Cross-Reactivity of Herpesvirus-Specific CD8 T Cell Lines Toward Allogeneic Class I MHC Molecules

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Abstract

Although association between persistent viral infection and allograft rejection is well characterized, few examples of T-cell cross-reactivity between self-MHC/viral and allogeneic HLA molecules have been documented so far. We appraised in this study the alloreactivity of CD8 T cell lines specific for immunodominant epitopes from human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV). CD8 T cell lines were generated after sorting with immunomagnetic beads coated with either pp65259–267/A*0201, BMLF1259–267/A*0201, or BZLF114–64/B*3501 multimeric complexes. Alloreactivity of the CD8 T cell lines against allogeneic class I MHC alleles was assessed by screening of (i) TNF-α production against COS-7 cells transfected with as many as 39 individual HLA class I-encoding cDNA, and (ii) cytotoxicity activity toward a large panel of HLA-typed EBV-transformed B lymphoblastoid cell lines. We identified several cross-reactive pp65/A*0201-specific T cell lines toward allogeneic HLA-A*3001, A*3101, or A*3201. Moreover, we described here cross-recognition of HLA-Cw*0602 by BZLF1/ B*3501-specific T cells. It is noteworthy that these alloreactive CD8 T cell lines showed efficient recognition of endothelial cells expressing the relevant HLA class I allele, with high level TNF-α production and cytotoxicity activity. Taken together, our data support the notion that herpes virus-specific T cells recognizing allo-HLA alleles may promote solid organ rejection.

Introduction

It is now well established that the memory subset of circulating T cells contribute to alloreponse, thus explaining that viral infections are associated with graft failure in human transplant recipients [1,2,3]. A range of acute viral infections, most particularly cytomegalovirus (HCMV) infection, has been linked with initiating the clinical complications that often follow transplantation [4]. The best evidence that HCMV is involved in acute and chronic rejection is based on studies with the anti-HCMV drug ganciclovir in humans and animal models that demonstrated a reduction in allograft failure in solid organ transplant patients [5]. HCMV could account for graft rejection by selective endothelial cell activation thereby attracting and activating alloreactive T cells [2]. Another factor of the association between HCMV infection and allograft rejection could be cross-reactivity of HCMV-specific T cells to allogeneic HLA molecules. Persistent viral infections have a profound impact on T cell repertoire, since they lead to long-term clonal expansions of virus-specific memory CD8 T cells. Large clonal expansions of αβ T cells within the human peripheral repertoire have been documented in several acute viral infections [6] and in healthy individuals [7]. In particular, human CD8 memory T cell repertoire is often dramatically skewed by predominant clones directed against HCMV or Epstein-Barr virus (EBV), which can persist unaltered for many years [8,9,10]. Through cross-reactivity, these memory T cells could contribute to the alloreponse, owing to their lack of requirement for co-stimulation, easy and rapid activation, and vigorous effector functions [11].

Though association between persistent viral infection and allograft rejection is well admitted, few examples of T-cell cross-reactivity between self-MHC/viral and allogeneic HLA molecules have been documented so far. The influence of antiviral T cell responses on the CD8+ T cell alloreactive repertoire was first described for an EBV T cell response specific to the EBNA3A325–333/B*0801 EBV epitope [12,13,14]. More recently, cross-reactivity of HCMV-specific and herpes simplex virus-specific CD8 T cells to allogeneic HLA alleles has been reported [15,16].

To appraise the contribution of EBV- or HCMV-specific CD8 T cell responses to the allogeneic repertoire, we screened a number of CD8 T cell lines, that had been sorted with recombinant peptide/MHC class I (pMHC) multimeric complexes, on a large panel of HLA class I alleles expressed either by transfected COS cells or by EBV-transformed B lymphoblastoid cell lines (LCL) for cross-reactivity to allogeneic class I HLA molecules. Our study was focused on the pp65259–267/A*0201 HCMV epitope (NLVPIVATV) [17] and two epitopes of early lytic EBV proteins [BZLF114–64/B*3501: EPLPQGQLTAY [18,19] and BMLF1259–267/A*0201:
GLCTLVAML [20,21], for which immunodominance [17,19,22,23] and high frequency [10,24] is well documented. This unveiled several allospecific CD8 T cell responses, leading to cytotoxicity and TNF-α production against primary endothelial cell cultures expressing the relevant allogeneic HLA alleles. This might have important physiopathological implications in an allograft setting, which are discussed.

