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Priming of Allo-HLA-DP-Specific Reactivity from the Naïve T Cell Compartment Is Not Exclusively Mediated by Professional Antigen-Presenting Cells

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Allogeneic (allo) stem cell transplantation is applied to patients suffering from hematologic malignancies to replace the diseased hematopoietic system with cells derived from a donor stem cell graft. The majority of 10/10-matched unrelated donors are HLA-DP-mismatched, and this may result in varying degrees of the graft-versus-leukemia (GVL) effect with or without the occurrence of graft-versus-host disease (GVHD). Allo-HLA-reactive T cells are commonly present in the donor T cell repertoire, and thus a very profound alloreactive immune response can be provoked in the HLA-DP-mismatched setting. The magnitude and the diversity of the allo-HLA-DP-specific immune response likely dictates the balance between the occurrence of GVL and/or GVHD after transplantation. To understand the nature of the allo-HLA-DP-specific immune response provoked under different stimulatory conditions, immune responses were induced from both the naïve and memory T cell compartments using either HLA-DP-mismatched professional antigen-presenting cells (APCs) (monocyte-derived dendritic cells [allo-DCs]) or HLA-DP-mismatched nonprofessional APCs (skin-derived fibroblasts [allo-fibroblasts]) as stimulator cells. In this study, we observed that allo-HLA-DP-reactive T cells could be provoked from both the naïve and memory compartments by both types of APCs. However, the magnitude of the allo-HLA-DP-specific immune response was greater when stimulation was performed with allo-DCs. Moreover, we found that the frequency of allo-HLA-DP-reactive T cells was greater in the naïve T cell compartment compared with the memory T cell compartment, but we observed a comparable lineage specificity of these allo-HLA-DP-specific reactivities. Overall, the data from this study illustrate that the presence of professional APCs of recipient origin will mostly dictate the magnitude of the allo-HLA-DP-specific immune response derived from both the naïve and memory T cell compartments, but does not exclusively mediate the induction of these immune responses.

INTRODUCTION

Patients suffering from hematologic malignancies can be treated with allogeneic stem cell transplantation (alloSCT) [1-4]. The aim of alloSCT is to replace the diseased hematopoietic system of the patient with a healthy donor-derived hematopoietic system [5-9]. Patients and donors are preferentially HLA-matched, but HLA-DP is often not taken into consideration in the HLA-matching procedure, and thus the majority of 10/10- matched unrelated donors are mismatched for 1 or 2 HLA-DP alleles [10-12]. Because no negative thymic selection takes place on non-self HLA alleles in the donor, any peptide that is presented in HLA alleles that are differentially expressed on cells of the donor and patient can potentially induce an alloreactive immune response from the donor T cell repertoire [13,14]. By coincidence, in any normal T cell repertoire, a high number of allo-HLA-reactive T cells are present, because T cells specific for a nonself peptide in the context of self-HLA can cross-react with nonself HLA presenting a self or nonself peptide [15-17].

In the HLA-DP-mismatched setting, a profound immune response can be provoked after alloSCT, resulting in graft-versus-host disease (GVHD) or graft-versus-leukemia (GVL) reactivity [18-20]. After transplantation in the HLA-DP-mismatched setting, the GVL effect may occur due to the presence of (residual) patient hematopoietic antigen-presenting cells (APCs) that induce a strong immune response reactive against HLA-DP-expressing hematologic malignancies. However, under inflammatory conditions, HLA class II expression can be up-regulated on nonhematopoietic cells, leading to GVHD responses by donor-derived allo-HLA-DP-reactive $CD4^+$ T cells [21,22].

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Allo-HLA reactivity is anticipated to be present in both the naïve and memory T cell compartments [13-15,23,24]. It is generally accepted that naïve T cells require priming by professional hematopoietic APCs in the induction phase of the immune response, whereas memory T cells can also be readily activated when exposed to nonprofessional APCs of nonhematopoietic origin [25-28]. The contribution of donor-derived naïve or memory T cells to induction of the allo-HLA-specific immune response in the patient depends on the immunologic history of the donor. In cases with young donors or umbilical cord blood transplantation, the allo-HLA-specific reactivity will be induced primarily from the naïve compartment and therefore is hypothesized to require the presence of professional patient-derived hematopoietic APCs for stimulation [1,3,27,29]. In grafts derived from adult donors, a higher frequency of allo-HLA-reactive T cells is likely to be present in the memory T cell compartment, and memory T cells can be readily activated even in the absence of professional APCs, like in the HLA-DP-mismatched setting, when the nonhematopoietic tissue expresses HLA-DP molecules under inflammatory conditions [23,24]. Differences in how the naïve and memory T cells become activated and also in how they expand in response to professional hematopoietic APCs or nonprofessional nonhematopoietic APCs will influence the outcome of HLA-matched, HLA-DP-mismatched, unrelated alloSCT.

In the present study, we studied the magnitude of allo-HLA-DP-specific immune responses derived from the naïve or memory T cell compartment upon stimulation with either professional hematopoietic APCs (HLA-DP-mismatched monocyte-derived dendritic cells [allo-DCs]) or nonprofessional APCs (HLA-DP-mismatched skin-derived fibroblasts [allo-fibroblasts] pretreated with IFNγ to induce expression of HLA-class II, adhesion, and costimulatory molecules). We observed that both APC types were capable of provoking an allo-HLA-DP-specific immune response from both the memory and naïve T cell compartments. Allo-DCs were found to induce a more profound allo-HLA-DP-specific immune response from both compartments compared with allo-fibroblasts. The frequency of allo-HLA-DP-reactive T cells was higher in the naïve T cell compartment compared with the memory compartment even after stimulation with allo-fibroblasts. Overall, our data illustrate that the nature of the APCs mostly dictates the magnitude of the allo-HLA-DP-specific immune response derived from both the naïve and memory T cell compartments, and that the naïve T cell repertoire contains the highest frequencies of allo-HLA-DP-reactive T cells.

