Isolation of Human CD4/CD8 Double-Positive, Graft-Versus-Host Disease–Protective, Minor Histocompatibility Antigen–Specific Regulatory T Cells and of a Novel HLA-DR7–Restricted HY-Specific CD4 Clone

Assia Eljaafari, Ozel Yuruker, Christophe Ferrand, Annie Farre, Caroline Addey, Marie-Laure Tartelin, Xavier Thomas, Pierre Tiberghien, Elizabeth Simpson, Dominique Rigal and Diane Scott

*J Immunol* published online 7 December 2012
http://www.jimmunol.org/content/early/2012/12/07/jimmunol.1201163

Supplementary Material  http://www.jimmunol.org/content/suppl/2012/12/07/jimmunol.1201163.DC1

Subscription  Information about subscribing to *The Journal of Immunology* is online at:  http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:  http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:  http://jimmunol.org/alerts
Isolation of Human CD4/CD8 Double-Positive, Graft-Versus-Host Disease–Protective, Minor Histocompatibility Antigen–Specific Regulatory T Cells and of a Novel HLA-DR7–Restricted HY-Specific CD4 Clone

Assia Eljaafari,*†‡ Ozel Yuruker,§ Christophe Ferrand,*‡ Annie Farre,* Caroline Addey,§ Marie-Laure Tartelin,*‡ Xavier Thomas,# Pierre Tiberghien,¶ Elizabeth Simpson,§ Dominique Rigal, and Diane Scott**††‡

Minor histocompatibility (H) Ags are classicaly described as self-peptides derived from intracellular proteins that are expressed at the cell surface by MHC class I and class II molecules and that induce T cell alloresponses. We have isolated three different T cell populations from a skin biopsy of a patient suffering from acute graft-versus-host disease following sex-mismatched HLA-identical bone marrow transplantation. The first population was: 1) CD4+/CD8+ double-positive; 2) specific for an HLA class I–restricted autosomal Ag; 3) expressed a Tr1 profile with high levels of IL-10, but low IL-2 and IFN-γ; and 4) exerted regulatory function in the presence of recipient APCs. The second was CD8+ positive, specific for an HLA class I–restricted autosomally encoded minor H Ag, but was only weakly cytotoxic. The third was CD4 single positive, specific for an HLA-DR7–restricted HY epitope and exerted both proliferative and cytotoxic functions. Identification of the peptide recognized by these latter cells revealed a new human HY epitope, TGKIIINFIKFDTGNL, encoded by RPS4Y and restricted by HLA-DR7. In this paper, we show human CD4/CD8 double-positive, acute graft-versus-host disease–protective, minor H Ag–specific regulatory T cells and identify a novel HLA-DR7 HY T cell epitope, encoded by RPS4Y, a potential new therapeutic target. The Journal of Immunology, 2013, 190: 000–000.
six—RPS4Y, UPS9Y, DDX3Y, UTY, TMSB4Y, and SMCY—encode epitopes that are clinically relevant. Most of these are HLA class I–restricted (18), and only two, DDX3Y and RPS4Y, encode HLA class II–restricted male-specific minor H Ags (6, 7, 19). To date, very few peptide epitopes have been characterized (20–23).

To identify T cells able to recognize re-emergent recipient leukemic cells, one line of investigation is to isolate donor T cells specific for mismatched minor H Ag expressed by recipient cells. Such T cells can be found within GVHD lesions (24). We initially isolated both CD8 single-positive (SP) and CD4/CD8 double-positive (DP) T cells from skin cultures of a male patient with mild GVHD following BMT from his HLA-identical sister. After several rounds of stimulation with recipient APCs, CD4 SP T cells exhibiting both helper and cytotoxic functions against HLA-DR7/HY–expressing cells emerged from this culture. Using retroviral gene expression followed by synthetic peptides designed using peptide–MHC binding databases, we identified a new HLA-DR7/HY peptide encoded by the RPS4Y gene.

In this study, we also functionally characterized the CD4/CD8 DP T cells, showing that they exerted regulatory function following recognition of their target autosomal HLA class I–restricted minor H Ag. This demonstrates the existence of minor H Ag–specific CD4/CD8 DP T cells with regulatory potential to control clinical acute GVHD. Minor H–specific regulatory CD8+ T cells with low avidity and the ability to diminish concurrent CD8+ T cell responses have been described in mouse and human models (25, 26). Moreover, adoptive transfer of such regulatory T cells (Treg) appeared to contribute to male graft rejection (27). We initially described CD4/CD8 DP T cells, showing that they exerted regulatory function following recognition of their target autosomal HLA class I–restricted male-specific minor H Ags expressed by recipient cells. This demonstrates the existence of minor H Ag–specific CD4/CD8 DP T cells with regulatory function with potentially controlling clinical acute GVHD. Minor H–specific regulatory CD8+ T cells with low avidity and the ability to diminish concurrent CD8+ T cell responses have been described in mouse and human models (25, 26).

