et al.*, 2012).

Despite enhanced viral suppression, the triple regimen is associated with an increased risk of adverse cutaneous reactions, with triple telaprevir-containing therapy causing a severe rash in 4.8% of patients compared with just 0.4% with the standard dual therapy (Roujeau *et al.*, 2013). Of more concern, a small subset of patients treated with telaprevir develop life-threatening cutaneous drug hypersensitivity reactions including drug rash with eosinophilia and systemic symptoms (DRESS) and Stevens Johnson syndrome (SJS) (Pavlos *et al.*, 2012; Roujeau, 2005; Roujeau *et al.*, 2013). These clinical diagnoses, alongside the lack of correlation between the severity of telaprevir-induced cutaneous reactions and drug plasma concentration, as well as the delayed onset (median 15 days) and slow resolution (median 44 days) after drug discontinuation, are indicative of a type IV hypersensitivity reaction (Roujeau *et al.*, 2013). Such delayed drug hypersensitivity reactions are thought to be mediated by the activation and subsequent cytotoxic action of drug-specific T-cells, which have been previously isolated from patients with hypersensitivity to a diverse array of drugs (Kim *et al.*, 2015; Lichtenfels *et al.*, 2014; Meng *et al.*, 2017; Usui *et al.*, 2017). Despite a reported correlation between the level of the T-cell-derived cytotoxic mediator granulysin and the severity of telaprevir-induced skin reactions (Suda *et al.*, 2015), telaprevir-specific T-cells have not been identified. Furthermore, no specific human leukocyte antigen (HLA) alleles are associated with telaprevir-induced skin reactions of any severity (Roujeau *et al.*, 2013).

To circumnavigate the inability of animal models to predict hypersensitivity, *in vitro* models that utilize T-cells from healthy human donors have been developed that are successful at generating and characterizing drug-specific T-cells (Bell *et al.*, 2013; Gibson *et al.*, 2017; Monshi *et al.*, 2013; Sullivan *et al.*, 2018). Critically, these assays enable the modulation of reported susceptibility factors and the identification of the antigen, whether parent compound or metabolic derivative, responsible for the initial, highly regulated activation of T-cells. Telaprevir undergoes extensive hepatic metabolism and forms a range of metabolites, including M11, which was identified as potentially immunogenic due to a positive read out for skin sensitizing potential in a guinea pig maximization test (FDA application Number 201917; Garg *et al.*, 2012) (Figure 1c). In order to provide an understanding of telaprevir immunogenicity, we utilized *in vitro* peripheral blood mononuclear cell (PBMC) drug bulk cultures to assess the propensity for telaprevir-derived antigens to activate T-cells isolated from drug-naïve healthy human donors.

MATERIALS AND METHODS

Isolation of PBMC from drug-naïve healthy human donors. Venous blood samples (120 ml) were taken from 7 telaprevir-naïve healthy human donors who had provided informed written consent as directed by the Liverpool local research ethics committee. A density gradient separation technique was performed to isolate the PBMC population from whole venous blood using lymphoprep (Axis-shield, Dundee).

PBMC bulk culture. PBMCs (1×10^6/well; 48-well plate; 660 μl total) were cultured for 14 days with either the S- or R-diastereomer of telaprevir (5–20 μM), or the M11 metabolite (20 μM; all compounds were a kind gift from Janssen Research and Development, Beerse, Belgium). Drug and metabolite concentrations were chosen after conducting a PBMC toxicity assay. The maximum concentration in the T-cell assays was nontoxic and associated with no >20% inhibition of mitogen-induced PBMC proliferation. All drug solutions were prepared fresh prior to use. Cultures were fed with R9 medium (RPMI 1640, 100 μg/ml penicillin, 100 U/ml streptomycin, 25 μg/ml transferrin, 10% human AB serum [Innovative Research], 25 mM HEPES buffer, and 2 mM L-glutamine) supplemented with IL-2 on days 6 and 9. On day 14, cultures for the same antigen but of differing concentrations were harvested and pooled. A sample of PBMCs were frozen for later use at 10–20 $\times$ 10^6 cells/ml at 1:1 ratio of R9 medium to 80% human AB serum, 20% DMSO (total volume, 1 ml). Cryovials were stored at −80°C for 24–48 h before transfer to −150°C for longer term storage. Any remaining PBMCs were used for functional studies. Briefly, 1×10^5/well antigen-exposed PBMCs were re-exposed in triplicate wells (96-well plate, 200 μl total) to either the S- or R-diastereomer of telaprevir for 48 h (37°C/5% CO_2). Antigen re-exposed cultures were then pulsed with [^{3}H] thymidine (0.5 μCi/well) and subject to a further 16 h incubation before analysis of incorporated radioactivity as a measure of drug-specific proliferation using a Microbeta Trilux 1450 LSC beta counter (PerkinElmer, Cambridge, UK).

Serial dilution and T-cell cloning. T-cell clones were generated from PBMC bulk cultures using serial dilution and mitogen-driven expansion (Mauri-Hellweg *et al.*, 1995). Briefly, cells were plated at

1 cell/well (96-well U-bottomed plate) in a restimulation cocktail (5×10^4 irradiated allogeneic PBMC/well, 10 μg/ml PHA, 5 μl/ml IL-2) and cultured for 14 days (37°C/5% CO_2). Cultures were fed on day 5 and then every 2 days subsequently with R9 medium supplemented with IL-2. Additionally, autologous EBV-transformed B-cells (EBV) were generated from PBMC to function as an immortalized antigen presenting cell line.

To probe for antigen-specificity, expanded T-cell clones (5×10^4/well; 96-well plate; total volume, 200 μl) were cultured (37°C/5% CO_2) in duplicate per experimental condition with irradiated autologous EBVs (1×10^4/well) ± the S- or R-diastereomer of telaprevir (10 μM) or the M11 metabolite (20 μM). After 48 h, [^{3}H] thymidine was added before a further 16 h culture prior to analysis of cellular proliferation by scintillation counting. T-cell clones with a stimulation index (mean counts per minute [cpm] drug-treated wells/mean cpm of control wells) of > 1.5 were repetitively stimulated with allogeneic PBMCs (5×10^4/well; 96-well plate; total volume, 200 μl) in R9 medium supplemented with PHA (5 μg/ml) and IL-2 for further expansion.

T-cell clone characterization assays. Those clones that responded to telaprevir-derived antigens in a second confirmatory proliferation assay were further expanded and characterized. To define cross-reactivity between telaprevir diastereomers and the M11 metabolite, T-cell clones (5×10^4/well; 96-well plate; total volume, 200 μl) were cultured (37°C/5% CO_2) in triplicate with autologous irradiated EBVs (1×10^4/well) and either diastereomer (5–20 μM) or M11 (20 μM) for 48 h prior to proliferative analysis as described earlier. In order to explore the requirement for antigen uptake and processing, EBVs were pulsed with telaprevir for 1–16 h. After the allotted exposure period, drug-exposed EBVs were washed in PBS and used to restimulate cells as above in the absence of soluble drug. Alternatively, to determine whether antigen was presented in the context of MHC, EBVs were first precultured with either MHC class I (clone DX17) or II (clone Tu39) blocking antibodies or their corresponding isotype controls (5 μl; BD Biosciences, Oxford, UK) for 30 min. MHC blocked EBVs were then washed and included in the proliferation assay.