Results

Screening of HCMV- or EBV-specific CD8 T cell lines for cross-recognition of allogeneic MHC molecules

To assess the influence of CD8 T cell responses specific to HCMV or EBV to the allogeneic repertoire, we screened CD8 T cell lines sorted with recombinant pMHC multimeric complexes specific to HCMV (pp65495–503/A*0201) or EBV (BMLF1259–267/A*0201 or BZLF154–64/B*3501) epitopes for cross-recognition of allogeneic MHC class I molecules, taking into account the immunodominance of those responses and the frequent expression of A*0201 and B*3501 alleles (Table 1). Most T cell lines analyzed were derived from PBL from healthy donors (D01 to D08, D12). Other T cell lines were derived from PBL from patients suffering from arthritis (D09 to D11, D13 to D16). The enrichment in EBV- or HCMV-specific T cells was checked by staining by ad hoc pMHC tetramers, and two successive sortings were made, when necessary, to achieve a purity between 89 to 100% (Table 1, Fig. 1.A, and data not shown). Staining of T cell lines before sorting indicated frequencies comprised between 0.1 and 5.6% (Fig. 1.B and data not shown). Two unsorted T cell lines, derived from A2-negative donors were also included in the screening.

T cell lines were screened both for TNF-α production against COS-7 cells transfected with as many as 39 individual HLA class I-encoding cDNA, and for cytotoxicity toward a panel of 30 HLA-typed LCLs (Table 2), in order to detect allo-MHC recognition. Data are summarized in Table 1.

### Table 1: Screening of CD8 T cell lines enriched in HCMV- or EBV-specific T cells for cross-reactivity to allogeneic MHC molecules.

| Donor# | Molecular HLA class I typing | % of tetramer-positive T cells | Reactivity to allogeneic HLA+ |
|--------|-----------------------------|-------------------------------|-------------------------------|
|        | HLA-A* | HLA-B* | HLA-Cw* |                              |

pp65495–503/A*0201-sorted T cell lines

| Donor# | Molecular HLA class I typing | % of tetramer-positive T cells | Reactivity to allogeneic HLA+ |
|--------|-----------------------------|-------------------------------|-------------------------------|
|        | HLA-A* | HLA-B* | HLA-Cw* |                              |
| D01    | 0201   | 0301   | 3501    | 4402 | 0401 | 0501 | 97 | A*3101 |
| D02    | 0201   | 33     | 08     | 14   | 07   | 08   | 98 | none   |
| D03    | 0201   | 0201   | 1302   | 5101 | 0202 | 0602 | 99 | A*3201 |
| D04    | 0201   | ND     | ND     | ND   | ND   | 98   | none |
| D05    | 0201   | ND     | ND     | ND   | ND   | 99   | none |
| D06    | 0201   | ND     | ND     | ND   | ND   | 99   | none |
| D07    | 0201   | 2301   | 2705   | 4402 | 0102 | 0509 | 98 | A*3001 |
| D08    | 0201   | 2301   | 4101   | 4402 | 0501 | 1701 | 89 | none   |
| D09    | 0201   | 30     | 08     | 44   | 04   | 07   | 98 | none   |
| D10    | 0201   | 0201   | 1501   | 4403 | 0304 | 0501 | 95 | none   |
| D11    | 0201   | 1101   | 0801   | 2705 | 01   | 07   | 97 | none   |

BMLF1/A*0201-sorted T cell lines

| Donor# | Molecular HLA class I typing | % of tetramer-positive T cells | Reactivity to allogeneic HLA+ |
|--------|-----------------------------|-------------------------------|-------------------------------|
|        | HLA-A* | HLA-B* | HLA-Cw* |                              |
| D12    | 0201   | 3201   | 15     | 15   | 0303 | 04   | 93 | none   |
| D13    | 0201   | 0201   | 2705   | 4002 | 0102 | 1501 | 95 | none   |