Materials and Methods

Medium and reagents

RPMI 1640 (Life Technologies, Eggenstein, Germany) was supplemented with 1-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), NaHCO3 (1.5 mg/ml), and 10% pooled, heat-inactivated human AB serum. Recombinant human (rh)GM-CSF, rhIL-2, rhIL-4, and rhTNF-α were purchased from R&D Systems (Abingdon, U.K.). HLA typing was assessed by serology followed by oligonucleotide typing. Anti–HLA class I (W632), anti–HLA-DR (L243), and anti–HLA-DP (B7-21) mAbs were purchased from BD Pharmingen (San Diego, CA). The cultured Abs used for flow cytometry were FITC- or PE-conjugated mouse anti-human (BD Biosciences, San Jose, CA). The Abs used for immunohistochemistry were FITC- or biotin-conjugated (USChrome, Los Angeles, CA) or biotinylated antibodies (DakoCytomation, Glostrup, Denmark). The tyramide signal amplification was performed using Duolink detection kits (Olink Bioscience, Uppsala, Sweden) and duolink detection kit (Olink Bioscience, Uppsala, Sweden).

Generation of minor H Ag–specific T cell lines and clones

The patient was grafted with the HLA-identical bone marrow from his sister: A*02:05/*68:01; B*14:01/*44:03; C*08:02/*16:01; DRB1*07:01; DQB1*02:02; DPB1*04:01/*11:01. The conditioning regimen consisted of cyclophosphamide 60 mg/kg/d, for 2 d, and total-body irradiation (12 Gy, fractionated). GVHD was controlled with methotrexate and cyclosporin A. In this study, we also functionally characterized the CD4/CD8 DP T cells, showing that they exerted regulatory function following recognition of their target autosomal HLA class I–restricted minor H Ag. This demonstrates the existence of minor H Ag–specific CD4/CD8 DP T cells with regulatory potential to control clinical acute GVHD. Minor H–specific regulatory CD8+ T cells with low avidity and the ability to diminish concurrent CD8+ T cell responses have been described in mouse and human models (25, 26).

RT-PCR amplification and sequencing protocols

Total RNA from clones or lines was isolated from 1 × 106 cells using the Reagent Kit (Promega, Madison, WI). Total RNA was converted into first-strand cDNA using an oligo(dT) primer (Amersham Pharmacia Biotech, Orsay, France) and avian myeloblastosis virus reverse transcriptase, according to the manufacturer’s specifications (Promega).

PCR amplification (30 cycles) was carried out using 25 V region sequence-specific sense and antisense primers for TCR V3β/Valpha and a 3’ antisense Cβ primer. As a positive internal control, 5’ sense and the 3’ antisense C region primers were included. Cycles consisted of 95˚C denaturation, 57˚C primer annealing, and 72˚C extension steps, 1 min each. PCR was carried out in a Biomed Thermocycler 60 (Biomed Instruments) using 2.5 U Taq DNA polymerase (Cetus) in a solution containing 4 μmol/ml primers, 0.5 μmol/ml each 2’-deoxynucleoside 5’-triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 4 mM MgCl2, and 5 μg sample. PCR products were sequenced by Genoscreen (Lille, France). The TCR sequences were analyzed using Internet ImMunoGeneTics (IMGT) database (http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanTcR).

ELISA

A total of 1.5 × 105/ml T cell lines or clones were stimulated with 3 × 105/ml EBV-B LCLs in X-VIVO-20 medium (Cambrex) without serum. Supernatant was removed after 24 h. Cytokine concentrations were evaluated by standard commercial ELISA following the manufacturers’ instructions: IL-4, IL-10, IL-2, IFN-γ (BioSource International), and TGF-β (R&D Systems).

T cell epitope identification

Two Y-chromosome genes, DDX3Y and RPS4Y, were cloned into the retroviral expression vector pMIGR1 and transfectioned into the Phoenix-AMPHO helper-free amphotropic cell line that had the internal ribosome entry site–CD8 surface marker introduced downstream of the gag-pol construct (28) using Lipofectamine 2000 (Invitrogen, Paisley, U.K.). Viral-containing supernatant was harvested from cells expressing high levels of CD8, filtered, concentrated by high-speed centrifugation, and used to transduce female HLA-DR7 EBV-B LCLs by spinfection. Briefly, 8 ml concentrated virus-containing supernatant was mixed with 8 μl
polybrene (4 mg/ml; Sigma-Aldrich) and added to \( \sim 15 \times 10^6 \) HLA-DR7 EBV-B LCLs. The cells were plated (1 ml/well) into a 24-well tissue-culture plate (BD Biosciences, Oxford, U.K.) and centrifuged for 90 min at 760 \( \times \) g. A total of 1 ml RPMI 1640 (Life Technologies) supplemented as described above but with 10% FCS instead of human AB serum was then added for 3-d culture. Cells were sorted for expression of high CD8, and the levels of DDX3Y and RPS4Y expression were confirmed by quantitative real-time PCR.

HY-specific CD4+ T-cells were stimulated with the DDX3Y and RPS4Y gene-transduced female EBV-B LCLs. Proliferation and IFN-\( \gamma \) production were, respectively, measured by \([^{3}H]\)thymidine incorporation at day 3 and ELISA at day 1. For mapping the MHC class II–restricted T cell epitope, several long peptides with potential DRB1*0701 binding were synthesized from the regions of RPS4Y differing from RPS4X. They were identified from MHC–peptide binding databases SYFPEITHI, Net-MHC, Propred, and HotSpot Hunter. The peptides were tested at concentrations from 100 \( \mu M \) to 10 nM using female EBV-B-LCL expressing HLA-DRB1*07:01 as APCs. The short peptide was identified by testing a series of 15–17 aa synthetic peptides from within the long peptide eliciting the positive response.