ELISpot was used to characterize the drug-specific release of specific cytokines and cytolytic molecules from $CD8^+$ T-cell clones as these are believed to be the main effectors in severe cutaneous hypersensitivity reactions. The release of IFN-γ, IL-13, IL-22, and granzyme B in response to telaprevir-derived antigens was visualized by the ELISpot procedure provided by the manufacturer (Mabtech, Nacka Strand, Sweden). The maximum spot count detected by the ELIspot reader is approximately 600 sfu and the reader settings were not adjusted when analyzing different clones. Thus, for strongly responding clones the reader becomes saturated. For this reason, spot counts are provided in the article text, while ELIspot images are shown in the figures. Flow cytometry was utilized to characterize T-cell clone phenotype, including clone $CD4^+$ or $CD8^+$ coreceptor expression, to assess the expression profile for a defined chemokine receptor panel (CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CCR9, CCR10, E-cadherin, CLA, CXCR3, and CXCR6), and to determine TCR Vβ protein expression using the IOTest Beta Mark TCR Vβ repertoire kit (Immunotech, Beckman Coulter, UK). Briefly, aliquots of T-cells were stained with fluorescence-conjugated antibodies before incubation on ice in the dark for 20 min. Cells were then washed with PBS and resuspended in 200 μl 10% FBS/PBS prior to data acquisition (minimum 5×10^4 events) using a FACS CANTO II flow cytometer. Clones were selected according to FS/SS characteristics; however, no additional gating strategy was used. All clones had viability of 95% or above. Data was analyzed using FACS DIVA or Cyflogic software (CyFlo Ltd., Finland).

Mass spectrometric characterization of the stability of S- and R-diastereomers of telaprevir in cell culture medium. Drugs were prepared in fresh media containing 1% DMSO and were incubated at 37°C for 10 min, 1, 16, 24, and 48 h. Samples were prepared for analysis based on the method described by Penchala *et al.* (2013) with some modifications. To measure diastereomer conversion, samples were acidified to 1% formic acid and immediately frozen (−20°C) at the end of each incubation time to prevent further epimerization. Extraction was performed by the addition of 950 μl of ice cold tert-butyl-methyl-ether to 50 μl of sample and was placed on a rotator for 30 min. Samples were centrifuged at 4500 RPM, 4°C for 10 min and 800 μl supernatant was removed and dried under nitrogen. Samples were resuspended in 40 μl of 50%ACN/0.1%FA for mass spectrometric analysis using a QTRAP 4000 mass spectrometer (AB Sciex) in positive ion mode paired with a Dionex Ultimate 3000 HPLC (Thermo Scientific). Mobile phase A consisted of water:ammonia (25%) 100:0.1 (v/v) mobile phase B was ACN:MeOH:ammonia (25%) 50:50:0.01 (v/v). A 10 μl sample was loaded onto an XBridge BEH C18 2.5 μm, 2.1 × 150 mm column (Waters) with the HPLC gradient (min, %B) 0, 45; 2, 70; 6.5, 70; 6.8, 95; 10, 95; 12, 45; and 15, 45. Each gradient was followed by a wash run with 5 min 100% water and 5 min 100% ACN before re-equilibrating the column. MRM transitions consisted of Q1:680.334, Q3:322.3 in positive ion mode. Relative peak intensity was analyzed using PeakView 1.2 (Ab Sciex).

RESULTS

Weak Telaprevir-Specific Proliferative Response From Healthy Donor-Derived T-Cell Cultures

PBMC bulk cultures established with either the S- or R-diastereomer of telaprevir were restimulated every 2–3 weeks to promote further expansion of antigen-specific T-cells. Cultures with high cell recovery were tested for antigen specificity before being subject to serial dilution for T-cell cloning. Although responses to the model drug immunogen nitroso sulfamethoxazole (SMX-NO) were clearly detectable from PBMC bulk cultures in all donors (Figure 2), the majority of cultures exposed to telaprevir diastereomers failed to proliferate (Figs. 2i and 2ii; representative donors 1–3). T-cells from donor 2 responded weakly in response to the R-diastereomer of telaprevir, but this did not reach statistical significance.

Identification of $CD4^+$ and $CD8^+$ Telaprevir Antigen-Specific T-Cells

To explore whether telaprevir-specific T-cells are present below the limit of detection in the PBMC assay, T-cell cloning was performed on cells derived from all 7 telaprevir-exposed cultures. Initial testing identified the drug-specific proliferation (SI > 1.5) of T-cell clones from 3/7 donors (Figure 3). After expansion, a further triplicate proliferation culture confirmed the presence of telaprevir-responsive T-cell clones. Five and thirty-five T-cell clones derived from PBMC cultures containing the S- and R-diastereomer, respectively remained drug-responsive during repetitive mitogen-driven expansions and were used for the mechanistic studies described below.

Irrespective of the antigen used for initial culture, all clones proliferated in the presence of either diastereomer to a similar extent at similar concentrations (Figure 4). Both compounds contained a single diastereomer of telaprevir when freshly