BZLF1/B*3501-sorted T cell lines

| Donor# | Molecular HLA class I typing | % of tetramer-positive T cells | Reactivity to allogeneic HLA+ |
|--------|-----------------------------|-------------------------------|-------------------------------|
|        | HLA-A* | HLA-B* | HLA-Cw* |                              |
| D14    | 0301   | 1101   | 0701   | 3501 | 04   | 07   | 92 | B*0602 |
| D15    | 2402   | 3101   | 3501   | 4001 | 03   | 07   | 97 | none   |
| D16    | 2402   | 3201   | 2705   | 3501 | 01   | 04   | 97 | none   |

*CD8 T cell lines were screened on COS-7 cells transfected with individual HLA-encoding cDNA and TNF-α production was measured after a 6h-coculture. All T cell lines were PBL-derived. ND : not determined.
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against COS cells expressing A*0201, and killing all A*0201+ LCL (data not shown). This indicates that results obtained from the two assays were fully consistent, thus validating the use of COS cells transfectsed with individual HLA allele to screen for allorecognition in the MHC class I context.

To appraise the polyclonality of the pp65/A2-specific T cell lines, their TCR Vβ repertoire was analyzed by immunofluorescence, using Vβ-specific mAb (Table 3). T cell lines derived from healthy donors used an heterogeneous set of TCR Vβ families. In contrast, T cell lines derived from RA patient PBL, that were analyzed in a previous study [25], showed a dramatic skewing of their TCR Vβ repertoire, as >95% of T cells for a given donor expressed the same Vβ region. Three TCR Vβ regions (TRBV10-3, TRBV12-4 and TRBV20-1) were preferentially expressed by most of the pp65/A2-specific T cell lines analyzed in the present study.

To assess the percentage of alloreactive T cells within a given pp65/A2-sorted T cell line and the TCR Vβ region used by the fraction of alloreactive T cells, we conducted a CD25 upregulation assay. T cell lines were stimulated overnight with LCL expressing, or not, the relevant allogeneic HLA allele and cells were triple stained by anti-CD3-APC, anti-CD25-FITC and by anti-Vβ-PE mAb specific to each one of the TCR Vβ used by this T cell line, as indicated in Table 3. Such an analysis was done for the D01 and D03 cell lines, a fraction of CD25+ cells was stained by anti-Vβ13.1 mAb (TRBV6-9; Fig. 3B), while a fraction of CD25+ cells was not stained by this mAb, nor by any of the other anti-Vβ mAb (data not shown). Due to the down-modulation of the TCR-CD3 complex following activation by Ag, most probably the whole fraction of CD25+ T cells activated by the allogeneic HLA molecule is included in the TRBV6-9 subset. This suggests that alloreactive T cells contained in the pp65/A2-specific T cell lines from D01 and D03 used the same TCR Vβ (TRBV6-9), though they did not recognize the same allogeneic HLA allele.

Allorecognition of HLA-Cw*0602 by T cells specific to the BZLF154–64/B*3501 epitope

Strong allorecognition of HLA-Cw*0602 was observed for the BZLF1/B*3501-sorted T cell line from D15. This T cell line killed selectively the four LCL of the panel (BAR, MHE, SBN, and TIM), that shared Cw*0602 expression (Fig. 2A) and produced TNF-α selectively against COS cells expressing Cw*0602 (Fig. 2B). The CD25 assay, performed as described above, indicated that at least 70% of T cells within this cell line acquired CD25 expression upon stimulation with Cw*0602+ LCL, but not with Cw*0602+ LCL (data not shown). The two other BZLF1/B35-sorted T cell lines did not respond to Cw*0602, though they were stained at >90% by the BZLF1/B35 tetramer (Table 1). All three BZLF1/B35-sorted T cell lines killed B*3501+ LCL loaded with the BZLF154–64 peptide (data not shown).