Results

Isolation of CD4/CD8 DP, CD8, and CD4 SP T cells from an acute GVHD skin biopsy

One month after BMT between the sex-mismatched HLA-identical siblings, the male recipient was diagnosed with acute skin GVHD. T cells were isolated from his skin biopsy following culture with 20 U/ml IL-2. After 14 d, phenotypic analysis revealed two populations of cells, one CD4/CD8 DP and the other CD8 SP. The CD4/CD8 DP population represented \( \sim 60\% \) of the culture. Overall, T cells isolated from the GVHD skin biopsy after 14 d culture were CD3+CD56- and 95.9% expressed TCR\( \alpha/\beta \) (Fig. 1A). CD8 SP and CD4/CD8 DP populations were separated using anti-CD4 magnetic beads. The sorted CD4/CD8 DP cells were stimulated with recipient APCs and cultured without IL-2 for another week, when they expressed CD25 (84%) and CD69 (58%) (Fig. 1B). Following further restimulations, a new population expressing only CD4 emerged. This new population was then isolated using anti-CD8 magnetic beads (Fig. 1C).

Thus, three different populations were obtained from the GVHD skin biopsy: CD8 SP cells, CD4 SP cells, and CD4/CD8 DP cells.

VB TCR spectratyping of the three populations

T cells were analyzed using immunoscope (Fig. 2). The GVHD skin-derived T cells after 14 d in culture (Fig. 2A) showed a skewed, oligoclonal VB TCR repertoire. Following further stimulation with recipient APCs, the VB TCR repertoire of the three subpopulations was more skewed. Six fewer VB TCR were present in the CD8 SP and CD4 SP populations compared with the CD4/CD8 DP. Furthermore, only one or two previously undetected VB TCR appeared in each respective population (Fig. 2B). Encircled panels in Fig. 2B indicate differences in the VB TCR profiles of CD4 or CD8 SP compared with the CD4/CD8 DP cells. Comparison of the long-term cultured and sorted CD4/CD8 magnetic beads. The sorted CD4/CD8 DP cells were stimulated with recipient APCs and cultured without IL-2 for another week, when they expressed CD25 (84%) and CD69 (58%) (Fig. 1B). Following further restimulations, a new population expressing only CD4 emerged. This new population was then isolated using anti-CD8 magnetic beads (Fig. 1C).

Thus, three different populations were obtained from the GVHD skin biopsy: CD8 SP cells, CD4 SP cells, and CD4/CD8 DP cells.

VB TCR spectratyping of the three populations

T cells were analyzed using immunoscope (Fig. 2). The GVHD skin-derived T cells after 14 d in culture (Fig. 2A) showed a skewed, oligoclonal VB TCR repertoire. Following further stimulation with recipient APCs, the VB TCR repertoire of the three subpopulations was more skewed. Six fewer VB TCR were present in the CD8 SP and CD4 SP populations compared with the CD4/CD8 DP. Furthermore, only one or two previously undetected VB TCR appeared in each respective population (Fig. 2B). Encircled panels in Fig. 2B indicate differences in the VB TCR profiles of CD4 or CD8 SP compared with the CD4/CD8 DP cells. Comparison of the long-term cultured and sorted CD4/CD8 magnetic beads. The sorted CD4/CD8 DP cells were stimulated with recipient APCs and cultured without IL-2 for another week, when they expressed CD25 (84%) and CD69 (58%) (Fig. 1B). Following further restimulations, a new population expressing only CD4 emerged. This new population was then isolated using anti-CD8 magnetic beads (Fig. 1C).

Thus, three different populations were obtained from the GVHD skin biopsy: CD8 SP cells, CD4 SP cells, and CD4/CD8 DP cells.

VB TCR spectratyping of the three populations

T cells were analyzed using immunoscope (Fig. 2). The GVHD skin-derived T cells after 14 d in culture (Fig. 2A) showed a skewed, oligoclonal VB TCR repertoire. Following further stimulation with recipient APCs, the VB TCR repertoire of the three subpopulations was more skewed. Six fewer VB TCR were present in the CD8 SP and CD4 SP populations compared with the CD4/CD8 DP. Furthermore, only one or two previously undetected VB TCR appeared in each respective population (Fig. 2B). Encircled panels in Fig. 2B indicate differences in the VB TCR profiles of CD4 or CD8 SP compared with the CD4/CD8 DP cells. Comparison of the long-term cultured and sorted CD4/CD8 magnetic beads. The sorted CD4/CD8 DP cells were stimulated with recipient APCs and cultured without IL-2 for another week, when they expressed CD25 (84%) and CD69 (58%) (Fig. 1B). Following further restimulations, a new population expressing only CD4 emerged. This new population was then isolated using anti-CD8 magnetic beads (Fig. 1C).

Thus, three different populations were obtained from the GVHD skin biopsy: CD8 SP cells, CD4 SP cells, and CD4/CD8 DP cells.