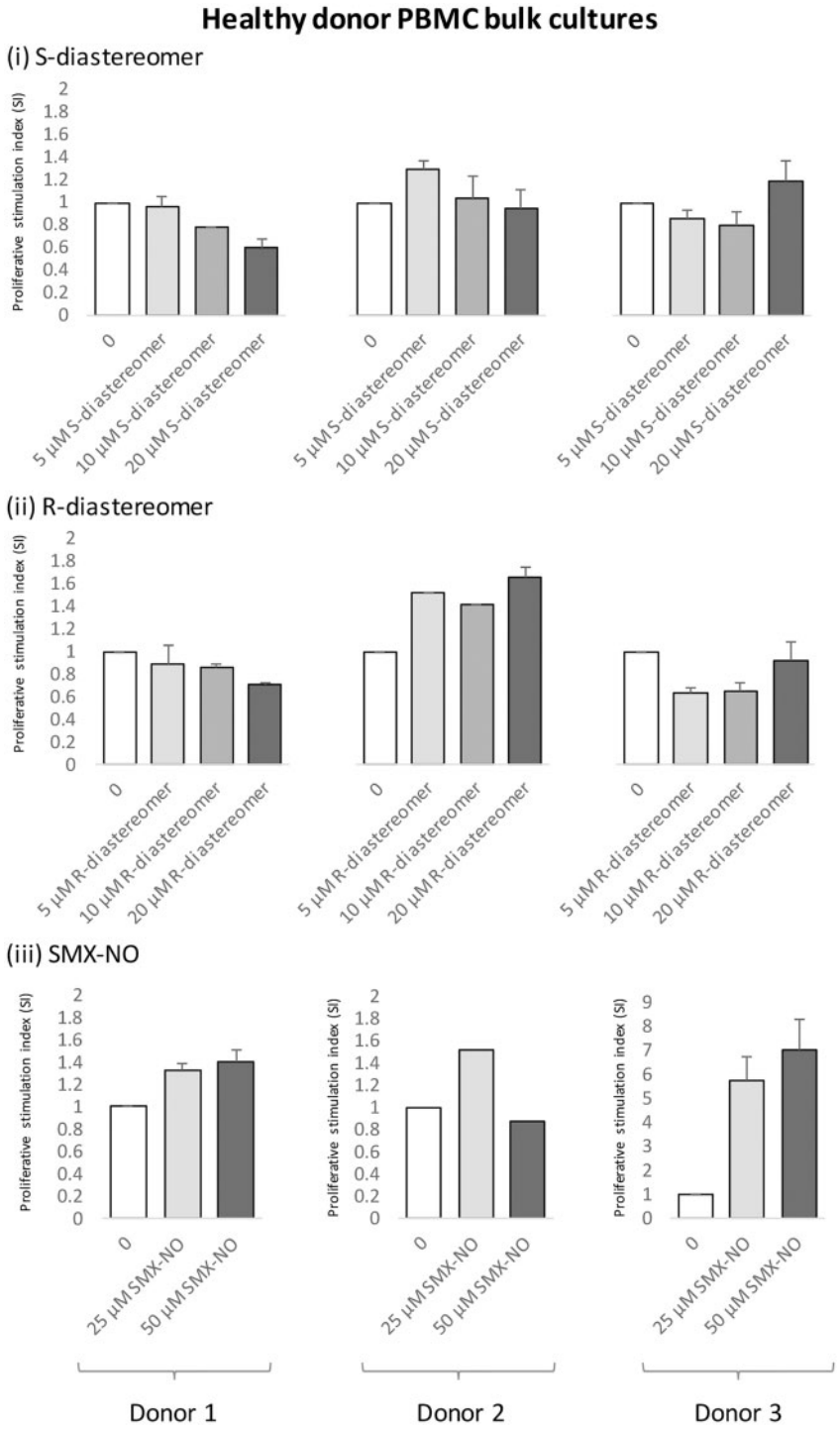


Figure 2. Healthy donor T-cell responses from PBMC bulk cultures with the telaprevir (i) S- diastereomer (ii) R-diastereomer (5–20 μM), or (iii) SMX-NO (25–50 μM; model drug-derived immunogen). PBMCs (1×10^6/well; 48-well plate; 660 μl total) were directly cultured with antigen for 14 days prior assessment of drug specificity. Briefly, 1×10^5/well antigen-exposed PBMCs were re-exposed in triplicate wells (96-well plate, 200 μl total) to either the S- or R-diastereomer of telaprevir for 48 h (37°C/5% CO_2). [^{3}H]thymidine (0.5 μCi/well) was added for the final 16 h of the incubation and then incorporated radioactivity was measured. Data shown as proliferative stimulation index (SI; average of drug-exposed wells/average of control wells). Error bars indicate standard deviation for the average of replicate cultures.

prepared. However, they underwent epimerization in culture medium to form almost equal quantities of the alternative diastereomer after 16 h (Figure 5). Flow cytometry determined that all telaprevir diastereomer-responsive T-cell clones from donor 3 were $CD8^+$ T-cells, while a mixed phenotype was observed from the 13 suitable for analysis from donor 2, with 10 $CD4^+$ (76.9%) and 3 $CD8^+$ (23.1%) T-cell clones identified. CD phenotyping was not performed with clones from donor 1. Figures 4C and 4D show flow cytometry traces of representative $CD4^+$ and $CD8^+$ clones.

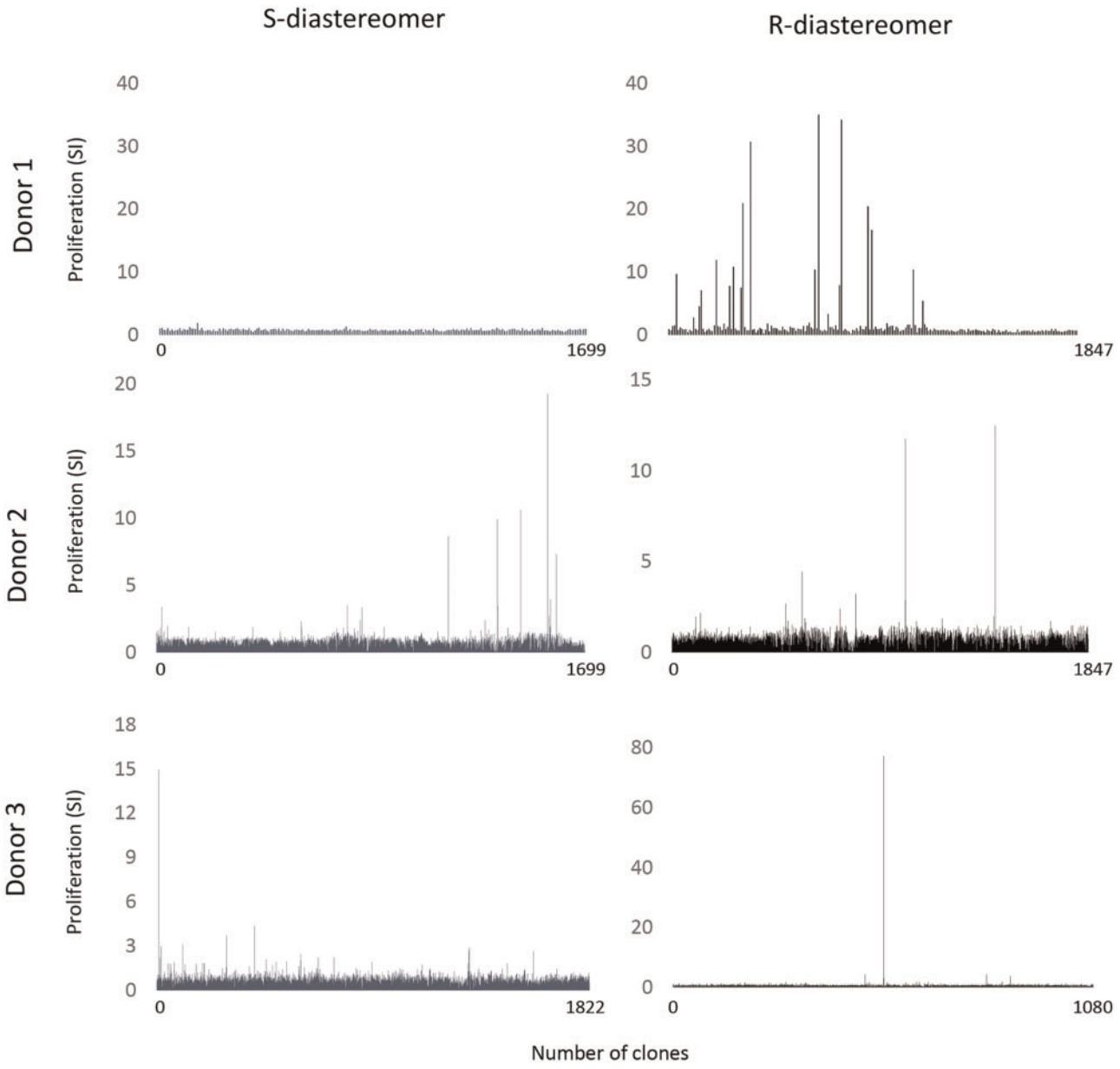


Figure 3. Generation of telaprevir-responsive T-cell clones. Telaprevir diastereomer-exposed T-cells from PBMC bulk cultures were subject to serial dilution and mitogen-driven expansion. Individual T-cell clones (5×10^4/well; 96-well plate; total volume, 200 μl) were cultured (37°C/5% CO_2) in duplicate per experimental condition with irradiated autologous EBVs (1×10^4/well) ± the drug antigen. After 48 h, [^{3}H] thymidine was added before a further 16 h culture prior to analysis of cellular proliferation by scintillation counting. T-cell clones with a SI (mean cpm drug-treated wells/mean cpm of control wells) of > 1.5 were selected as drug-responsive and subject to further expansion and investigation.

In stark contrast, cloning performed on PBMC bulk cultures with the M11 metabolite from 3 healthy donors failed to identify M11-responsive T-cell clones.