METHODS

Naïve and Memory T Cell Isolation Using Magnetic Cell Separation

Unmanipulated peripheral blood (without previous granulocyte colony-stimulating factor stimulation) was obtained from healthy donors after informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Isopaque separation. CD4 T cells were enriched by untouched magnetic cell separation (MACS) using the CD4 T cell isolation kit with additionally added CD14 beads for more profound monocyte depletion (Miltenyi Biotec, Bergisch Gladbach, Germany). To obtain memory CD4 T cells, naïve and effector cells were depleted using CD45RA beads (Miltenyi Biotec), and to obtain naive T cells, CD4 T cells were untouched isolated using the Naive Pan T Cell Isolation Kit (Miltenyi Biotec). Isolation purities were analyzed by counterstaining with CD45RO-FITC (Invitrogen, Grand Island, NY), CD3-PerCP (BD Biosciences, San Jose, CA), CD45RA-PE (BD Pharmingen, San Diego, CA), and CD27-PE (BD Pharmingen) monoclonal antibodies. Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences), CellQuest (BD Biosciences), and FlowJo (FlowJo, Ashland, OR).

Naïve and Memory T Cell Isolation Using FACS

In a selected number of experiments, we attempted to increase the purity of the isolated naïve and memory T cell populations using another isolation procedure: initial enrichment of CD4 T cells using MACS followed by flow cytometric cell sorting (FACS) of the naïve and memory CD4 T cell subsets using a FACSAria cell sorter (BD Biosciences). First, peripheral blood was obtained from healthy donors after informed consent. PBMCs were isolated by Ficoll-Isopaque separation. CD4 T cells were enriched from total PBMCs using untouched isolation with the CD4 T Cell Isolation Kit (Miltenyi Biotec). The obtained CD4 T cells were labeled with a mix of antibodies needed to set up the dump channel in the gating procedure to deplete unwanted cells: CD8-FITC (BD Pharmingen), CD14-FITC (BD Pharmingen), CD16-FITC (BD Biosciences), CD19-FITC (BD Pharmingen), CD56-FITC (BD Biosciences), TCR-$\gamma\delta$-FITC (BD Biosciences) and CD235-FITC (Invitrogen). For the gating of naïve CD45RA$^+$CD27$^+$ CD4 T cells and memory CD45RO$^+$ CD4 T cells, the following labeling mixture was used: CD4-Pacific Blue (BD Biosciences), CD45RA-PE (BD Pharmingen), CD27-V500 (BD Pharmingen), and CD45RO-APC antibodies (BD Pharmingen).

Generation and Culture of Stimulator Cells

Skin-derived fibroblasts and peripheral blood from patients (pretransplantation) and bone marrow-derived fibroblasts and peripheral blood from healthy donors (for generation of autologous monocyte-derived DCs) were obtained after informed consent was obtained. Fibroblasts were cultured up to 90% confluency in Dulbecco's Modified Eagle's Medium (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS; Bodinco, Alkmaar, The Netherlands) and were pretreated for 3 to 4 days with 200 IU/mL IFN-γ (Boehringer Ingelheim, Rijnland-Palts, Germany) to up-regulate HLA class II expression as described previously [30].

To generate monocyte-derived DC, peripheral blood mononuclear cells (PB-MNC) were isolated using Ficoll-Isopaque separation. Monocyte isolation was performed using magnetic CD14 beads (Miltenyi Biotec) and MACS LS columns (Miltenyi Biotec) according to the manufacturer's instructions. The isolated monocytes were transformed into immature monocyte-derived DCs by culturing for 2 days at a concentration of 2×10^6 cells/mL in Iscove's modified Dulbecco's medium (IMDM; Lonza) containing 10% heat-inactivated human serum (ABOS) supplemented with 100 ng/mL GM-CSF (Novartis Sandoz Pharmaceuticals, Rotkreutz, Switzerland) and 500 IU/mL IL-4 (Schering-Plough, Innishammon, Cork, Ireland). To generate mature DCs, immature DCs were cultured for an additional 2 to 3 days in IMDM/10% ABOS supplemented with 100 ng/mL GM-CSF, 10 ng/mL TNF-α (CellGenix, Freiburg, Germany), 10 ng/mL IL-1β (Sclavo Diagnostics International, Siena, Italy), 10 ng/mL IL-6 (Novartis Sandoz Pharmaceuticals), 1 μg/mL PGE-2 (Sigma-Aldrich/Merck, Darmstadt, Germany), and 500 IU/mL IFN-γ (Boehringer Ingelheim). The phenotypes of fibroblasts and monocyte-derived DCs were analyzed using flow cytometry by labeling with CD54-FITC (Bio-Rad, Hercules, CA), CD80-PE (BD Pharmingen), CD86-FITC (BD Pharmingen), HLA-I-FITC (Bio-Rad), HLA-DP-PE (Bio-Connect, Huissen, The Netherlands), HLA-DQ-PE (Bio-Connect), and HLA-DR-FITC (BD Biosciences).

Both K562 (an immortalized chronic myelogenous leukemia cell line) and HeLa (an immortalized cervical cancer cell line) were cultured in IMDM supplemented with 10% FCS. The cell lines were retrovirally transduced to induce the expression of specific HLA-DP molecules. In short, different HLA-DP alleles were cloned into a pLZRS retroviral vector, and the constructs were verified by sequencing. To retrovirally transduce the different cell lines, Retronectin (recombinant human fibronectin; Takara Bio USA, Mountain View, CA) was used [31]. The expression level of HLA-DP on the transduced cell lines was measured using HLA-DP-PE monoclonal antibodies.

Induction of Allo-HLA-DP-Specific Immune Responses from the Naïve and Memory CD4 T Cell Compartments

Naïve and memory T cells were stimulated with irradiated (25 Gy) HLA-DP-mismatched allo-DCs or HLA-DP-mismatched allogeneic fibroblasts (allo-fibroblasts) at a 10:1 responder T cell:stimulator cell ratio. At the initiation and restimulation of the allo-HLA-DP-specific immune responses, the percentages of CD3$^+$TCR-$\alpha\beta^+$ T cells were measured in the responder populations to determine the correct ratio of stimulation. Flow cytometry was performed using counterstaining with TCR-$\alpha\beta$-FITC (BD Biosciences), CD4-PE (BD Pharmingen), CD3-PerCP (BD Biosciences), and CD8-APC (BD Pharmingen) monoclonal antibodies. The expression level of HLA-DP on the stimulator cells was measured using HLA-DP-PE. Fluorescent events were analyzed using a FACSCalibur flow cytometer and CellQuest and FlowJo software.