VB TCR spectratyping of the three populations

T cells were analyzed using immunoscope (Fig. 2). The GVHD skin-derived T cells after 14 d in culture (Fig. 2A) showed a skewed, oligoclonal VB TCR repertoire. Following further stimulation with recipient APCs, the VB TCR repertoire of the three subpopulations was more skewed. Six fewer VB TCR were present in the CD8 SP and CD4 SP populations compared with the CD4/CD8 DP. Furthermore, only one or two previously undetected VB TCR appeared in each respective population (Fig. 2B). Encircled panels in Fig. 2B indicate differences in the VB TCR profiles of CD4 or CD8 SP compared with the CD4/CD8 DP cells. Comparison of the long-term cultured and sorted CD4/CD8 magnetic beads. The sorted CD4/CD8 DP cells were stimulated with recipient APCs and cultured without IL-2 for another week, when they expressed CD25 (84%) and CD69 (58%) (Fig. 1B). Following further restimulations, a new population expressing only CD4 emerged. This new population was then isolated using anti-CD8 magnetic beads (Fig. 1C).

Thus, three different populations were obtained from the GVHD skin biopsy: CD8 SP cells, CD4 SP cells, and CD4/CD8 DP cells.
DP cells with the unsorted T cells after 14 d culture showed the sorted CD4/CD8 DP population losing just one V\(\beta\) TCR, probably related to the CD8 profile, because it was found in the CD8 SP, but not the CD4 SP population. A previously undetected V\(\beta\) TCR was also observed in the sorted CD4/CD8 DP cells and in the CD4 SP cell immunoscope profile. The finding of so few V\(\beta\) TCR profile modifications at the time of the initial CD4/CD8 DP cell screening suggested the GVHD skin T cells isolated after 14 d culture were already oligoclonal, perhaps specific for one or a small number of minor H Ags.

**CD4/CD8 DP cells proliferate in response to but have no cytotoxic activity against recipient PBMC and recognize an autosomal minor H Ag presented by the HLA-B*14:01 molecule**

To evaluate the function of the CD4/CD8 DP cells, we stimulated them with donor, recipient, or HLA-mismatched PBMC. High levels of proliferation against recipient, but not donor or mismatched PBMC, were observed. Cytotoxic function was tested in parallel, showing only weak CTL activity against recipient but not donor or HLA class I–mismatched targets (Fig. 3). We determined which HLA molecule was the restriction element of cloned CD4/CD8 DP T cells. Twelve recipient-specific clones were isolated, of which five, along with the cell line, were stimulated with recipient APCs in the presence of mAb against HLA class I, -DR, -DQ, or -DP molecules. Results showed that anti–HLA class I, but not anti–HLA class II mAb, anti-CD4, nor anti-CD8 inhibited the proliferation of CD4/CD8 DP cloned T cells and the line (Table I). Using anti-Bw6 and anti-Bw4 sera, HLA-B*14:01 was identified as the restriction molecule (Table I). PBMC from male and female donors sharing HLA-B*14:01 with recipient APCs were used to investigate possible HY specificity: APCs from three HLA-B*14:01 males were tested. Only one was recognized by the CD4/CD8 DP T cell line, indicating that the specificity was for an

![FIGURE 3. CD4/CD8 DP T cells are able to proliferate but not to exert CTL activity following recognition of a minor H Ag on recipient cells. Sorted CD4/CD8 DP T cells were stimulated with donor, recipient, or cells from two to three HLA-mismatched controls (A). T cell proliferation or cytolytic activity was measured by either [\(3^H\)]T incorporation or [\(51^Cr\)] release (B). Results of proliferation assays are the mean ± SD of three experiments. Results of cytolytic assays are representative of two experiments.](http://www.jimmunol.org/)

| Table 1. The CD4/CD8 DP T cells recognize an HLA-B*14:01–restricted autosomal Ag |
|-----------------------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| **Responder** | **APC** | **Anti–HLA DR** | **Anti–HLA DQ** | **Anti–HLA DP** | **Anti–HLA DQ** | **Anti–HLA DR** | **Anti–HLA DQ** | **Anti–HLA DP** | **Anti–HLA DQ** |
| **Cell line** | **Recipient** | **Donor** | **Recipient** | **Donor** | **Recipient** | **Donor** | **Recipient** | **Donor** | **Recipient** |
| **Clone 1** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 2** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 3** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 4** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 5** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 6** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 7** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 8** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 9** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 10** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 11** | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **Clone 12** | Male | Female | Male | Female | Male | Female | Male | Female | Male |

*Results in boldface represent statistically significant results with two-tailed unpaired *t* test (*p*, 0.05).*
autosomal Ag, not HY (Table I). Vβ spectratyping of three of the clones, 2, 7, and 10 (Fig. 2), indicated a high degree of similarity with the DP cell line, perhaps not unsurprisingly because the three clones were also CD4/CD8 DP (not shown).

At the clonal level, clones 2 and 7 appeared to share the same TCRBV, TCRBV 7, whereas clone 10 expressed TCRBV 9.