Telaprevir-Responsive T-Cells Secrete Cytotoxic and Proinflammatory Mediators

ELISpot was utilized to probe for the telaprevir-induced secretion of cytokines and cytolytic molecules from the $CD8^+$ T-cell clones. All clones secreted IFN-γ to a similar degree upon exposure to either telaprevir diastereomer (Figure 6—average spot count across 7 clones shown: IFN-γ; medium, 81 ± 47.9; 10 μM S-diastereomer, 282 ± 77.4; 10 μM R-diastereomer, 288 ± 53). Drug-induced secretion of IL-13 (medium, 27.3 ± 20.6; 10 μM S-diastereomer, 101.8 ± 79.2; 10 μM R-diastereomer, 19.3 ± 19.4) and the cytotoxic mediator granzyme B (12.1 ± 4.2; 10 μM S-diastereomer, 55.4 ± 20.2; 10 μM R-diastereomer, 46.0 ± 18.4) was similarly observed. In stark comparison, none of the aforementioned mediators were secreted when telaprevir-responsive T-cell clones were exposed to the M11 metabolite (Figure 7a). The lack of cross-reactivity was further confirmed by a negative proliferative response to the M11 metabolite in these clones (Figure 7b). Secretion of IL-22 was not detected in response to telaprevir diastereomers, despite its reported involvement in inflammatory skin conditions (Figs. 6 and 7).

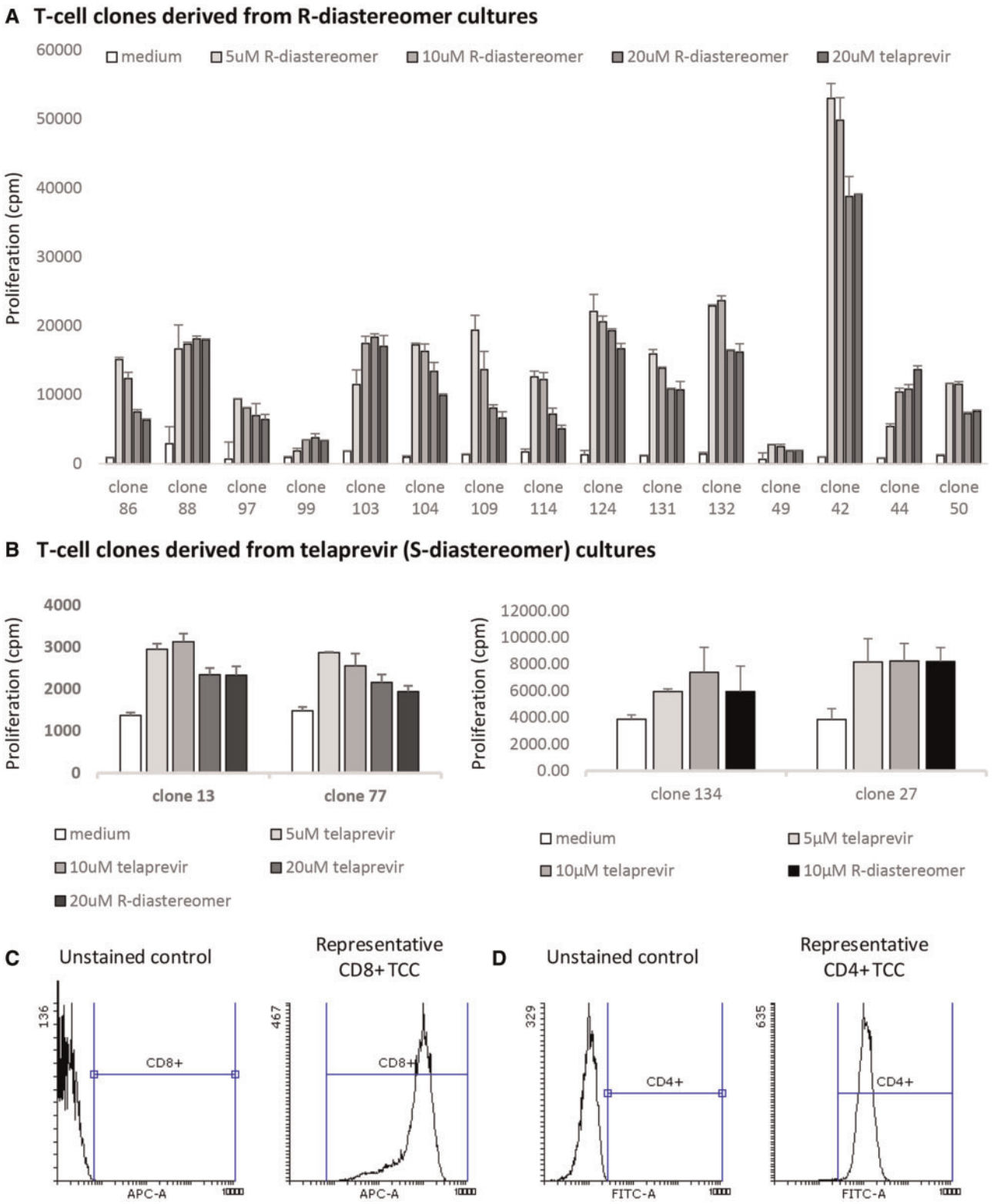


Figure 4. Cross-reactivity between telaprevir-diastereomers. Drug-responsive T-cell clones (5×10^4/well; 96-well plate; total volume, 200 μl) generated from initial cultures with either the (A) R- or (B) S -diastereomers were cultured (37°C/5% CO_2) in triplicate with autologous irradiated EBV-transformed B-cells (1×10^4/well) and either diastereomer (5–20 μM) for 48 h prior to pulsing with [^{3}H]thymidine (0.5 μCi/well). After a further 16 h incubation, incorporated radioactivity was counted as a measure of proliferation. Data presented as radioactive cpm; error bars indicate the standard deviation for the average of triplicate cultures. Flow cytometry histograms detailing the gating strategy used to determine T-cell phenotype. Representative T-cell clones shown that were deemed (C) $CD8^+$ via positive CD8-APC staining or (D) $CD4^+$ via positive CD4-FITC staining.