Unstimulated and autologous DC-stimulated naïve and memory T cells served as control responses. The cells were cultured in IMDM containing 10% heat-inactivated ABOS supplemented with IL-7 (10 ng/mL; Miltenyi Biotec), IL-15 (.1 ng/mL; Miltenyi Biotec), and IL-2 (50 IU/mL; Novartis Sandoz). After 14 days, the cell cultures were harvested to quantify the expansion of T cells after primary stimulation with allo-DCs or allo-fibroblasts. The cells were counted manually using Eosin Y (Sigma-Aldrich) and a hemocytometer, and fold expansion was quantified by dividing the total number of viable cells at day 14 by the number of viable cells at start of the in vitro responses. To investigate the antigen-specific expansion/maturation, T cells were analyzed by labeling with CD45RO-FITC (Invitrogen), CD3-PerCP (BD Biosciences), CD45RA-PE (BD Pharmingen), and CD27-PE (BD Pharmingen) to assess the

proportion of total T cells displaying a naïve ($CD45RA^+CD27^+$), memory ($CD45RO^+$), or effector ($CD45RA^+CD27^-$) phenotype.

The cell cultures were restimulated at day 14 with either HLA-DP-mismatched DCs or HLA-DP-mismatched fibroblasts. At 24 to 36 hours after restimulation, reactive CD4 T cells were quantified using flow cytometry and clonally isolated by single-cell flow cytometry based on staining with CD4-PE (BD Pharmingen) and CD137-APC (BD Pharmingen) using a FACSAria (BD Biosciences). CD8-AF700 (Thermo Fisher Scientific, Waltham, MA), CD14-FITC (BD Pharmingen), CD16-FITC (BD Biosciences), CD19-FITC (BD Pharmingen), and TCR-$\gamma\delta$-FITC (BD Biosciences) antibodies were used to set up the dump channel for exclusion. Fluorescent events were analyzed and collected using a FACSAria cell sorter, FACSDiva software (BD Biosciences), and FlowJo. The T cell clones were expanded for 2 weeks using an allogeneic feeder mixture consisting of IMDM containing 5% heat-inactivated ABOS and 5% FCS supplemented with 5× irradiated (35 Gy) allogeneic feeder cells, .5× irradiated (60 Gy) allogeneic Epstein-Barr virus-transformed lymphoblastoid cells, 100 IU/mL IL-2, and 800 ng/mL phytohemagglutinin (PHA-HA16; Oxoid, Altrincham, UK). A schematic overview of the complete procedure is shown in Supplementary Figure S1.

Recognition Assay

To investigate the reactivity of the expanded T cell populations at 2 weeks after primary stimulation with either DCs or fibroblasts, their recognition profiles were analyzed using stimulator cells with the expression of matched or mismatched HLA-DP alleles. The T cell populations were stimulated with K562 and HeLa cells transduced with specific HLA-DP alleles at a 10:1 responder:stimulator ratio. Approximately 24 hours later, CD137 upregulation was measured on the CD4 T cells using a FACSCalibur, CellQuest, and FlowJo.

To investigate the allo-HLA-DP-specific reactivity of the sorted and expanded T cell clones, T cells were stimulated with different matched and mismatched HLA-DP-expressing hematopoietic cells (DCs and K562) and with matched and mismatched HLA-DP expressing nonhematopoietic cells (fibroblasts and HeLa cells). The CD4 T cell clones recognizing only stimulator cells expressing the mismatched target HLA-DP allele(s) and not stimulator cells expressing the autologous HLA-DP allele(s) were categorized as allo-HLA-DP reactive. The assay was set up at a 1:5 responder:stimulator ratio for cytokine production and at a 1:3 responder:stimulator ratio when stimulated with fibroblasts and HeLa cells. The supernatants were harvested after overnight incubation of the cells in IMDM/10% ABOS supplemented with 25 IU/mL IL-2, and the amounts of IFNγ and IL-4 in the supernatants were quantified using a standard enzyme-linked immunosorbent assay (ELISA; Sanquin Reagents, Amsterdam, The Netherlands).

RESULTS

Expansion of Naïve and Memory T Cells after Stimulation with HLA-DP-Mismatched DCs or Fibroblasts

Four donor-patient pairs with different HLA-DP mismatches were selected for this study (Table 1): 1 pair with a targeted permissive HLA-DPB1*01:01 mismatch, 1 pair with a targeted nonpermissive HLA-DPB1*03:01 mismatch, and 2 nonpermissive pairs with 2 HLA-DPB1 mismatches, including a permissive approach and a nonpermissive mismatch according to the classification proposed by Fleischhauer et al [32]. To study the allo-HLA-DP-specific immune responses from the naïve and memory T cell compartments, naïve ($CD45RO^-$ or $CD45RA^+CD27^+$) and memory ($CD45RO^+$ or $CD45RA^-$) CD4 T cells were isolated from donor PBMCs using either magnetic cell separation (MACS) or fluorescence-activated cell sorting (FACS), resulting in comparable isolation purities, and stimulated with APCs generated from the corresponding HLA-DP-mismatched patients. The purities after isolation of naïve T cells (94.8% to 99.7%; median, 97.6%) and memory T cells (99.1% to 100%; median, 99.8%) were checked by flow cytometry (Table 1; representative dot plots of the isolated naïve and memory T cells are shown in Supplementary Figure S2). Representative histograms of the expression of adhesion molecule CD54, costimulatory molecules CD80 and CD86, and HLA class I and II molecules are shown for both DCs and fibroblasts in Supplementary Figure S3.