The CD4/CD8 DP cells exhibit specific regulatory effects in the presence of recipient APCs

We analyzed the cytokine profile of the CD4/CD8 DP T cell line following stimulation with recipient APCs. The cells secreted low levels of IFN-γ and TGF-β, moderate levels of IL-4, and high levels of IL-10, even at 24 h poststimulation, but no IL-2 (Table II). These CD4/CD8 DP T cells were therefore tested for regulatory function. A population of CD3⁺ T cells, T90, obtained from a fully HLA-mismatched blood donor and therefore able to respond to both donor and recipient APCs, was used in a primary MLR. Their proliferative response was measured in the presence or absence of different ratios of CD4/CD8 DP T cells. The T90 cells showed a strong alloresponse against donor, recipient, and third-party APCs, as expected. However, a clear dose-dependent inhibition was observed when CD4/CD8 DP T cell line (Fig. 4A) or clones (not shown) were added to the MLR in the presence of recipient but not donor or HLA-mismatched APCs (Fig. 4A is representative of the cell line and clones). This demonstrated that once activated by recognition of the specific minor H Ag, the CD4/CD8 DP T cells exerted regulatory function. However, anti-IL-10 mAb did not reverse inhibition (data not shown).

### Table II. Cytokine expression pattern of the CD4/CD8 DP T cells

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>IL-2 (IU/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IFN-γ (IU/ml)</th>
<th>TGF-β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor 24 h</td>
<td>Recipient 24 h</td>
<td>Donor 48 h</td>
<td>Recipient 48 h</td>
<td>Donor 72 h</td>
</tr>
<tr>
<td>APC</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>7</td>
<td>1370</td>
<td>10.8</td>
</tr>
<tr>
<td>IL-2 (IU/ml)</td>
<td>7</td>
<td>299</td>
<td>7</td>
<td>1276</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>&lt;5</td>
<td>242</td>
<td>&lt;5</td>
<td>816</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>&lt;5</td>
<td>206</td>
<td>&lt;5</td>
<td>1276</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>IFN-γ (IU/ml)</td>
<td>&lt;0.2</td>
<td>10.8</td>
<td>7</td>
<td>1370</td>
<td>10.8</td>
</tr>
<tr>
<td>TGF-β (pg/ml)</td>
<td>60</td>
<td>267</td>
<td>60</td>
<td>295</td>
<td>64</td>
</tr>
</tbody>
</table>

In the top portion of the table, the CD4/CD8 DP T cell line was stimulated with recipient or donor EBV-BLCL for 24, 48, or 72 h. In the bottom portion of the table, results represent mean ± SEM of four different experiments in which the CD4/CD8 DP cell line was stimulated through their TCR with recipient EBV-BLCL or anti-CD3/CD28 mAbs for 24 h. Cytokine secretion was measured by ELISA and expressed in IU/ml or pg/ml.
the DP cells expressed quite high levels of CD25 (Fig. 1B), we measured the IL-2 responsiveness of both CD4/CD8 DP T and T90 cells and found that of the CD4/CD8 DP T cell line much higher than that of the T90 cells (Fig. 4B). Moreover, using the helper T lymphocyte precursor frequency (HTLP) assay, IL-2 levels were significantly reduced in the presence of the CD4/CD8 DP cells. Even with low numbers of CD4/CD8 DP cells, almost all of the 16 wells were negative for IL-2 (Fig. 4C). Comparison of the CD4/CD8 DP cells and CD8 SP cells isolated from the skin biopsy also showed that the IL-2 responsiveness was much higher in the CD4/CD8 DP cell line (Fig. 4D). Finally, stimulation of the CD4/CD8 DP cells with PHA showed these cells to be dependent on rIL-2. Irrespective of PHA source, and in contrast to the T90, CD4/CD8 DP cells did not proliferate in the presence of PHA alone. When 20 UI/ml rIL-2 was added, proliferation of the CD4/CD8 DP T cell line matched that of T90 cells (Fig. 4E). These data indicate that the CD4/CD8 DP cells are able to regulate T cell proliferation, probably due to their high responsiveness to, and therefore consumption of, IL-2.

**CD4* T cells proliferate and exhibit cytotoxic activity on recognition of an HLA-DR7–restricted HY epitope**

The CD4 SP T cell line isolated from the skin biopsy was analyzed following stimulation with recipient APCs in the presence of anti-HLA class II mAbs. Anti–HLA-DR, but not anti–HLA-DQ or HLA-DP mAb, significantly inhibited proliferative responses (Table III). The recipient was homozygous for HLA-DRB1*07:01: following stimulation of the CD4 SP cell line or clones with APCs from HLA-DRB1*07:01 males or females, we observed a clear DR7-restricted anti-HY response because the T cells responded to each male but not to female APCs (Table III). CTL activity against recipient target cells by the CD4 SP T cells was found and the restriction element confirmed as HLA-DRB1, as cytotoxicity was significantly blocked by anti–HLA-DR but not anti–HLA class I mAb (p = 0.0143). Cells from an unrelated HLA-DRB1*07:01 male donor were also lysed by the CD4 SP T cells (Table III).