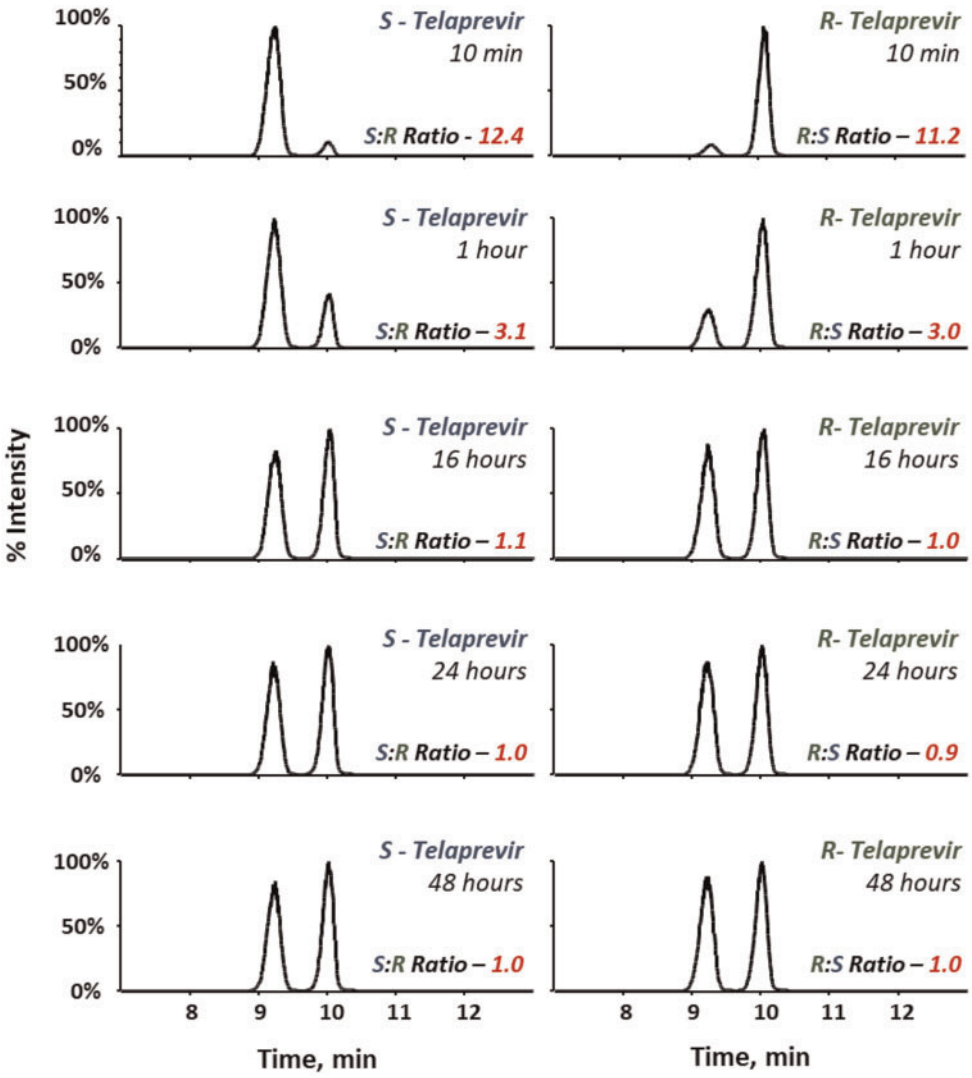


Figure 5. Mass spectrometric analysis of the epimerization of S to R telaprevir and vice versa in cell culture media. Drugs were prepared directly in cell culture media (1% DMSO per cell culture conditions) and were acidified to 1% FA at each incubation time point to prevent further epimerization. Mass analysis was performed using LC/MRM mass spectrometry in positive ion mode selecting for the Q1/Q3 transitions 680.334/322.3. Ratios were calculated based on relative peak intensity.

Telaprevir-Induced T-Cell Responses Are MHC-Restricted and Occur Independent of Antigen Presenting Cell Processing

To assess the presentation of telaprevir to T-cells, telaprevir-specific T-cell clones were first cultured with telaprevir-pulsed autologous irradiated EBV-transformed B-cells free of soluble drug. Although T-cells strongly proliferated in response to soluble drug, they were not stimulated by drug-pulsed antigen presenting cells at either time point (1 or 16 h; Figure 8a). Further investigation using HLA blocking antibodies focused on the requirement for HLA in the activation of T-cells. The telaprevir-induced $CD8^+$ T-cell proliferative response was diminished by blocking HLA class I molecules (Figure 8b), but not the corresponding isotype control. These data infer that telaprevir is directly presented on HLA class I molecules to passing $CD8^+$ T-cells, without a need for antigen uptake and processing.

Telaprevir Responsive T-Cells Express Distinct TCR Vβ and Chemokine Receptors

To induce keratinocyte death, telaprevir-responsive T-cells must express specific homing receptors to promote migration to the skin. The expression of a diverse array of tissue-homing chemokine receptors was analyzed on the drug-responsive T-cell clones, which expressed the T-cell activation marker CD69. Of the 13 migratory markers assessed, telaprevir-responsive T-cells most highly expressed CCR4 (skin homing; mean fluorescence intensity [MFI]: 3.40 ± 1.04) and CXCR3 (migration to peripheral tissue; MFI: 3.16 ± 1.52) (Figure 9a). Further T-cell surface expression analysis revealed a restricted pattern of TCR-Vβ expression, in which there was a high expression of TCR-Vβ 22 ($n = 6$, 46%), with fewer clones expressing TCR-Vβ 2 ($n = 5$, 38%) and TCR-Vβ 5.1 ($n = 1$, 8%). Of note, one clone expressed no identifiable TCR Vβ covered by the kit, which recognizes 24 specificities that account for 70% of the total repertoire (Figure 9b). Thus, it is likely this T-cell clone expressed another alternative TCR-Vβ.

DISCUSSION

As the acute stage of HCV is largely asymptomatic, the majority of patients develop a chronic infection that leads to long term, life-threatening liver complications. An adaptation of the standardized dual therapy, by coadministration of telaprevir,