To investigate the magnitude of the allo-HLA-DP-specific immune responses, T cells from both the naïve and memory compartments were exposed to allo-DCs or allo-fibroblasts pretreated with IFN-γ. The magnitude of the T cell responses was quantified by calculating the fold expansion of CD4 T cells at day 14 after initial stimulation with the HLA-DP-mismatched APCs. Both naïve (Figure 1A) and memory (Figure 1B) CD4 T cells expanded by 10- to 20-fold after stimulation with allo-DCs. Upon stimulation with allo-fibroblasts, both naïve (Figure 1C) and memory (Figure 1D) T cells expanded by 2- to 4-fold. Because we previously have illustrated that autologous donor-derived DCs (auto-DCs) are capable of inducing an auto-reactive CD4 T-cell response, the expansion was also analyzed on stimulation with auto-DCs [33,34].

After stimulation with auto- or allo-DCs, naïve (Figure 1A) and memory (Figure 1B) T cells from the single HLA-DP-mismatched responses showed comparable expansion, indicating that a high proportion of the expansion observed upon stimulation with allo-DCs was the result of a non-alloreactive response. When 2 HLA-DP mismatches were present, the responses against allo-DCs were greater than the responses against auto-DCs, except for 1 response from the memory compartment (Figure 1A and B).

The huge expansion of naïve CD4 T cells upon stimulation with HLA-DP-mismatched DCs was unexpected and not in line with the expected allo-HLA-DP-specific T cell frequencies. Therefore, we analyzed whether the observed expansion in the immune responses starting with naive T cells was the result (in part) of an antigen-driven response or cytokine-mediated proliferation. To assess this, the absolute numbers of T cells with a naïve ($CD45RA^+CD27^+$) or antigen-experienced ($CD45RO^+$) phenotype were determined using flow cytometry

Table 1
Overview of the Donor/Patient-Pairs and Isolation Purities of Naïve and Memory T Cells Isolated from Donor PBMCs

Response	Donor			Patient: HLA-DP-typing	Donor-Patient Pair		Donor PBMCs		
	HLA-DP Typing	Sex	Age, yr		HLA-DP Mismatch, GVH Direction	HLA-DP Disparity, GVH Direction	Isolation Method	Naïve T Cell Isolation Purity, %*	Memory T Cell Isolation Purity, %*
1	DPB1*04:01 and DPB1*14:01	Male	28	DPB1*01:01 and DPB1*04:01	DPB1*01:01	Permissive	MACS	94.8	100
2	DPB1*04:01 and DPB1*04:02	Female	49	DPB1*03:01 and DPB1*04:01	DPB1*03:01	Nonpermissive	MACS	97.2	99.8
3	DPB1*02:01:02 and DPB1*04:01:01	Male	22	DPB1*01:01 and DPB1*03:01	DPB1*01:01 and DPB1*03:01	Nonpermissive	MACS	99.2	99.6
4	DPB1*01:01 and DPB1*02:01:02	Male	30	DPB1*03:01 and DPB1*04:01	DPB1*03:01 and DPB1*04:01	Nonpermissive	FACS	98	99.7

* Percentage of the CD4 T cell populations.

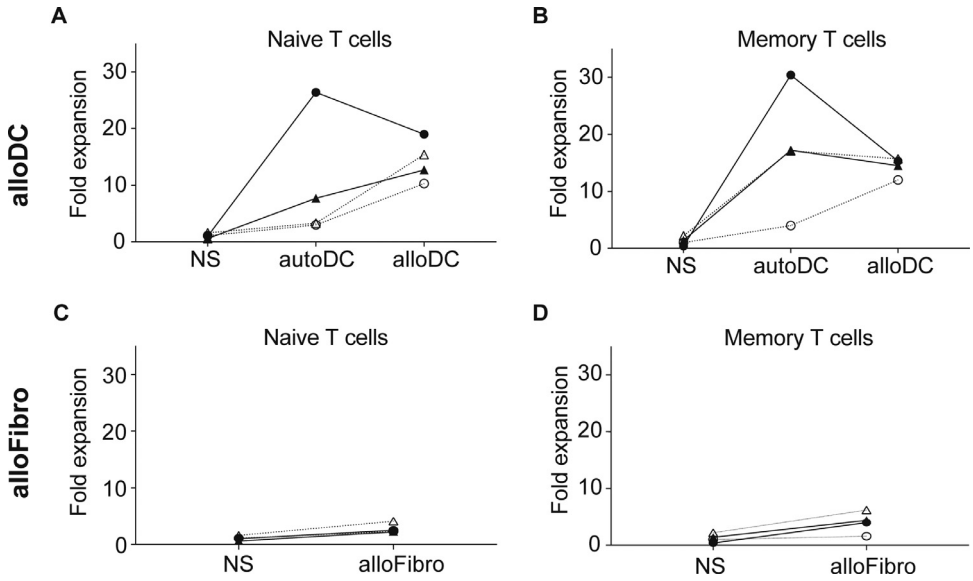

Figure 1. The fold expansion of naïve and memory T cells after stimulation with HLA-DP-matched or -mismatched DCs or fibroblasts. The fold expansion was quantified by dividing the CD4 T cell number at 2 weeks by the CD4 T cell number at day 0. The fold expansion was then compared between stimulation with APCs and no stimulation. The fold expansion of naïve (A) and memory (B) T cells after stimulation with professional APCs, auto-DCs, or allo-DCs and the fold expansion of naïve (C) and memory (D) T cells after stimulation with nonprofessional APCs, skin-derived fibroblasts (allo-fibroblasts). The dashed lines and open symbols represent the responses when 2 HLA-DP mismatches were targeted. Closed circle = response 1; closed triangle = response 2; open circle = response 3; open triangle = response 4. Single analyses are shown of the expanded T cell populations from either the naïve (n = 4) or memory (n = 4) compartment.

for the not stimulated, allo-DC-stimulated, and allo-fibroblast-stimulated conditions at day 14 after initial stimulation (representative dot plots shown in Figure 2A). After stimulation with allo-DCs, only a minority of T cells retained their naïve phenotype (Figure 2B), whereas most expanding T cells markedly acquired an antigen-experienced phenotype (Figure 2C). In contrast, when allo-fibroblasts were used as stimulator cells, the majority of T cells retained their naïve phenotype (Figure 2D) and did not acquire an antigen-experienced phenotype (Figure 2E). Based on the differentiation of the T cells from a naïve into an antigen-experienced phenotype, it can be concluded that in the case of stimulation with allo-DCs, an antigen-driven response is induced. Analysis of the phenotype at 14 days after stimulation of naïve T cells with auto-DCs illustrated that also under these conditions, the vast majority of the naïve T cells had acquired an antigen-experienced phenotype (Supplementary Figure S4). These data illustrate that the induction of coinciding autoreactive, allo-HLA-DP-independent reactivity hindered proper identification of the allo-HLA-DP-specific T cell responses.