Analysis of TCR Vβ repertoire of BZLF1/B*3501-sorted T cell lines showed a limited set of TCR Vβ regions, with a preferential usage of Vβ12 (TRBV10-3) for the three cell lines (Table 3). Repertoire skewing in favor of Vβ12 expression was the most

Figure 1. Enrichment in pp65495–503/A*0201- or BZLF154–64/B*3501-specific T cells after sorting of CD8 T cells with pMHC magnetic multimers. (A) CD8 T cells, derived from the PBL from D01 and D03 were sorted with 245V mutated pp65495–503/A*0201 multimers, then expanded in culture, and stained with PE-conjugated pp65495–503/A*0201 tetramers and FITC-conjugated anti-CD3. (B) CD8 T cells derived from the PBL from D15 were sorted with 245V mutated BZLF154–64/B*3501 multimers, and then expanded in culture. Unsorted and sorted T cells were stained with PE-conjugated BZLF1/B35 tetramers and FITC-conjugated anti-CD3. The percentage of positive cells is indicated in the upper right quadrant.

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striking in D15-derived T cell line, since almost all T cells use this VB region gene. The two other BZLF1/B35-sorted T cell lines comprised about 50% of VB12 T cells, though they did not exhibit allorecognition of Gw*0602, thus indicating that alloreactive T cells within D15-derived T cell line correspond to specific clonotype(s) not found in the two other cell lines.

Alloreactive CD8 T cells killed efficiently endothelial cells expressing the relevant class I HLA molecules

Endothelial cell cultures were tested for their capacity to activate alloreactive CD8 T cell lines. As endothelial cell cultures, expressing A*31, A*32 or Gw*0602 were available, we tested their capacity to activate CD8 T cell lines that had shown allorecognition toward these HLA alleles: T cell line from D01, alloreactive to A*3101, T cell line from D03, alloreactive to A*9201 and T cell line from D15, alloreactive to Gw*0602. While endothelial cell cultures alone did not produce TNF-α, they induced TNF-α responses from T cell lines specific for the relevant allogeneic MHC allele (Fig. 4A). Moreover, alloreactive CD8 T cell lines killed the endothelial cells expressing the relevant allogeneic MHC molecules, but not endothelial cells with irrelevant allogeneic MHC alleles (Fig. 4B and Table 4).

Table 2. HLA class I typing of LCL.

| LCL  | HLA-A* | HLA-B* | HLA-Cw* |
|------|--------|--------|---------|
| ADA  | 0201   | 2705   | 4002    | 0102 | 1502 |
| AK1  | 0201   | 0301   | 0702    | 1402 | 0702 | 0802 |
| BAR  | 3001   | 2402   | 4403    | 5101 | 0602 | 1601 |
| BAX  | 0101   | 0301   | 0702    | 1501 | 0303 | 0702 |
| BOI  | 2402   | 3101   | 3501    | 4003 | 03   | 07   |
| COL  | 0102   | 0801   | 3501    | 0401 | 07   | 07   |
| CRE  | 0101   | 2301   | 0801    | 4403 | 0701 | 0401 |
| D1   | 0201   | 0301   | 3501    | 4402 | 0401 | 0501 |
| D2   | 0201   | 33     | 08     | 14    | 07   | 08   |
| D4S  | 0201   | 2902   | 0702    | 0801 | 0701 | 0702 |
| DAB  | 0201   | 1101   | 1801    | 5501  | 03   | 07   |
| DUC  | 3002   |        | 1801    |       | 0501 |       |
| GAS  | 0301   | 2402   | 2705    | 5101 | 0202 | 1602 |
| HOB  | 0101   |        | 0801    |       | 0701 |       |
| HV1  | 0101   | 0201   | 0801    | 1501 | 0304 | 0701 |
| JES  | 0201   | 2705   |         |       | 01   |       |
| JHAF | 3101   |        | 5101    |       | 08   |       |
| KER  | 0101   | 6801   | 0801    | 5101 | 07   | 15   |
| LEE  | 0301   | 15     | 27      |       | ND   |       |
| LEP  | 0201   | 2501   | 1801    | 4402 | 0501 | 1203 |
| MHE  | 0201   | 33     | 14      | 57    | 0602 | 0801 |
| NJU  | 0201   | 33     | 08      | 58    | 0302 | 0304 |
| PAU  | 0201   | 1801   | 4001    | 0304 | 1203 |       |
| PIP  | 0102   | 0201   | 5701    | 0402  | ND   |       |
| SBN  | 0201   | 3201   | 1402    | 5701 | 0602 | 0802 |
| SYL  | 0301   |        | 0801    | 1801 | 0701 |       |
| TAY  | 0101   | 1101   | 0702    | 3501 | 0401 | 0702 |
| TIE  | 0201   | 2402   | 2705    | 35    | 0202 | 0401 |
| TIM  | 0201   | 2601   | 2703    | 4501 | 0102 | 0602 |
| YOU  | 0102   | 2402   | 2702    | 3501 | ND   |       |