**Different patterns of minor H Ag recognition and cytokine mRNA expression by CD4/CD8 DP T cells and CD4 SP T cells**

The different pattern of minor H Ag recognition by CD4/CD8 DP and CD4 SP T cell lines was confirmed in proliferative assays with either HLA-B*14:01 or HLA-DRB1*07:01 PBMC as stimulators. As shown in Fig. 5A, HLA-B*14:01 APCs specifically activated the CD4/CD8 DP T cells, whereas HLA-DRB1*07:01 male APCs activated the CD4 SP T cells, confirming that distinct minor H Ags were recognized by the two subpopulations of T cells. The same differential patterns were obtained with T cell clones (data not shown). Furthermore, following activation by their relevant APC, the mRNA profile of these two subpopulations of T cells was quite distinct. The CD4 SP T cells expressed higher levels of IFN-γ, IL-2 and FOXP3, but lower levels of IL-10 mRNA, whereas the CD4/CD8 DP cells expressed higher levels of IL-10, but lower levels of FOXP3, IL-2, and IFN-γ mRNA (Fig. 5B). Although, at lower levels, IL-4 and TGF-β mRNA were not differentially expressed by CD4/CD8 DP T cells.

**The HY epitope recognized by HLA-DRB1*07:01–restricted CD4* T cells is encoded by RPS4Y**

To define which HY gene encoded the HY epitope recognized by the CD4 SP T cells, we transduced female EBV cell lines with the RPS4Y or DDX3Y genes. IFN-γ production and proliferation measured T cell responses of the clone X2. The results indicated that RPS4Y, but not DDX3Y, was able to stimulate the proliferation

---

### Table III. Functional characterization of the CD4 T cell line

<table>
<thead>
<tr>
<th>Responder</th>
<th>Cell line</th>
<th>APC</th>
<th>HLA-DRB1*07:01</th>
<th>Anti-HLA class I</th>
<th>Anti-HLA class II</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>Cell line</td>
<td>#1</td>
<td>12.875 ± 5.12</td>
<td>0.0, 0.0</td>
<td>0.0, 0.0</td>
</tr>
<tr>
<td>#2</td>
<td>Cell line</td>
<td>#2</td>
<td>10.796 ± 4.10</td>
<td>0.0, 0.0</td>
<td>0.0, 0.0</td>
</tr>
<tr>
<td>#3</td>
<td>Cell line</td>
<td>#3</td>
<td>19.080 ± 8.88</td>
<td>0.0, 0.0</td>
<td>0.0, 0.0</td>
</tr>
</tbody>
</table>

**a** CD4 SP T cells were stimulated with donor or recipient EBV-BLCL. Anti–HLA class I or class II mAbs were added to inhibit T cell proliferation.

**b** HY specificity was assessed using male or female HLA-DRB1/B*14:01 or HLA-DRB1/B*07:01 or HLA-DQ male or female PBMC as APCs. Anti–HLA class I or class II mAbs were added as indicated.

**c** CTL experiments were performed in a [3H] release assay. APCs from donor, recipient, or unrelated males sharing HLA class I or class II HLA molecules were used as targets of CD4 SP effector cells. Anti–HLA class I or class II mAbs were added as indicated.
of CD4 SP T cells and production of IFN-γ (Fig. 6A, 6B). From peptide–MHC binding databases, we designed peptides of 25–40 aa in length covering regions spanning one or more candidate HLA-DRB1*07:01 peptide-binding motifs (Supplemental Table I), using them to pulse female HLA-DR7 EBVAPCs. One peptide, QR-40, stimulated IFN-γ production by the CD4 SP T cells (Fig. 6C). Several potential 15-mer HLA-DRB1*07:01 binding peptides within the QR-40 sequence were tested. One, TL15, specifically induced IFN-γ and weak IL-2 mRNA expression by the CD4 SP cells (Table IV). We thus identified TGKIINFIKFDTGNL as the HY peptide epitope recognized. Although FOXP3 was nonspecifically upregulated in the presence of every peptide, TL15 induced the highest FOXP3 mRNA response. TL15 also induced higher levels of IFN-γ than QR40 (Table IV).

**RPS4Y-specific CD4+ T cells express TCRVB19*01**

To identify the TCR Vβ expressed by the HY-specific CD4 SP T cells, the Vβ TCR of the T cell line and clones was sequenced. There was a single CDR3 rearrangement in each (Fig. 6D), suggesting the initial CD4 SP T cell line was already clonal. Sequencing the TCRVβ/JB/DB regions of the CD4 SP T cell line and clone showed them to be identical, homologous to TRBV19*01 and TRBJ2-3*01 of the IMGT database (Supplemental Table II).

**Discussion**

In contrast to immature thymic T cells, expression of the CD4 and CD8 coreceptors on mature T cells is generally mutually exclusive. However, peripheral CD4/CD8 DP T cells have been described in certain pathological as well as normal conditions (29–31). For example, a subset of peripheral blood CD4/CD8 DP T cells has been reported in autoimmune and chronic inflammatory disorders, like thyroiditis, multiple sclerosis, and systemic sclerosis (32–34). They have also been found in patients with Kawasaki syndrome and Hodgkin lymphoma (35, 36). Following transplantation, only a single study has described them in organ biopsies, but not formally demonstrated coexpression of CD4 and CD8 (37). In animal transplantation models, only one study has found such cells in the periphery associated with cyclosporin A treatment (38).