HLA-DP-Reactive T Cells Can Be Activated by Both HLA-DP-Mismatched DCs and Fibroblasts

To study the presence of allo-HLA-DP-specific T cells within the immune responses induced by allo-DCs or allo-fibroblasts, we tested the allo-HLA-DP-specific reactivities within the expanded T cell populations. Both hematopoietic (K562) and nonhematopoietic (HeLa) cell lines transduced with matched (auto-HLA-DP, serving as a negative control) or the mismatched HLA-DP alleles were used as stimulator cells. (HLA-DP expression on transduced cell lines is shown in Supplementary Figure S5.) Allo-HLA-DP-specific reactivity was assessed by analysis of activation marker CD137 expression after overnight stimulation with the transduced cell lines. The frequencies of $CD137^+$ T cells within the CD4 T cell population after stimulation with the transduced cell lines were quantified. For the double HLA-DP-mismatched responses, the cumulative reactivities against both mismatched allo-HLA-DP alleles are shown. Similarly, the cumulative reactivities against the auto-HLA-DP allele(s) are shown for all responses.

The T cell populations expanded from the naïve compartment after primary stimulation with allo-DCs showed higher frequencies of $CD137^+$ CD4 T cells after restimulation with K562 expressing the mismatched HLA-DP alleles (K562 allo-DP) compared with restimulation with K562 expressing the autologous/matched HLA-DP alleles (K562 auto-DP; Figure 3A). These differences were more profound for the responses with the double HLA-DP mismatches (open symbols). Similar differences in the frequencies of $CD137^+$ CD4 T cells were found when the same T cell populations were tested against the nonhematopoietic HeLa cell line expressing the mismatched (HeLa allo-DP) versus the autologous/matched HLA-DP alleles (HeLa auto-DP; Figure 3B). For the T cell populations that were initially stimulated with allo-fibroblasts (Figure 3C and D), the frequencies of $CD137^+$ T cells on stimulation with allogeneic stimulator cells was lower than after primary stimulation with allo-DCs. The differences in frequencies of reactive CD4 T cells after stimulation with K562 or HeLa cells expressing the mismatched allo-HLA-DP allele(s) compared with the HLA-DP-matched stimulation controls (auto-HLA-DP) were only visible for the 2 responses where 2 HLA-DP alleles were targeted (open symbols in Figure 3C and D).

The expanded T cell populations from the memory compartment after primary stimulation with allo-DCs showed increased frequencies of $CD137^+$ CD4 T cells after restimulation with K562 allo-DP compared to restimulation with K562 auto-DP (Figure 3E). Only the responses with 2 HLA-DP mismatches (open symbols) showed higher frequencies of $CD137^+$ CD4 T cells when restimulated with HeLa allo-DP compared with restimulation with HeLa auto-DP (Figure 3F). After the initial stimulation with allo-fibroblasts, only very minimal frequencies of $CD137^+$ CD4 T cells were detected when the T cell responses were tested against K562 (Figure 3G) or HeLa (Figure 3H) expressing the mismatched allo-HLA-DP alleles. In addition to