ND : Not determined. LCL and the HLA triggering allorecognition are indicated in bold. doi:10.1371/journal.pone.0012120.t002

Discussion

We identified in this study the cross-reactivity of herpesvirus-specific CD8 T cell lines with allogeneic HLA class I molecules. The rationale for analyzing these particular HCMV and EBV epitopes was that their immunodominance has been well documented [17,19,22]. CD8 T cell lines were screened on a large panel of class I HLA alleles expressed either by LCL or by COS-7 cells transfected with HLA-encoding cDNA. Identical results were obtained in both assays, thus validating the use of COS cells transfected with individual HLA allele to screen for allorecognition in the MHC class I context. The interest to use T cell lines sorted out with recombinant p/MHC multimeric complexes was that they were 90 to 100%-enriched for T cells of a given specificity, so that we could clearly link alloresponse to a given viral epitope.

In agreement with a recent report by Amir et al. [26], our data bring evidence that the pp65/A2 T cell response contributes to the alloreactive repertoire. Cross-recognition of allogeneic HLA-A molecules was found for three out of the eleven T cell lines tested that showed cytolytic activity and cytokine production toward cells expressing the relevant allogeneic HLA allele. This is in accordance with previous data, obtained with EBV-specific CD8 T cells, which showed that allogeneic responses are considerably stronger than syngeneic responses [12,13,14]. In contrast to the strong cytotoxicity toward allogeneic HLA molecules exhibited by the T cell lines we analysed, the pp65/A*0201-specific CD8 T cell cross-reactive to allogeneic molecules described by Gamadia et al. were not able to kill allogeneic LCLs, though they proliferated and produced IFN-γ against allogeneic LCL and killed autologous LCL loaded with the pp65/A2 peptide [15]. We did not find any recurrent cross-reactivity to allogeneic HLA alleles as each one of these three alloreactive T cell lines recognized a different allogeneic HLA-A allele: A*3001, A*3101, or A*3201. Remarkably, the pp65/A2-specific T cell line from D08 that showed alloreactivity toward A*3001 did not respond to A*3002, though these two allotypes differ from each other by only four amino acid within α2 and α3 domains, indicating exquisite HLA specificity for this allorecognition. Despite lack of recurrence, alloreactivity of pp65/A2-specific T cells was focused against three HLA-A alleles, that share >95% homology between each other and with HLA-A*0201. This is in accordance with a retrospective survey of a large cohort of hematopoietic stem cell donor/patient pairs with single HLA mismatch, that had shown that allogeneic HLA class I molecules with large sequence differences do not elicit a CTL response [27]. In light of the A*3001, A*3101 and A*3201-cross-recognition by pp65/A2-specific T cell lines, we tried to determine retrospectively whether single HLA-A2-A30, A2-A31, and A2-A32 mismatch combinations could be associated with increased renal graft loss [28] or higher incidence of severe GVHD [29]. However no conclusive results could be drawn yet from such an analysis, owing to the scarcity of such alleles, which are expressed by at most 4% of the caucasian population.

We could have missed alloreactive clonotypes poorly represented within the polyclonal T cell line. However, our data indicate that when as few as 3% of alloreactive T cells were present in the polyclonal population, alloresponse was detected, thus documenting the exquisite sensitivity of the functional assays herein used. In accordance with previous studies [17,24,25], most pp65/A*0201-specific CD8 T cell lines screened for allorecognition showed a
diversified TCR V\(\beta\) repertoire, so that a large number of clonotypes were screened concomitantly. Though alloreactive T cells contained in the pp65/A2-specific T cell lines from D01 and D03 used the same TCR V\(\beta\) (V\(\beta\)13.1), they did not recognize the same allogeneic HLA allele. Moreover, despite pp65/A2-specific T cell lines from D04 and D11 used preferentially the same TCR.
Diversity is unclear, it is possible that pre-existing expansions of one year has been reported [32]. Although the basis for this limited in vivo during acute GVHD and persistence of these T cells for up to one year has been reported has shown highly biased TCR usage, associated in some cases with predominance of a single clone [31]. Alloreactive T cell clonal expansions have also been identified in vivo during acute GVHD and persistence of these T cells for up to one year has been reported [32]. Although the basis for this limited diversity is unclear, it is possible that pre-existing expansions of primed alloreactive T cells could play a role in graft rejection or GVHD.