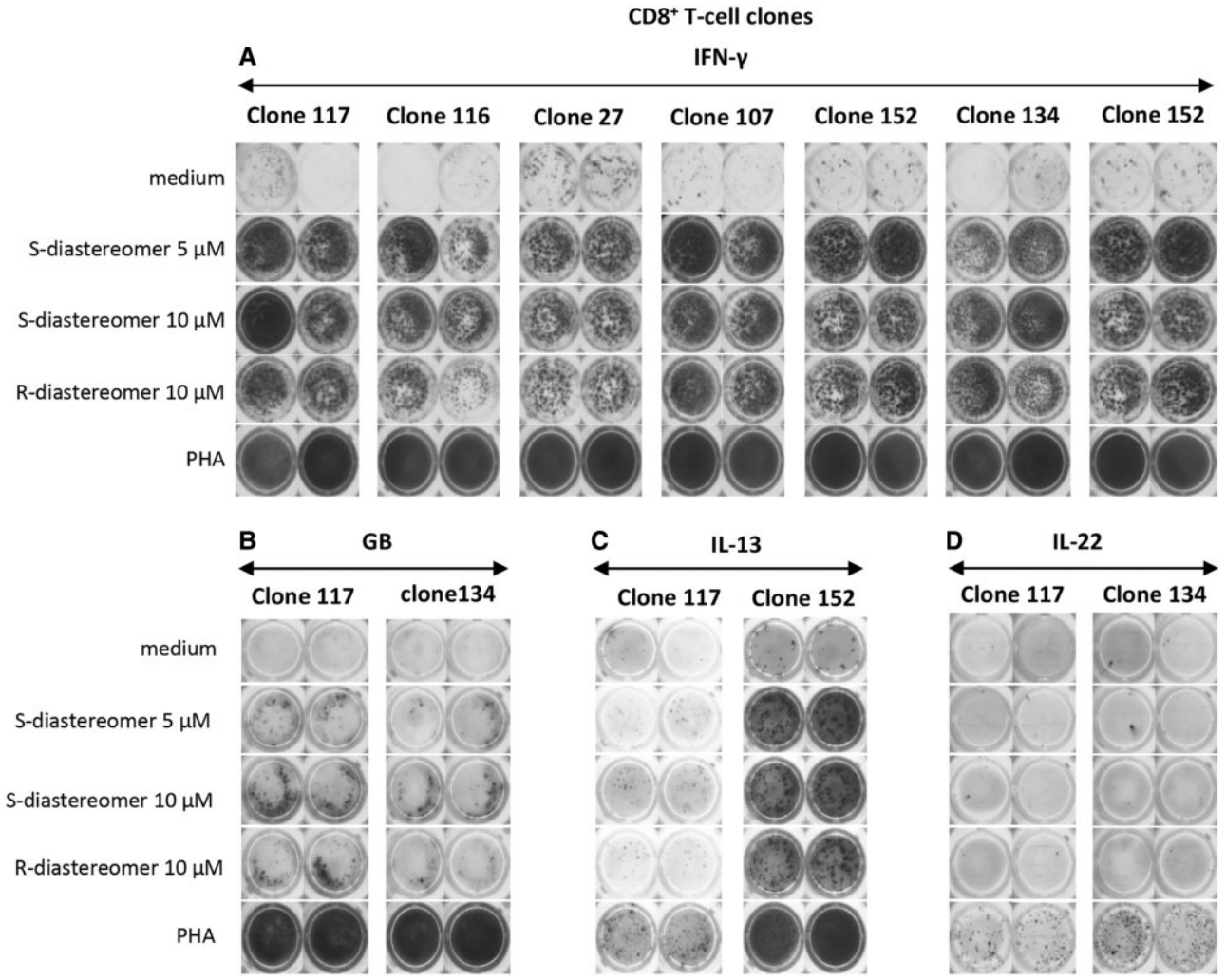


Figure 6. Cytokine and cytolytic molecule secretion from telaprevir-responsive T-cell clones. ELISpot plates were coated with (A) IFN-γ, (B) granzyme B, (C) IL-13, or (D) IL-22 capture antibody and incubated at 4°C overnight. Wells were then washed and blocked with R9 medium. Seven T-cell clones from 2 donors (5×10^4/well; total volume, 200 µl, 96-well U-bottomed ELISpot plate) were cultured with the S- (5-10 µM) or R-diastereomer of telaprevir and autologous irradiated EBV-transformed B-cells (1×10^4/well). After 48 h incubation, the plates were washed and developed in concordance with the manufacturer's instructions. Spot forming units (SFUs) counts were analyzed from dry wells using an ELISpot reader.

led to >30% increase in therapeutic response rate (Lang, 2007). However, telaprevir also enhanced the incidence of mild skin reactions, and led to a number of patients developing life-threatening hypersensitivity reactions including DRESS and SJS (Pavlos *et al.*, 2012; Roujeau, 2005; Roujeau *et al.*, 2013). The appearance of new drugs with improved safety profile resulted in telaprevir being withdrawn from the market.

Drug-specific T-cells have been isolated from the blood and blister fluid of patients with other forms of severe hypersensitivity reaction, and these T-cells are thought to mediate tissue destruction (Mauri-Hellweg *et al.*, 1995; Meng *et al.*, 2017; Sullivan *et al.*, 2018). However, as yet telaprevir-responsive T-cells have not been identified or characterized in terms of phenotype and function. Although samples from hypersensitive patients are informative for clinical diagnosis, they represent a memory T-cell response, and give no indication regarding the ability of the antigen to activate the T-cells in the more highly regulated drug-naïve human population. Thus, using PBMC drug bulk cultures we probed the immunogenicity of the telaprevir S- and R-diastereomers.

Telaprevir-responsive T-cell clones were generated from 3 out of 7 healthy volunteers; however, the frequency of identifiable drug-responsive T-cell clones was low. Although administered as the S-diastereomer, telaprevir spontaneously converts to the R-diastereomer *in vivo*. A study on Japanese HCV patients showed that the mean C_{max} for telaprevir was 5.4 µM at steady state (Yamada *et al.*, 2012), where maximal plasma concentrations of the R-diastereomer were almost equivalent to those of the S-diastereomer (Nakada *et al.*, 2014). In our study, T-cell clones were derived from PBMC cultured with both the S- and R-diastereomer. In contrast to the diastereomer-specific actions of telaprevir at its pharmacological target, the HCV enzyme NS3/4A serine protease, a high degree of T-cell cross reactivity was observed with both diastereomers. In fact, the same concentrations of S- and R-diastereomer were capable of inducing proliferate responses to a similar degree. Mass spectrometry was used to monitor the stability of S- and R-diastereomers of telaprevir in culture. Both compounds contained a single diastereomer when added to cells; however, after 24 h, epimerization had occurred and the culture medium contained equal concentrations of both diastereomers. Although these data imply the parent compound is responsible for T-cell activation, other metabolites formed in patients might also interact with HLA

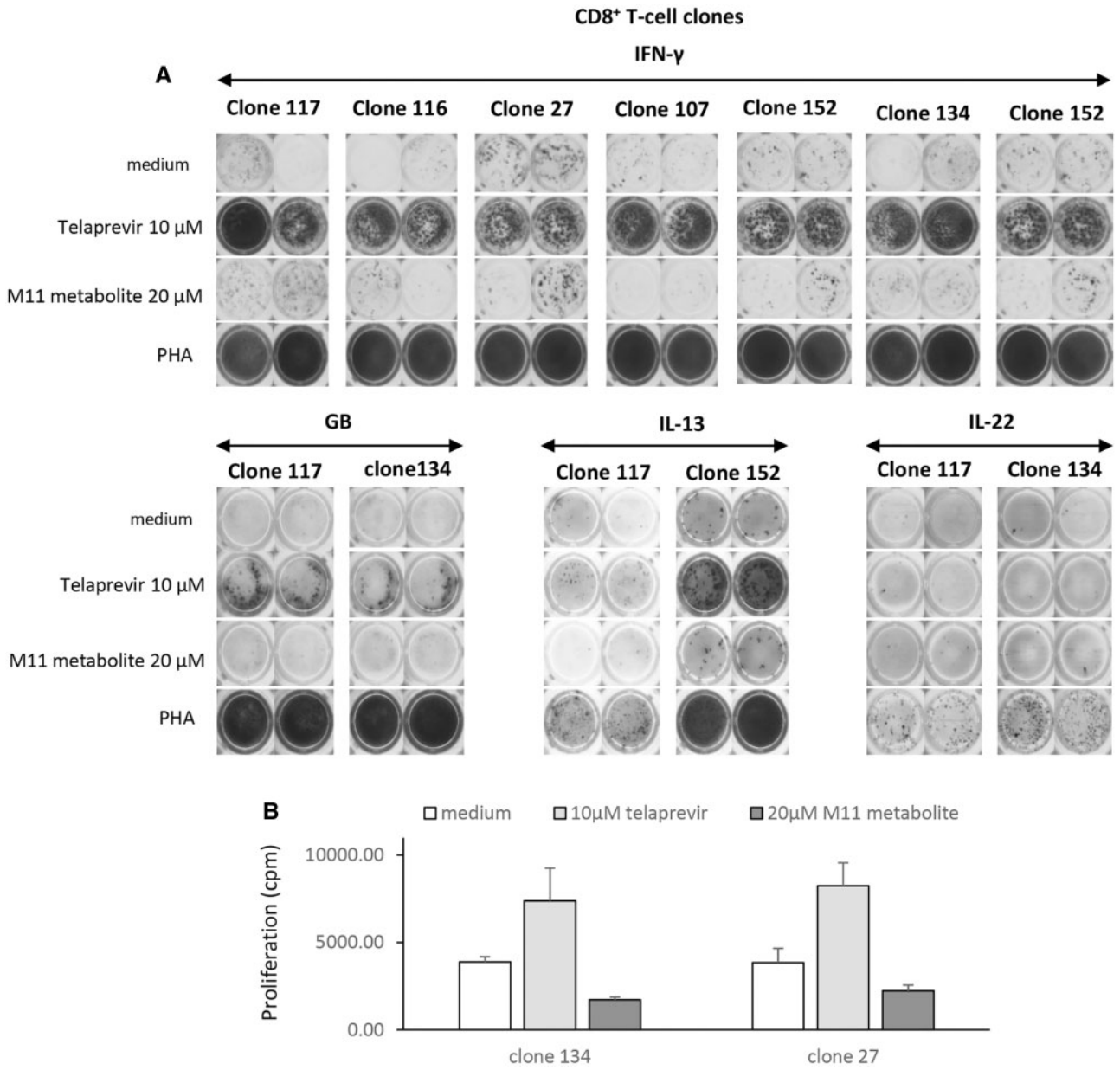


Figure 7. Ability of the M11 metabolite to induce (A) cytokine or cytolytic molecule secretion or (B) a proliferative response in telaprevir-responsive T-cell clones. ELISpot plates were coated with IFN-γ, IL-13, IL-22, or granzyme B capture antibody and incubated at 4°C overnight. Wells were then washed and blocked with R9 medium. For both proliferation and ELISpot assays, 7 T-cell clones (5×10^4/well; total volume, 200 μl, 96-well plate) were then cultured with telaprevir (10 μM) or the M11 metabolite (20 μM) and autologous irradiated EBV-transformed B-cells (1×10^4/well). After a 48 h incubation, the ELISpot plates were washed and developed in concordance with the manufacturer's instructions. SFU counts were analyzed from dry wells using an ELISpot reader. Alternatively, plates for proliferation analysis were pulsed with [^{3}H] thymidine (0.5 μCi/well) and subject to a further 16 h incubation before measurement of incorporated radioactivity. Data presented as radioactive cpm, error bars indicate the SD for the average of triplicate cultures.