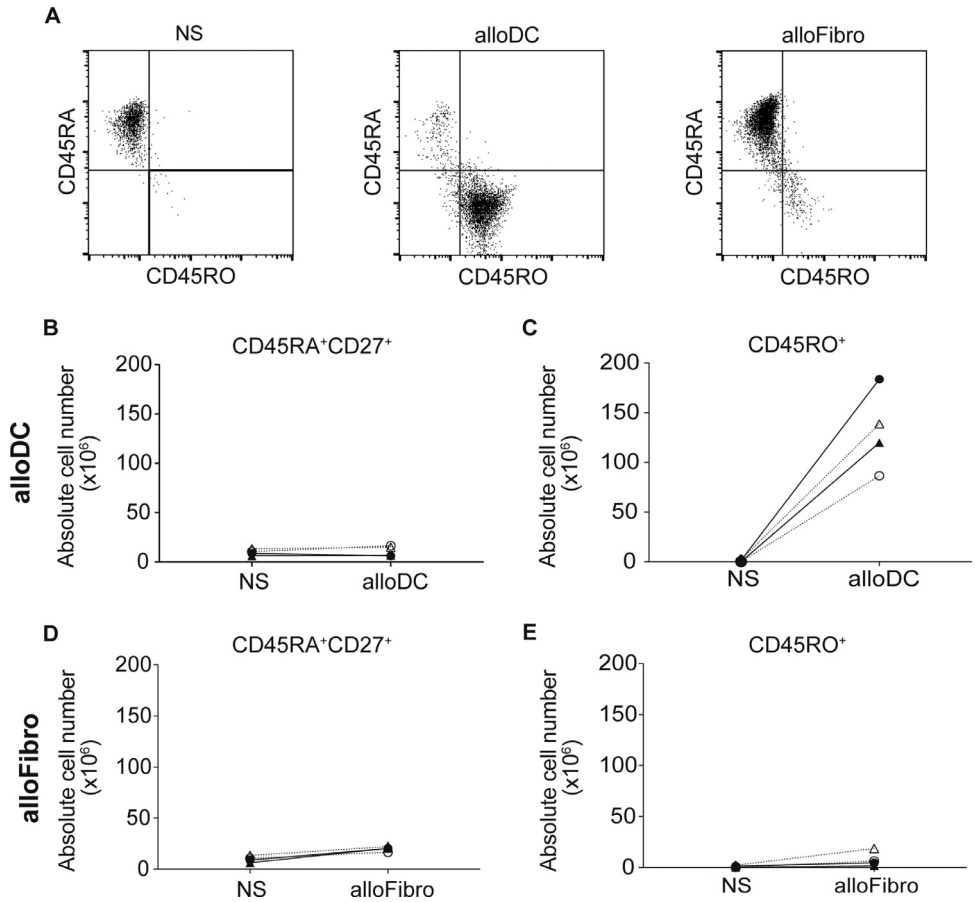

Figure 2. Differentiation of CD4 T cells from a naïve to an antigen-experienced phenotype after stimulation with HLA-DP-mismatched DCs. (A) Representative dot plots showing the naïve (CD45RA) and antigen-experienced (CD45RO) phenotypes of expanded CD4 T cells derived from the naïve compartment without stimulation and after stimulation with HLA-DP-mismatched DCs (allo-DCs) or HLA-DP-mismatched fibroblasts (allo-fibroblasts), respectively. Quantification was performed to obtain the absolute numbers of CD4 T cells with a naïve ($CD45RA^+CD27^+$) phenotype (B) or antigen-experienced ($CD45RO^+$) phenotype (C) in response to stimulation with allo-DCs. The same quantification was done after stimulation with allo-fibroblasts of CD4 T cells with a naïve (D) or antigen-experienced (E) phenotype. The dashed lines and open symbols represent the responses when 2 HLA-DP mismatches were targeted. Closed circle = response 1; closed triangle = response 2; open circle = response 3; open triangle = response 4. These are single analyses of the expanded T-cell populations from the naïve compartment (n = 4).

the cumulative reactivities, the reactivities against the individual autologous/matched and allogeneic/mismatched HLA-DP alleles are shown in Supplementary Figure S6.

From these data, it can be concluded that allo-HLA-DP-specific reactivity could be detected in the bulk T cell populations from both the naïve and memory compartments when primary stimulation was performed with allo-DCs. After primary stimulation with allo-fibroblasts, the allo-HLA-DP-specific reactivity was clearly shown only in the case of the double HLA-DP-mismatched responses from the naïve T cell populations and could not be clearly visualized in the responses derived from the memory T cell populations. The overall frequencies of allo-HLA-DP-reactive CD4 T cells were higher in the expanded T-cell populations from the naïve compartment compared with those from the memory compartment. In line with the expansion results, the hematopoietic allo-DCs were capable of provoking a more profound immune response than the nonhematopoietic allo-fibroblasts, as was similarly observed when restimulation was performed with the hematopoietic K562 cell line versus the nonhematopoietic HeLa cell line.

Both HLA-DP-mismatched DCs and fibroblasts provoked allo-HLA-DP-specific T cell responses with differential lineage recognition profiles from both the naïve and memory T cell compartments.

To further determine the allo-HLA-DP-restricted specificities within the expanded T cell populations from both the naïve and memory compartments under the different stimulation conditions, we analyzed the reactivities of sorted CD4 T cell clones against hematopoietic versus nonhematopoietic cells. The activated ($CD137^+$) CD4 T cell clones were obtained by single-cell/well FACS sorting at 36 hours after restimulation with either allo-DCs or allo-fibroblasts. After in vitro expansion, the CD4 T cell clones were tested in a recognition assay measuring the production of IFN-γ and IL-4 by ELISA after stimulation with different stimulator cells.

Within all in vitro responses (both single and double HLA-DP-mismatched responses) from both the naïve and memory compartments, allo-HLA-DP-reactive CD4 T cell clones showing different lineage recognition patterns were present. A detailed overview of the reactivities of the T cell clones derived from the individual T cell responses is presented in Supplementary Table S1). The allo-HLA-DP-restricted reactivity of a T cell clone was defined by the recognition of stimulator cells expressing only the allo-HLA-DP-allele(s). The majority of T cell clones showed broad recognition (representative T cell clones with this recognition profile shown in Supplementary Figures S7A for IFN-γ and S8A for IL-4) of both hematopoietic cells (DCs and/or K562) and nonhematopoietic cells (fibroblasts and/or HeLa cells) expressing the mismatched HLA-DP