A range of acute viral infections have been linked with initiation of complications that often follow transplantation, and most attention has focused on the role of herpesvirus infections [1,2,3,33]. In this context, the influence of the public CD8 T cell response to the EBNA3A 325–333/B*0801 EBV epitope on the alloergic repertoire has been well documented [12,13,14]. Clones were isolated, that displayed dual specificity for the EBNA3A 325–333/B*0801 EBV epitope and for HLA-B*14, B*44 or B*35 alleles, as alloantigens [12,13]. Each distinct pattern was found to be associated with a public TCR. The public TCR associated with B44 allorecognition was shown to be alloreactive against B*4402 and B*4405, but not B*4403 [12,33]. These three allotypes differ from each other by only one or 2 amino acids; yet this difference is enough to invoke a substantial difference in T cell recognition. These reports were the first to demonstrate that a history of EBV infection can augment responsiveness to particular alloantigens. Interestingly, HLA-B44 was identified as a ‘taboo mismatch’ for HLA-B8 renal transplant recipients [34].

While it has long been established that human vascular endothelium can activate alloreactive CD8 T cell in vitro [35] very few studies have examined in detail the response of alloreactive CD8 T cells toward endothelial cells. Here we show that alloreactive CD8 T cell lines efficiently killed endothelial cells expressing the relevant alloegenic HLA allele, and produced high level TNF-α in response to these alloegenic endothelial cells. These data clearly indicate that endothelial cells cultivated in vitro are targets for alloreactive CD8 T cells. Allograft rejection often involves injury of graft endothelium lining both large and small vessels. Human vascular endothelial cells display both class I and class II MHC molecules and are directly recognized in vitro by CD8 and CD4 alloreactive T cells, respectively [35,36,37].

| TCR Vβ specific mAb | 2 | 3-1 | 4-1 | 4-3 | 5-1 | 5-5 | 6-2 | 6-6 | 6-9 | 9 | 10-3 | 11-2 | 12-4 | 13 | 14 | 18 | 19 | 20-1 | 25-1 | 27 | 28 | 29-1 | 30 |
|---------------------|---|-----|-----|-----|-----|-----|-----|-----|-----|---|------|------|------|---|---|---|---|------|------|---|---|-----|-----|
| pp65/A*0201-sorted T cell lines | D01 | 18.2 | 57.4 | 10.6 | 12.3 |
|                     | D02 | 1.6  | 8.2  | 89.2 |     |
|                     | D03 | 1    | 1.1  |    | 2    | 2.2  | 20.8 | 2.2 | 1.3 | 6.9 | 10.3 | 1.1 | 1.2 | 13.2 | 2.7 |
|                     | D04 | 91.5 | 6.1  | 89.5 |     |
|                     | D05 | 6.9  | 1.1  |    | 2    | 20.9 | 5.3  | 2.4 | 1   |     | 6.8  | 24.7 | 2.3 |
|                     | D06 | 9.5  | 3.4  | 2.3 |     | 41.4 | 1.3  | 1.4 | 11.7 | 277 |     |
|                     | D07 | 28.4 | 21.1 | 3.9 | 8.7  | 12.1 | 1.6  | 2.5 | 10.2 |     |
|                     | D08 | 1.8  | 1.7  | 2.6 | 42.3 |     | 1    | 8.5 |     |
|                     | D09 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|                     | D10 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|                     | D11 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| BZLF1/B*3501-sorted T cell lines | D14 | 1.1  | 2.9  | 48.8 | 23  | 36.6 | 5.2 |
|                     | D15 | 1.2  | 96.4 |     |     |     |     |
|                     | D16 | 3.9  | 55.2 | 26.3 | 16.4 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

The percentage of pp65/A*0201- or BZLF1/B*3501-specific T cells stained by the various anti-TCR Vβ mAb is mentioned according to the IMGT nomenclature (Beckman Coulter anti-TCR Vβ name is indicated in bracket). All T cell lines were derived from PBL, either from healthy donors or from RA patients.