molecules to stimulate a T-cell response. One potentially immunogenic metabolite is M11, as it has previously been reported to induce a positive guinea pig maximization test. Telaprevir-responsive T-cell clones failed to respond to M11, and moreover, cloning directly using the M11 metabolite was unsuccessful in 3 donors, including the donors who provided clones responsive towards the parent compound. Although this interspecies differential response may relate to the small number of animals or humans tested between the 2 studies, it nonetheless stresses the importance of assessing antigenicity and immunogenicity using human models. In this respect, it is possible that M11-responsive T-cells are only detected in patients with telaprevir hypersensitivity.

The culture conditions used herein have been optimized to detect drug-specific T-cell responses. Thus, the number of telaprevir responders (3 out of 7 healthy donors) should not be related directly to the frequency of reactions seen in drug-exposed patients. However, one must consider the reason why clones were not detected in PBMC from 4 donors. It is possible that telaprevir has a different binding affinity at HLA molecules and/or a preference for specific T-cell receptors as indicated in the T-cell receptor Vβ analysis. However, the absence of a response might also relate to an active tolerance mechanism. For example, Tregs were not depleted from PBMC prior to telaprevir exposure. Furthermore, our study did not separate PBMCs into

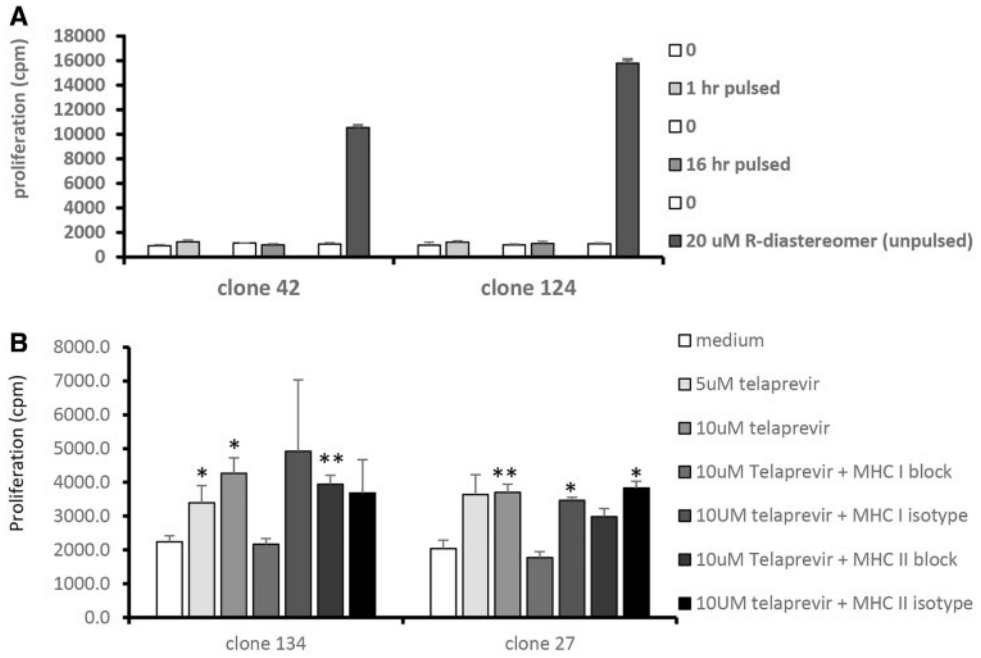


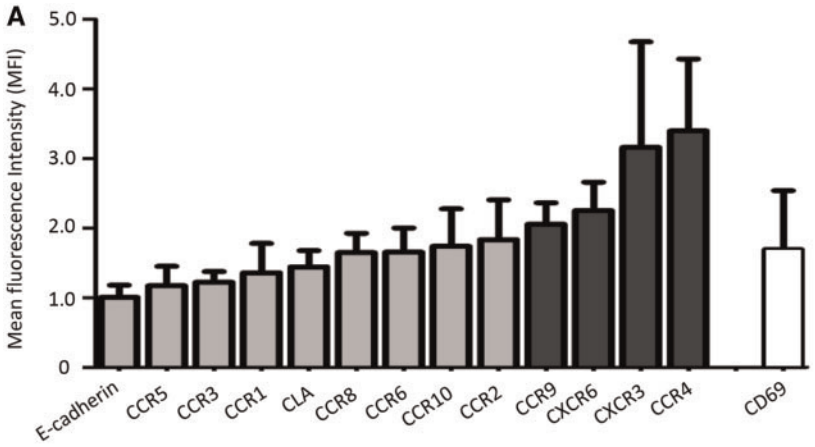
Figure 8. Requirement for (A) antigen uptake and (B) HLA restriction for T-cell activation. Autologous EBV were either (A) pulsed with soluble drug for 1–16 h prior to washing to remove free drug, or (B) cultured with HLA blocking antibodies or their respective isotype controls for 30 min. Four T-cell clones (5×10^4/well; total volume, 200 μl, 96-well plate; results from 4 representative clones shown in figure) were then cultured with telaprevir (10–20 μM) and preconditioned autologous irradiated EBV-transformed B-cells (1×10^4/well). After a 48 h incubation, cultures were pulsed with [^{3}H] thymidine (0.5 μCi/well) and subject to a further 16 h incubation before measurement of incorporated radioactivity. Data presented as radioactive cpm, error bars indicate the SD for the average of triplicate cultures. Statistical analysis (paired T test) compares treated conditions to medium control (*$p < .05$; **$p < .005$).

naïve and memory T-cells prior to telaprevir exposure. Thus, future experiments should aim to identify whether the telaprevir-specific T-cells derive from the naïve or memory compartment, or both. Telaprevir could activate naïve CD4$^+$ and CD8$^+$ T-cells or alternatively stimulate T-cells deriving from the pathogen-specific memory T-cell pool.