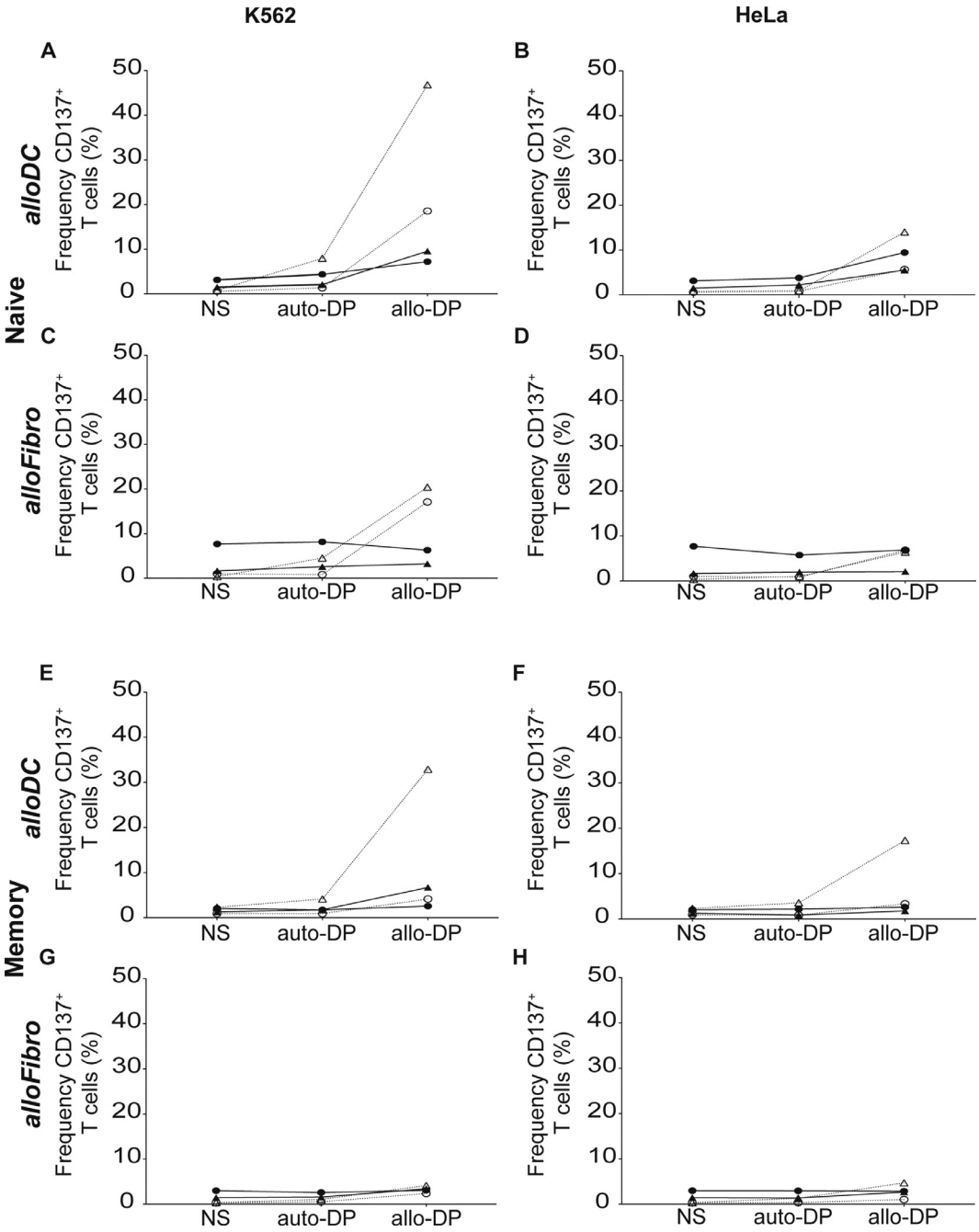

Figure 3. Allo-HLA-DP-specific reactivity in the expanded T cell populations. The allo-HLA-DP-specific reactivities were analyzed at 2 weeks after primary stimulation with either HLA-DP-mismatched DCs (allo-DCs) or HLA-DP-mismatched fibroblasts (allo-fibroblasts) using an assay with hematopoietic (K562) and nonhematopoietic (HeLa) cell lines transduced with either the matched (auto-DP) or mismatched (allo-DP) allele(s). The cumulative reactivities to auto-DP or allo-DP allele(s) are illustrated by the frequencies of $CD137^+$ T cells within the CD4 T cell populations. The figure shows the reactivities of the expanded T cell populations (n = 4) from the naïve compartment after primary stimulation with allo-DCs when tested against K562 (A) and HeLa (B) cells and of the expanded T cell populations after primary stimulation with allo-fibroblasts when tested against K562 (C) and HeLa (D) cells. In addition, the frequencies of $CD137^+$ T cells are shown for the expanded T cell populations (n = 4) from the memory compartment after primary stimulation with allo-DCs when tested against **E.** K562 (E) and HeLa (F) cells and of the expanded T cell populations after primary stimulation with allo-fibroblasts when tested against K562 (G) and HeLa (H) cells. The dashed lines and open symbols represent the responses when 2 HLA-DP mismatches were targeted. Closed circle = response 1; closed triangle = response 2; open circle = response 3; open triangle = response 4. Single analyses were performed for all the expanded T cell populations.

alleles. The second group of T cell clones recognized only nonhematopoietic cells (fibroblasts and/or HeLa cells expressing the mismatched HLA-DP alleles); representative T cell clones with this recognition profile are shown in Figures S7B (for IFN-γ) and S8B (for IL-4). The third group of T cell clones recognized only hematopoietic cells (DCs and/or K562 cells expressing the mismatched HLA-DP alleles); representative recognition profiles are shown in Supplementary Figures S7C and D and S8C and D. The stimulation with allo-DCs resulted in a trend toward greater enrichment of allo-HLA-DP-reactive T cells with specificity for hematopoietic cells, along with an enrichment of T cells specific for nonhematopoietic cells after stimulation with allo-fibroblasts (Supplementary Table S1).

Table 2
Frequencies of CD4 T Cell Clones with Different Recognition Profiles*

Primary Stimulation	T Cell Compartment†	Allo-HLA-DP-Specific T Cell Clones, n	Hematopoietic and Nonhematopoietic HLA-DPB1-Expressing Cells, %‡	Nonhematopoietic HLA-DPB1-Expressing Cells, %‡	Hematopoietic HLA-DPB1- Expressing Cells, %‡
Allo-DCs	Naïve	385	73.2	1.0	25.7
	Memory	285	70.2	2.1	27.7
Allo-fibroblasts	Naïve	121	66.1	19.0	14.9
	Memory	75	66.7	5.3	28.0

* Quantification is based on 3 independent analyses of the T cell clones.
† Cumulative data of 4 responses for each condition.
‡ Percentage of total allo-HLA-DP-specific CD4 T cells.

The cumulative numbers of T cell clones from the naïve and memory compartments under the different stimulation conditions are shown in Table 2. These data are in line with our previous finding that allo-HLA-DP-specific T cells can be provoked from both the naïve and memory compartments independent of the type of APC used, although the magnitude of the immune responses differs between the responses, as illustrated by the number of allo-HLA-DP reactive T cell clones. The representation of the different lineage-specificities within the allo-HLA-DP T cell repertoire is similar in the naïve and memory T cell compartments. From the total number of CD4 T cell clones (Supplementary Table S1 and Table 2), it can be observed that also on a clonal level, the allo-HLA-DP-specific response was higher in the double HLA-DP mismatched responses, higher for the naïve compartment compared with the memory compartment, and higher after stimulation with allo-DCs compared with stimulation with allo-fibroblasts.