*Percentage determined by TCR sequencing (ref. 28). T cell lines exhibiting alloreactivity are marked in bold.

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Table 3. Analysis of the TCR Vβ repertoire of pp65 495–502/A*0201- or BZLF1/B*3501-sorted T cell lines, using TCR Vβ-specific mAb.
Moreover, experiments using immunodeficient mouse hosts have revealed that human endothelial cells are capable of triggering graft rejection by adoptively transferred alloreactive T cells [38]. This issue is important in transplantation because, unlike hemopoietic APCs, allogeneic vascular endothelium remains in the allograft indefinitely.

Materials and Methods

Ethics Statement

PBL from healthy donors were obtained from the blood bank (EFS Pays de la Loire), which informed the healthy donors about the final use of their blood. Based on their choice regarding the destination of their blood (research vs. medical purpose), healthy donors signed a consent statement. The approval of an ethical committee was thus not necessary. A signed convention was established between our institution (INSERM) and the blood bank (EFS Pays de la Loire) to have access to PBL from healthy donors for research purpose.

T cell lines and culture

T cell lines were derived from PBL, originating either from healthy donors (D01 to D08, and D12) or from patients suffering from arthritis (D09 to D11, and D13 to D16) that were recruited by the department of Rheumatology (Centre Hospitalier de l’Université de Nantes, Nantes, France), as previously described [20]. PBMC were separated by Ficoll density centrifugation (LMS Eurobio). HLA class I genotyping of donors (Table 1) was performed by the Etablissement Français du Sang (Nantes, France).

T cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 1mM L-glutamine and 150 U/mL recombinant IL-2 (hereafter referred to as IL-2/CM). CD8\(^+\) T lymphocytes were positively selected by magnetic cell sorting from PBL using anti-CD8 mAb, then expanded in vitro under nonspecific stimulation in IL-2/CM supplemented with leukoagglutinin at 1 mg/mL, irradiated allogeneic PBL and LCL as described [39]. T cells were maintained for at least 3 weeks without restimulation.

Target cells

A panel of thirty LCL was used. They were generated by exogenous transformation of peripheral B cells with EBV-containing supernatant from the virus-producing B95.8 marmoset cell line and were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS). Human arterial endothelial cells (HAEC) were isolated from renal artery patches collected at the time of kidney transplantation, harvested according to good

Table 4. HLA class I typing of endothelial cells.

| Endothelial cells | HLA-A* | HLA-B* | HLA-Cw* |
|-------------------|--------|--------|---------|
| HAEC 1415         | 01     | 02     | 07      | 08      | 0702 |
| HAEC 3315         | 30     | 31     | 37      | 39      | ND   |
| HAEC 3376         | 24     | 31     | 35      | 60      | ND   |
| HAEC 3643         | 01     | 02     | 15      | 57      | 0602 |
| HAEC 4373         | 02     | 32     | 44      | 55      | ND   |

ND : Not determined.

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medical practice and stored in the DIVAT Biocollection (French Health Minister Project n°2G55), and cultured as described previously [40]. All target cells were typed by HLA class I DNA sequencing (Table 2 and 4).

Immunomagnetic cell sorting

The following antigenic peptides were used: pp65495–503/A*0201 from the HCMV pp65 protein; BZLF154–64/B*3501, BMLF1259–267/A*0201, from lytic EBV proteins (Genosys).

Soluble pMHC monomers were synthesized as previously described [41,42]. They comprised mutated HLA I heavy chains, in vitro expanded sorted T lymphocytes, as described [41,42]. T cells were then expanded in vitro under non-specific stimulation, as described above, and maintained for 3 weeks without restimulation before analysis. The purity of Ag-specific T cells after one sorting was checked by tetramer staining. No major perturbation in the TCR Vβ repertoire is induced by the culture system [43].