Telaprevir is a reversible covalent binding inhibitor of its pharmacological target. The drug interacts with a high degree of specificity with serine in position 139, which resides in the catalytic area of the protease enzyme. Dissociation of the covalently bound drug has a half-life of 58 min (Fowell and Nash, 2010). To investigate whether the telaprevir-responsive T-cells were activated through the formation of protein adducts by a hapten mechanism or through direct HLA binding, antigen presenting cell pulsing experiments were conducted. Haptenic drugs bind irreversibly to antigen presenting cells in the pulsing assay and stimulate T-cells after repeated washing to remove the noncovalently bound drug. In contrast, drugs that bind directly to HLA through a reversible bond yield a negative result (Alzahrani *et al.*, 2017; Castrejon *et al.*, 2010; Schnyder *et al.*, 2000). Although the telaprevir-specific T-cell response was MHC restricted, the T-cells were only activated in the presence of soluble drug, highlighting that formation of a covalent adduct and antigen-uptake is not a requirement for T-cell activation. These data indicate that telaprevir may be able to activate T-cells expressing distinct TCR Vβs via a direct HLA-binding interaction. Interestingly, telaprevir is relatively large, with a molecular weight of 680 Da. This equates to the over 50% of mass of a typical HLA class I binding peptide (assuming an average amino acid mass of 130 Da and 9–10 amino acids in the peptide sequence). Thus, it will be intriguing to discover the structure of the telaprevir HLA peptide-binding interaction. It seems unlikely that telaprevir will fit into a binding pocket under an HLA binding peptide. The only other possibilities are that telaprevir (1) interacts with the HLA-binding peptide and projects from the HLA molecule, (2) replaces the requirement for an HLA binding peptide, or (3) binds elsewhere on the HLA molecule altering the structure of the HLA-binding cleft. Alternatively, telaprevir might interact with conserved sites on the HLA molecule forming a bridge to the T-cell receptor via a superantigen-like mechanism. Future experiments should use protease inhibitors or other chemical means to block antigen processing to explore whether new peptide sequences need to be displayed on the surface of antigen presenting cells to activate telaprevir-specific clones.

Flow cytometric analysis of telaprevir-specific clones revealed a restricted TCR Vβ usage. In total 11 out of 13 clones tested expressed either Vβ 2 or 22. This suggests that telaprevir interacts with only a small subset of available T-cell receptors. However, additional studies using PBMC and cloned T-cells from hypersensitive patients are required to support this tentative conclusion.

Telaprevir-specific CD8$^+$ clones were generated in relatively high numbers from healthy donors, when compared with other drugs associated with hypersensitivity such as the β-lactam antibiotics and sulfamethoxazole (Gibson *et al.*, 2017; Sullivan *et al.*, 2018). The reason for this is unclear; however, it might relate to the selective binding of telaprevir to HLA class I molecules. CD8$^+$ T-cells are designed to inflict damage and thus are the most likely mediators of keratinocyte death in patients with telaprevir hypersensitivity. Thus, CD8$^+$ cells were analyzed for their cytokine secretion profile and the secretion of cytolytic molecules using ELISpot. Previously Suda *et al.* (2015) reported a correlation between the level of the cytotoxic mediator granulysin and the severity of telaprevir-induced skin reactions. Moreover, they describe an early rise in serum granulysin levels



CD69	1.7	±	0.8
Chemokines	MFI		
CCR4	3.40	±	1.04
CXCR3	3.16	±	1.52
CXCR6	2.25	±	0.41
CCR9	2.06	±	0.31
CCR2	1.83	±	0.57
CCR10	1.74	±	0.54
CCR6	1.66	±	0.35
CCR8	1.65	±	0.28
CLA	1.44	±	0.23
CCR1	1.36	±	0.42
CCR3	1.22	±	0.15
CCR5	1.17	±	0.28
E CAD	1.01	±	0.17

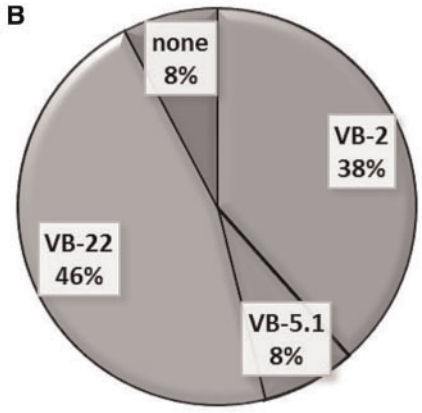


Figure 9. TCR Vβ and chemokine profile of telaprevir-responsive T-cell clones. T-cell clones (5×10^4) were washed and stained with (A) individual Fluorochrome-conjugated antibodies specific for chemokine receptors or (B) combined Fluorochrome-conjugated antibodies specific for TCR Vβ specificities. Samples were left in the dark for 20 min at 4°C to aid antibody binding. After which samples were washed to remove unbound antibody and fixed in 4% PFA prior to analysis using a BD FACS CANTO II flow cytometer. Data analysis was performed using cyflogic software.

with the onset of severe symptoms which fades within 6 days and therefore concluded that granulysin can be utilized as an early predictive marker for telaprevir-induced skin reactions. In agreement with the induction of a cytotoxic response, telaprevir-responsive $CD8^+$ T-cell clones not only secreted IFN-γ and IL-13, but also the cytotoxic mediator granzyme B in response to culture with either diastereomer. Upon release from cytotoxic T-cell granules, granzyme B enters target cells to cleave caspases and initiate apoptosis. The secretion of IL-22 was additionally monitored due to its proposed role in inflammatory skin conditions, including psoriasis. Furthermore, IL-22-secreting cells have been identified in patients with allergic contact dermatitis and β-lactam hypersensitivity reactions (Akdis *et al.*, 2012; Cavani *et al.*, 2012; Eyerich *et al.*, 2010; Sullivan *et al.*, 2018). IL-22 was not secreted by telaprevir-responsive T-cells in this study.

We have previously demonstrated that drug antigen-specific and nondrug antigen-specific clones express chemokine receptors at different levels (Sullivan *et al.*, 2018). Furthermore, chemokine receptors such as CXCR6 are detected on drug-specific clones from patients with certain forms of hypersensitivity, but not others (Monshi *et al.*, 2013; Sullivan *et al.*, 2018). Thus, chemokine receptor pattern analysis was conducted on telaprevir-responsive T-cell clones. The analysis detected the skin-homing receptor CCR10; however, CCR4 and CXCR3 were also present. CXCR3 is predominantly expressed by Th1 T-cells, whereas CCR4 is expressed by Th2 T-cells (Kim *et al.*, 2001). These findings, along with the antigen-specific secretion of IFN-γ (Th1) and IL-13 (Th2), suggest the presence of a mixed Th1/2 T-cell population. CCR9, characterized as a gut homing receptor for T-cells with a role in intestinal inflammation (Agace, 2008; Bekker *et al.*, 2015), was also relatively highly expressed in comparison to other chemokine receptors. Interestingly, adverse intestinal effects with telaprevir hypersensitivity are very common ranging from diarrhea to hemorrhoids in 25% and 12% of patients, respectively. As yet, the underlying mechanism of intestinal disruption is not defined, but this data indicate the migration of T-cells may play a role. It is important to emphasize that these phenotypic analyses (cytokine release and chemokine receptor expression) are conducted on drug-specific T-cells generated from healthy donors. Thus, their role in the iatrogenic disease (ie, telaprevir hypersensitivity) should be considered with some caution until a similar confirmatory analysis has been conducted on T-cells from hypersensitive patients.

This study identifies and characterizes the telaprevir-induced activation of T-cells from 3 out of 7 healthy drug-naïve donors that develop into skin-homing, cytotoxic T-cells, which are activated by an HLA-restricted but processing-independent

mechanism. Drug-specific T-cells responded to either telaprevir diastereomer and expressed a restricted pattern of TCRs. Drug-responsive T-cells were induced using cultures from drug-naïve healthy donors and so define the utility of *in vitro* human platforms to explore the requirements for T-cell activation. As studies have previously failed to associate telaprevir hypersensitivity with HLA risk alleles (Roujeau *et al.*, 2013), the development of *in vitro* human assays to allow (1) modulation of other immune parameters and (2) inclusion of autologous keratinocytes will be key to understand the inter-individual skin-specific targeting by the immune system.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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