Alloreactive and leukemia-reactive T cells are preferentially derived from naïve precursors in healthy donors: implications for immunotherapy with memory T cells

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ABSTRACT

Background
HLA mismatch antigens are major targets of alloreactive T cells in HLA-incompatible stem-cell transplantation, which can trigger severe graft-*versus*-host disease and reduce survival in transplant recipients. Our objective was to identify T-cell subsets with reduced *in vitro* reactivity to allogeneic HLA antigens.

Design and Methods
We sorted CD4 and CD8 T-cell subsets from peripheral blood by flow cytometry according to their expression of naive and memory markers CD45RA, CD45RO, CD62L, and CCR7. Subsets were defined by a single marker to facilitate future establishment of a clinical-grade procedure for reducing alloreactive T-cell precursors and graft-*versus*-host disease. T cells were stimulated in mixed lymphocyte reactions against HLA-deficient K562 cells transfected with single HLA-A/-B/-C/-DR/-DQ mismatch alleles. Alloreactivity was measured by interferon-γ spot production and cell proliferation.

Results
We observed that allogeneic HLA-reactivity was preferentially derived from subsets enriched for naïve T cells rather than memory T cells in healthy donors, irrespective of the HLA mismatch allele. This separation was most efficient if CD45RA (*versus* other markers) was used for sorting. The numbers of allogeneic HLA-reactive effector cells were in median 7.2-fold and 16.6-fold lower in $CD45RA^{neg}$ memory CD8 and CD4 T cells than in entire CD8 and CD4 T cells, respectively. In contrast, proliferation of memory T cells in response to allogeneic HLA was more variably reduced (CD8) or equivalent (CD4) when compared to that of naïve T cells. We also demonstrated in HLA-matched donor-patient pairs that leukemia-reactive CD8 cytotoxic T-lymphocytes were mainly derived from subsets enriched for naïve T cells compared to memory T cells.

Conclusions
Memory T-cell subsets of most healthy individuals showed decreased allogeneic HLA-reactivity, but lacked significant anti-leukemia responses *in vitro*. The clinical use of memory or naïve-depleted T cells might be beneficial for HLA-mismatched patients at high risk of graft-*versus*-host disease and low risk of leukemia relapse. Preferred allografts are those which contain leukemia-reactive memory T cells. Alternatively, replenishment with leukemia-reactive T cells isolated from naïve subsets is desirable.

Key words: alloreactivity, leukemia-reactive T cells, T-cell subsets, naïve T cells, immunotherapy.

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The online version of this article has a Supplementary Appendix.

Introduction

Separation of graft-*versus*-leukemia from graft-*versus*-host immune reactions remains the major challenge in allogeneic hematopoietic stem-cell transplantation (HSCT).[1] Both effects are mainly mediated by T cells that are contained in donor-derived stem-cell grafts and lymphocyte products with a very broad repertoire of allospecificities.[2] Therefore, refined *in vitro* strategies have been developed that selectively deplete graft-*versus*-host-reactive (i.e. alloreactive) donor T cells while sparing beneficial reactivity of T cells to infectious agents and leukemia.[3] Although several of these approaches have already been demonstrated to be feasible and produce significant reductions of graft-*versus*-host disease (GvHD) in clinical trials,[4-8] selective depletion of alloreactive T cells is still a complex technical approach and requires the *in vitro* culture of donor T cells for several days.

A less complex, culture-independent approach for GvHD prophylaxis would be to eliminate naïve T cells from the allograft, because they should contain most alloreactive precursors due to the enormous diversity of the naive T-cell receptor (TCR) repertoire.[9] Studies in mice have established this concept by demonstrating a much higher rate of GvHD if naive T cells compared to memory T cells of unprimed animals were adoptively transferred into allogeneic recipients.[10-12] *In vitro* experiments with human T cells confirmed that the $CD62L^{pos}$ subset containing naïve and central memory T cells showed stronger alloresponses than the $CD62L^{neg}$ effector memory counterpart.[13] These data, however, raise important questions. First, which marker(s) of T-cell differentiation should be used clinically for depleting naïve T cells with the aim of minimizing alloreactive precursors in human allografts? Second, is the superior alloresponse of naïve precursors similar in CD4 and CD8 T-cell subsets? Lastly, will residual memory T cells still mediate reactivity to leukemia?

Naïve T cells have the phenotype $CD45RA^{pos}$ $CD45RO^{neg}$ $CCR7^{pos}$ $CD62L^{pos}$; CCR7 and CD62L are also expressed on central memory T cells.[14,15] We, therefore, decided to investigate *in vitro* alloreactivity of CD4 and CD8 T-cell subsets which were enriched for naïve (i.e. $CD45RA^{pos}$ and $CD45RO^{neg}$) as well as for naïve and central memory T cells (i.e. $CCR7^{pos}$ and $CD62L^{pos}$) by flow cytometric cell sorting. The counterpart fractions mainly containing memory T cells as well as entire CD4 and CD8 T cells were also included in the experiments. Because alloreactivity is very complex and can be directed to a very diverse panel of major and minor histocompatibility antigens mismatched between donor and recipient, we chose single non-self-HLA (allo-HLA) molecules as surrogate alloantigens. To detect pure alloreactive T-cell responses against an individual HLA mismatch allele and to minimize the interference by other T-cell specificities, HLA-deficient K562 cells were applied as standard recipient cells and were transfected with single HLA class I (-A/-B/-C) or class II (-DR/-DQ) molecules before use.