Flow cytometric analysis

T cell lines were phenotyped for 24 TCR Vβ expression (JOTest Beta Mark PN IM3497) and anti-CD3-PE-Cy5 mAb (Beckman Coulter). Staining with pp65495–503/A*0201, BZLF154–64/B*3501 or BMLF1259–267/A*0201 tetramers was performed at 4°C with PE-labeled pMHC complexes at 10 μg/mL and anti-CD3-FTTC mAb (Beckman Coulter) for 30 minutes at 4°C, as described previously [41,42]. Double stained cells were analyzed by flow cytometry (BD FACSCalibur; Becton Dickinson). Data analysis was performed using CellQuest Pro software (Becton Dickinson).

Plasmids

Plasmids encoding the following HLA antigens were included in the analysis: A*0101, A*0201, A*0301, A*1101, A*2402, A*3201, A*3402, A*6801, B*0702, B*0801, B*1302, B*1401, B*1516, B*1801, B*2701, B*2702, B*2704, B*2705, B*2706, B*2709, B*3501, B*3508, B*4001, B*4002, B*4102, Cw*0102, Cw*0201, Cw*0302, Cw*0303, Cw*0401, Cw*0501, Cw*0602, Cw*0701, Cw*0702, Cw*0802, Cw*1402, Cw*1501, Cw*1601. Plasmids encoding BMLF1, BZLF1, and pp65 were also used.

Transient transfection of COS-7 cells and TNF-α assay

Transfection of COS-7 cells was performed by the DEAE-dextran-chloroquine method, as described [20]. COS-7 cells were either transfected with 100 ng of cDNA encoding one of the 39 HLA class I-encoding cDNAs, that were available, or cotransfected with 100 ng of an expression vector encoding the protein and 100 ng of an expression vector encoding the restricting HLA allele. Transfected COS-7 cells were tested 48 h after transfection for their ability to stimulate the production of TNF-α by CD8 T cell lines. Briefly, 5×10^3 cells from a CD8 T cell line were added to transfected COS-7 cells, culture supernatants were harvested 6 h later and their TNF-α content was determined by measuring their cytotoxicity on WEHI-164 clone 13 cells in a MTT colorimetric assay [44,45].

Lymphocyte functional assays

Cytotoxicity of T cell lines against LCL or primary cultures of endothelial cells was evaluated in a standard 4h 51Cr-release assay, as previously described [46]. When used, synthetic peptides were directly added to 10^5 Cr-labeled targets and incubated for 1 h before excess unbound peptide was washed off. Results are expressed as percent specific 51Cr lysis = (experimental release - spontaneous release)/(maximum release - spontaneous release)×100%. Maximum and spontaneous release were determined by respectively adding 1% Triton X-100 or medium to target cells in the absence of effector cells. Each test was performed in triplicate at the indicated E:T ratio.

In TNF-α release assays, 5×10^3 T cells were incubated with cultured HAEC (3×10^4 cells/well) generated from different donors and the amount of TNF-α released in the supernatant was estimated after 6 hours by the WEHI-164 cytotoxicity assay [44].

To assess the percentage of alloreactive T cells within a given T cell line, T cells (1.5×10^5 cells/well) were stimulated overnight with allogeneic LCL cells expressing or not the relevant allogeneic HLA allele (E:T = 1:1). Sixteen hours later, cells were double stained by anti-CD25-FTTC (BD Pharmingen) and anti-CD3-APC mAb (Beckman Coulter), and analyzed by flow cytometry using a BD FACSCalibur (Becton Dickinson, san Jose, CA). To determine the TCR Vβ region used by the fraction of alloreactive T cells, the same protocol of stimulation with allogeneic LCL was followed and cells were triple stained by anti-CD3-APC, anti-CD25-FTTC and by anti-Vβ-PE mAb specific to each one of the TCR Vβ used by those T cell lines, (Beckman Coulter).

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Author Contributions

Conceived and designed the experiments: AM BC SB JPS EH ND. Performed the experiments: AM BC BN MB. Analyzed the data: AM SB JPS EH ND. Contributed reagents/materials/analysis tools: BC BN MB. Wrote the paper: AM JPS MB EH ND.

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