DISCUSSION

In this study, we analyzed the magnitude of allo-HLA-DP-specific immune responses derived from the naïve or memory T cell compartment upon stimulation with either professional hematopoietic APCs (HLA-DP-mismatched monocyte-derived DCs [allo-DCs]) or nonprofessional APCs (HLA-DP-mismatched skin-derived fibroblasts [allo-fibroblasts], pretreated with IFN-γ). We observed a profound expansion of naïve and memory T cells in response to stimulation with allo-DCs and limited expansion of naïve and memory T cells upon stimulation with allo-fibroblasts. We demonstrated that the observed expansion was caused mainly by a non-alloreactive antigen-driven response after allo-DC or auto-DC stimulation. Further analyses showed that both allo-DCs and allo-fibroblasts were capable of provoking allo-HLA-DP reactivity from both the naïve and memory T-cell compartments. The allo-HLA-DP-specific immune responses were more profound when stimulation was performed with allo-DCs versus with allo-fibroblasts, and the frequency of the allo-HLA-DP-reactive T cells was higher in the T cell population derived from the naïve compartment compared with that derived from the memory compartment, even after stimulation with nonhematopoietic APCs.

Clinical outcome after HLA-DP-mismatched alloSCT has been shown to be influenced by the magnitude and specificity of the allo-HLA-DP-specific immune response [3,18-22,31]. The magnitude of the allo-HLA-DP-specific response has been shown to differ depending on the HLA-DP expression profiles of the donor-patient pairs. Permissive HLA-DP mismatches are predicted to induce a tolerable T cell response with a lower amplitude, whereas nonpermissive HLA-DP mismatches are anticipated to induce stronger T cell responses [32,35-37]. In this project, we were able to include only 1 permissive donor-patient pair, so a difference between the permissive and nonpermissive HLA-DP mismatches cannot be concluded. However, there was a clear difference between the single and double HLA-DP-mismatched responses in our analyses, demonstrating more profound allo-HLA-DP-specific reactivity when 2 allogeneic HLA-DP alleles were targeted within 1 response. This finding is in line with the clinical observations that multiple mismatches at the low-expression HLA loci, including HLA-DP, are associated with worse clinical outcome [10].

The specificity of the allo-HLA-DP-specific immune response has been shown to result in different clinical outcomes. For some patients, profound HLA-DP-specific CD4 T cell responses were induced resulting in GVHD by targeting inflamed HLA-class II-expressing nonhematopoietic tissues [22]. The patient from which we used material for the in vitro response 3 in our study has been described previously. This patient received a prophylactic CD4-selected donor lymphocyte infusion after transplantation and developed severe acute GVHD coincided by expansion of CD4 T cells targeting both mismatched HLA-DP alleles that were up-regulated on the nonhematopoietic cells [22]. However, in other patients, it has been found that the allo-HLA-DP-specific CD4 T cell response induced a beneficial GVL effect [21,31]. These clinical observations indicate that also the conditions within the patient determine the clinical outcome. It is not possible to deduce the origin of the stimulator cell(s) that initiate(s) the immune response in the clinical setting. This kind of information can only come from in vitro analyses, like ours.

After alloSCT, the presence or absence of residual patient hematopoietic APCs and the inflammatory status of the patient dictate the magnitude and the diversity of the immune response from the donor T cell repertoire. Allo-HLA reactive T cells are anticipated to be derived from both the naïve and memory T cell compartments, depending on the donor's immunologic history [13-15,23,24]. It can be assumed that in cases with a young donor or an umbilical cord blood transplantation, the allo-HLA-DP-specific reactivity will be induced primarily from the naïve compartment, whereas in case of adult donors, a higher frequency of allo-HLA-DP-reactive T cells is likely to be present in the memory T cell compartment. In the clinical setting, worse outcomes have been reported when grafts from older donors were used [38,39].

Naïve and memory T cells differ in how they become activated and also in how they expand in response to either professional hematopoietic APCs or nonprofessional nonhematopoietic APCs. It is generally thought that the naïve T cell compartment is composed of a broad and diverse T cell repertoire that has never been exposed to antigens and requires the presence of professional hematopoietic APCs to become activated [40]. After expansion and the contraction phase, a subset of antigen-experienced T cells becomes the more restricted memory T cell repertoire [1,3,27,29]. The relative contribution of naïve and memory T cells in the balance of GVL and GVHD and the possible clinical application of this knowledge remains

a challenge. The present study demonstrates that priming of allo-HLA-DP-specific reactivity from the naïve T cell compartment is not mediated exclusively by professional hematopoietic APCs, but can also be induced by nonprofessional, nonhematopoietic APCs like HLA-II-expressing skin-derived fibroblasts, although resulting in an immune response with a lower magnitude.

Previous studies have suggested that because of the much more diverse T cell repertoire in the naïve compartment, alloreactive T cells reside primarily in this T cell subset, and thus the depletion of the naïve T cells would prevent GVHD. Indeed, when the naïve and memory T cells were tested in different in vitro analyses, greater alloreactivity was found in the naïve T cell subsets [28,41-44]. We made the same observation in the present study. Several clinical trials have investigated the safety and efficacy of CD45RA-depleted grafts [45-48]; however, the clinical effectiveness could not be determined from the data, although some of the in vivo and also in vitro studies indicate a reduction of GVHD reactivity. It could be hypothesized that after alloSCT, when professional hematopoietic APCs of recipient origin are absent or present only in low numbers, memory T cells are the major contributors to an alloimmune response, because they also can be more readily activated by nonprofessional APCs. However, our data illustrate induction of allo-HLA-DP-specific T cell responses of similarly low magnitude provoked from both the naïve and memory compartments on stimulation with nonhematopoietic allo-HLA-DP-expressing APCs.

In summary, the magnitude and diversity of allo-HLA-DP-specific immune response and the balance between the induction of GVL and GVHD after HLA-DP-mismatched alloSCT is dictated by the interplay between donor T cell composition and numbers, the presence of professional APCs of recipient origin, and the presence of an inflammatory environment in the patient.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.bbmt.2020.03.001.

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