It is not known whether peripheral CD4/CD8 DP T cells are a result of a failure of thymic selection or if the second coreceptor is expressed in response to an immunogenic stimulus that enhances intracellular signaling by recruiting p56 lck (29). The relative levels of CD4 and CD8 on these cells might provide insight into this: CD4high/CD8low DP T cells are found in small numbers in normal individuals and in somewhat higher numbers of those with autoimmunity (29, 33). However, with the exception of a single study that demonstrated such T cells infiltrating cutaneous lymphoma and exerting tumor-specific MHC class I–restricted lysis, no function could be attributed to them (39). With the exception of that study, CD4high/CD8low DP T cells were found resistant to apoptosis, to proliferate poorly upon CD3/TCR stimulation, and unable to produce IL-2 (29). In contrast, CD4high/CD8high T cells are more likely to be Ag-specific effector cells, rather than immature cells released from the thymus. Indeed, such cells have been described to exert antiviral and antitumor immunity following contact with the cognate Ag (40–44).

The CD4high/CD8high DP T cell population described in this study was isolated from skin of a patient with grade 2 acute GVHD that resolved quickly. The donor/recipient pair was HLA identical, sex-mismatched in the direction favoring the activation of HY-specific female donor T cells by recipient male cells. This CD4/CD8 DP population was coisolated together with a CD8 SP T cell population (Fig. 1). We were readily able to separate these two populations, but not able to culture the CD8 SP T cells because after sorting, CD4/CD8 DP T cells reappeared in the cul-
This could be due either to acquisition of the CD4 coreceptor upon stimulation or competitive proliferation of small numbers of CD4/CD8 DP T cells that remained after sorting. The results of proliferation assays (Fig. 4D) suggested the latter was the case. This was supported by blocking experiments showing that neither anti-CD4 or anti-CD8 mAbs inhibited the CD4/CD8 DP specific alloresponse (Table I). Similar results have been reported using tumor-specific DP T cells (39). The CD4/CD8 DP T cell subset was only found in the patient’s skin, not peripheral blood (data not shown). These cells specifically proliferated in response to an autosomally encoded Ag expressed by recipient cells, but produced negligible amounts of IL-2 and rather high levels of IL-10, together with lower levels of IFN-γ and IL-4 (Table II). These cells exerted regulation in vitro in the presence of donor APCs (Fig. 4A). A similar finding has been reported for intraintestinal CD4/CD8 DP T cells: secretion IL-10 and regulatory function (45). However, anti–IL-10 did not reverse our inhibitory effect (data not shown), suggesting IL-10 played no role in the regulatory activity. IFN-γ has been shown by us and others to reduce T cell responses by inhibiting APC maturation (46) or by inducing inducible NO synthase production (47, 48). However, in this study, this was excluded because IFN-γ was secreted at low levels, and anti–IFN-γ Ab did not block inhibition. Furthermore, inducible NO synthase was not increased upon stimulation of these cells (data not shown), and they expressed low levels of FOXP3 (Fig. 5), suggesting another inhibitory mechanism was involved. Because the CD4/CD8 DP T cells expressed high levels of IL-2R, even 7 d after activation (Fig. 1), and did not produce IL-2 (Table II), we hypothesized that suppression was due to consumption of IL-2. Indeed, such a suppressor mechanism has been previously shown to be used by Treg (49, 50). This mechanism being likely in this study was shown by: 1) their high IL-2 responsiveness, greater than the CD8 SP T cells (Fig. 4D) and an unrelated population of CD3+ primary T90 T cells, (Fig. 4B); 2) their consumption of the IL-2 produced by third-party cells in an HTLP assay (Fig. 4C); and 3) their unresponsiveness to PHA in the absence of rIL-2 (Fig. 4E). These observations might further explain why the acute GVHD episode was brief and weak in this patient, suggesting control in vivo by these Treg. Further studies are needed to analyze the tissue distribution of this minor H Ag, as, because of its low frequency of expression, only one of six individuals with HLA-B*14:01 expressed this Ag, we were unable to evaluate this in our present study.

After repeated rounds of stimulation with recipient APCs, we isolated a subset of CD4 SP T cells (Fig. 1C). These produced IL-2 and IFN-γ and were cytotoxic (Fig. 5B, Table III). Further char-
Table IV. Determination of the T cell epitope recognized by the CD4+ T cell clone

<table>
<thead>
<tr>
<th>Peptide</th>
<th>mRNA Concentration</th>
<th>IL-2/CD3</th>
<th>IFN-γ/CD3</th>
<th>FOXP3/CD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAbs</td>
<td>No.</td>
<td>Sequence</td>
<td>IL-2/CD3</td>
<td>IFN-γ/CD3</td>
</tr>
<tr>
<td>TGKIINFIKFDTGNL</td>
<td>0.009</td>
<td>0.012</td>
<td>0.009</td>
<td>0.012</td>
</tr>
<tr>
<td>GV17</td>
<td>0.009</td>
<td>0.012</td>
<td>0.009</td>
<td>0.012</td>
</tr>
<tr>
<td>GV17.1</td>
<td>0.009</td>
<td>0.012</td>
<td>0.009</td>
<td>0.012</td>
</tr>
<tr>
<td>GV17.2</td>
<td>0.009</td>
<td>0.012</td>
<td>0.009</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Results in boldface represent the highest levels that were obtained in the same experiment. This experiment is representative of two different experiments.

a mRNA concentration ratios were used to determine which peptides were recognized.

b A dose-response curve of IFN-γ secretion was then measured in the presence of different concentrations of TL15, the specific epitope, or QR-40.

disciplines.