Design and Methods

Primary cells and cell lines

This study was approved by the local Ethics Committee and was performed according to the Declaration of Helsinki. Healthy peripheral blood mononuclear cell (PBMC) donors were selected based on their HLA type (*Online Supplementary Table S1*). PBMC were isolated from buffy coats by Ficoll separation. Primary AML blasts were separated by Ficoll centrifugation from leukapheresis products of patients with a white blood cell count greater than $10^5/\mu L$.

The K562 cell clone transfected with HLA-A*02:01 cDNA has been previously described.[16] Similarly produced K562 cell clones for HLA-B*35:03 and HLA-C*03:03 were provided by Dr. T. Wölfel (Mainz, Germany). Cells showed stable HLA transgene expression in G418-containing medium [median positive cells, 83 (44-98) %]. K562-HLA class II transfectant cells were generated by mRNA transfection. Briefly, 2×10^7 K562 cells were electroporated (400V, 5ms; Gene Pulser Xcell™, Bio-Rad, Hercules, CA, USA) with 10 μg RNA encoding the α and β chains of HLA-DR (DRA1*01:01, DRB1*07:01) and HLA-DQ (DQA1*01:02, DQB1*06:02) molecules, respectively. RNA was synthesized by *in vitro* transcription from pST1 cDNA vectors containing full-length HLA class II genes (provided by Dr. U. Sahin, Mainz, Germany) according to the manufacturer's instructions (mMESSAGE mMACHINE T7 Ultra, Ambion, Austin, TX, USA). The procedure resulted in transient HLA class II expression for up to 1 week with reliably strong levels 12 h after electroporation [median positive cells, 70 (22-85) %]. Aliquots of K562-HLA class II transfectant cells were frozen and used directly after thawing and lethal irradiation (100 Gy). Epstein-Barr virus-transformed B-lymphoblastoid cell lines were generated according to standard *in vitro* procedures.

Cell sorting and allostimulation of CD8 and CD4 T-cell subsets in vitro

Cryopreserved PBMC were thawed and stained with fluorochrome-conjugated monoclonal antibodies for CD3 and CD8 or CD4, in combination with those for CD45RA, CD45RO, CCR7, or CD62L (Beckman Coulter, Brea, CA; BD Biosciences, San Jose, CA, USA; R&D Systems, Minneapolis, MN, USA). After washing, cells were sorted on a BD FACSAria cell sorter (BD Biosciences) into the indicated T-cell subsets (Figure 1C). Allogeneic mixed lymphocyte reactions (MLR) were performed at 37°C in AIM-V medium (GIBCO-BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated human serum. At day 0 (d0), 5×10^5 sorted CD8 or CD4 T cells were stimulated with 5×10^4 lethally irradiated mismatched K562-HLA transfectant cells in 48-well plates. Irradiated (35 Gy) autologous PBMC (5×10^5/well) were added as feeder cells. MLR responder populations were re-stimulated weekly with irradiated K562-HLA cells at a responder:stimulator ratio of 10:1. Cultures contained 10 ng/mL human interleukin (hIL)-7, 1 ng/mL hIL-12, and 5 ng/mL hIL-15 (all from Miltenyi Biotec, Bergisch-Gladbach, Germany) during the first week. From d7 onwards, IL-12 was omitted, and hIL-2 was added at 100 IU/mL (ProleukinTM; Novartis, Nürnberg, Germany).

For the generation of leukemia-reactive CD8 T-cell lines, 5×10^5 cells of sorted CD8 T-cell subsets from healthy donors were stimulated in allogeneic mixed lymphocyte-leukemia cultures (MLLC) with 5×10^5 irradiated (35 Gy) HLA class I-matched primary acute myeloid leukemia (AML) blasts. Irradiated autologous PBMC (5×10^5/well) of donor origin were added as feeder cells. MLLC were supplemented with the same cytokines as described for the MLR. Additionally, 10 ng/mL hIL-21 (Biomol, Hamburg, Germany) was added to improve *in vitro* priming of leukemia-reactive T-cell precursors.[17] Cultures were re-stimulated weekly with irradiated AML blasts at a responder:stimulator ratio of 1:1.

T-cell expansion was measured by counting viable cells every week with trypan blue staining. Statistical analysis between different experimental arms was performed with SPSS15.0 software. Wilcoxon's signed-rank test was used to calculate *P* values.