In conclusion, we describe in this study evidence for the existence of CD4/CD8 DP T cells in the skin of an acute GVHD patient and show that these cells exert regulatory function, probably as a result of IL-2 consumption. We suggest these cells may have had a role in the reduction of the pathogenic response causing acute GVHD in this patient. We also identify a novel HLA class II-restricted minor H Ag, its HLA-restriction molecule, and its TCR profile of these two populations was distinct, because some Vβ TCR were lost in the CD4 SP population compared with the CD4/CD8 DP T cells (Fig. 2).

The CD4 SP T cells were HY specific and HLA-DRB1*0701 restricted; we were able to identify the gene and peptide encoding this HY epitope, with a candidate gene approach similar to that previously used to identify murine MHC class II-restricted HY epitopes (51). We retrovirally transduced female DR7-EBV cells with the candidate genes DDX3Y and RPS4Y. Using our CD4 SP T cell clone or the line from which clones were derived, we identified RPS4Y as the gene encoding the DR7-restricted HY epitope. The peptide epitope itself was identified first using peptides of 25–40 aa, designed to incorporate candidate DRB1*0701-binding peptides selected from several databases. This allowed us to pinpoint one peptide, QR40 (Fig. 6), and subsequently the specific 15-mer peptide, TGKIINFIKFDTGNL, as the HY epitope recognized by the CD4 SP T cells (Table IV). Comparing the response of the CD4 SP T cell clone to QR40 with TL15, the shorter peptide, presumably with no need for further processing, induced higher responses (Table IV). This reflects recent findings that long peptides have a lower affinity for MHC than short peptides (52). The TL15 peptide represents a novel HY epitope, which may indeed represent a new target for GVL. Although RPS4Y has already been shown to encode an HLA class II-restricted HY epitope able to induce helper and cytolytic activity, in that study, the epitope was restricted by HLA-DRB3. Not surprisingly, the amino-acid sequence was different from the one identified in this study, as was the Vβ TCR of the clone that recognized it (7).

In conclusion, we describe in this study evidence for the existence of CD4/CD8 DP T cells in the skin of an acute GVHD patient and show that these cells exert regulatory function, probably as a result of IL-2 consumption. We suggest these cells may have had a role in the reduction of the pathogenic response causing acute GVHD in this patient. We also identify a novel HLA class II-restricted HY minor H Ag, its HLA-restriction molecule, and its amino acid sequence and describe the CDR3 region and Vβ restricted HY minor H Ag, its HLA-restriction molecule, and its TCR profile of these two populations was distinct, because some Vβ TCR were lost in the CD4 SP population compared with the CD4/CD8 DP T cells (Fig. 2).

The CD4 SP T cells were HY specific and HLA-DRB1*0701 restricted; we were able to identify the gene and peptide encoding this HY epitope, with a candidate gene approach similar to that previously used to identify murine MHC class II-restricted HY epitopes (51). We retrovirally transduced female DR7-EBV cells with the candidate genes DDX3Y and RPS4Y. Using our CD4 SP T cell clone or the line from which clones were derived, we identified RPS4Y as the gene encoding the DR7-restricted HY epitope. The peptide epitope itself was identified first using peptides of 25–40 aa, designed to incorporate candidate DRB1*0701-binding peptides selected from several databases. This allowed us to pinpoint one peptide, QR40 (Fig. 6), and subsequently the specific 15-mer peptide, TGKIINFIKFDTGNL, as the HY epitope recognized by the CD4 SP T cells (Table IV). Comparing the response of the CD4 SP T cell clone to QR40 with TL15, the shorter peptide, presumably with no need for further processing, induced higher responses (Table IV). This reflects recent findings that long peptides have a lower affinity for MHC than short peptides (52). The TL15 peptide represents a novel HY epitope, which may indeed represent a new target for GVL. Although RPS4Y has already been shown to encode an HLA class II-restricted HY epitope able to induce helper and cytolytic activity, in that study, the epitope was restricted by HLA-DRB3. Not surprisingly, the amino-acid sequence was different from the one identified in this study, as was the Vβ TCR of the clone that recognized it (7).

In conclusion, we describe in this study evidence for the existence of CD4/CD8 DP T cells in the skin of an acute GVHD patient and show that these cells exert regulatory function, probably as a result of IL-2 consumption. We suggest these cells may have had a role in the reduction of the pathogenic response causing acute GVHD in this patient. We also identify a novel HLA class II-restricted HY minor H Ag, its HLA-restriction molecule, and its amino acid sequence and describe the CDR3 region and Vβ TCR of the clone that recognizes this peptide. Because HLA class II-restricted minor H Ags are important potential targets expressed by leukemic cells in vivo, due to their expression by hematopoietic cells, our findings could contribute to specifically target HLA-DR7 male leukemic cells in cell therapy programs.

Acknowledgments

We thank Jeanine Bernaud (Etablissement Français du Sang Rhone Alpes) for excellent technical assistance in flow cytometry.

Disclosures

The authors have no financial conflicts of interest.