Flow cytometric analysis

Cells were incubated for 20 min at 4°C with fluorochrome-conjugated monoclonal antibodies (Beckman Coulter, BD Biosciences, R&D Systems). Staining was analyzed on a BD FACSCanto flow cytometer (BD Biosciences). After gating on viable lymphocytes, 10^4 events were evaluated by BD FACSDiva™ software (BD Biosciences) and EXPO32™ software (Beckman Coulter) for re-analysis. To determine the percentage of regulatory T cells (T_{reg}) in CD4 T cells, co-staining for CD25, CD127 (Beckman Coulter), and FOXP3 (eBioscience, San Diego, CA, USA) was performed.

Interferon-γ enzyme-linked immunosorbent spot assay

Twenty-hour interferon-γ (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assays were performed as described previously.[18] T cells were seeded at 1×10^3 to 4×10^4/well and target cells at 5×10^4/well in AIM-V medium. To demonstrate HLA-restricted T-cell reactivity, the following murine monoclonal antibodies were added:[19] W6/32, an anti-HLA class I IgG2a, PA2.1, an anti-HLA-A2 IgG1, B1.23.2, an anti-HLA-B and -C IgG2a, L243, an anti-HLA-DR IgG2a, and SPV-L3, an anti-HLA-DQ IgG2a.[20] Allo-HLA reactivity of MLR cultures was determined by subtraction of spot numbers in wells with parental K562 cells from those with K562-HLA transfectant cells. The results shown are means ± standard deviation (SD) of duplicate experiments. Statistical analysis was performed with SPSS15.0 software and Wilcoxon's signed-rank test.

^{51}Chromium-release assay

^{51}Chromium-release assays with an effector-to-target (E/T) incubation of 5 h were conducted as previously described.[19] Data shown data are means ± SD of duplicate experiments.

Results

Measuring alloreactivity of CD8 T-cell subsets to single HLA mismatch alleles

We separated CD8 T cells from PBMC of healthy donors by flow cytometric sorting according to the strong or absent expression of CD45RA, CD45RO, CD62L, or CCR7, respectively (Figure 1). Eight different CD8 T-cell subsets with a median purity of 98.7 (77.1-100) % (n=56) were obtained and were analyzed for alloreactivity to single HLA-A, HLA-B, and HLA-C mismatch alleles by allogeneic MLR *in vitro*. Sorted total CD8 T cells were also included in the MLR experiments. For *in vitro* stimulation against allo-HLA, we used HLA-deficient K562 cells expressing single HLA molecules (K562-HLA) after transfection. MLR populations were regularly screened for allo-HLA-reactive CD8 T cells by IFN-γ ELISpot assay on d12 of cultures. We detected HLA-mismatch reactivity primarily in MLR that were initiated with CD8 subsets enriched for naïve T cells (Figure 2A). Recognition was clearly restricted to the allo-HLA mismatch allele used during primary *in vitro* stimulation. This was demonstrated by including parental K562 cells and K562 transfectant cells expressing third-party HLA class I alleles as control targets in all screening assays. In addition, allorecognition could be regularly blocked by monoclonal antibodies binding to the allo-HLA stimulator allele (Figure 2A).

Superior allogeneic HLA reactivity in subsets enriched for naïve compared to memory CD8 T cells

We next used the MLR-ELISpot test system to screen subsets enriched for naïve or memory CD8 T cells for reactivity to single HLA-A/-B/-C mismatch alleles in six different donors (for HLA types see *Online Supplementary Table S1*). We observed that much higher numbers of HLA mismatch-reactive CD8 T cells were derived from the $CCR7^{pos}$ subset than from the $CCR7^{neg}$ counterpart [median IFN-γ spot-forming cells (SFC) 2500 *versus* 74 per 10^4 MLR responder cells; Figure 2B,C]. This difference was detected in all donor/allo-HLA combinations tested (P=0.008; Figure 2C). Other subsets enriched for naïve CD8 T cells (i.e. $CD45RA^{pos}$, $CD45RO^{neg}$, $CD62L^{pos}$) were also more responsive to allo-HLA stimulation than related memory fractions, with the difference being statistically significant for CD45RA (P=0.011) and CD45RO (P=0.005). The median level of allo-HLA reactivity was higher for the HLA-B/-C used than for the HLA-A mismatch alleles (2000 *versus* 556 SFC per 10^4 MLR responders for all naïve-enriched CD8 subsets). In single subsets enriched for memory CD8 T cells, however, alloreactivity could exceed that of naïve counterparts (e.g. Don 804/A02 for CD62L). Most importantly, the alloreactivity of MLR cultures derived from memory-enriched CD8 subpopulations was considerably lower than that of cultures of entire CD8 T cells. Median numbers of SFC per 10^4 MLR/allo-HLA I responder cells were 74 (range, 15-219) for memory $CCR7^{neg}$ CD8 T cells, 79 (range, 6-484) for memory $CD45RA^{neg}$ CD8 T cells, and 350 (range, 5-3500) for entire CD8 T cells.

As a further parameter of the alloreactive CD8 T-cell response, we measured the expansion of MLR populations during 3 weeks of *in vitro* stimulation with single HLA class I mismatch alleles. Again, 11 donor/allo-HLA combinations were analyzed. The strongest alloproliferation was observed for $CD62L^{pos}$ and $CCR7^{pos}$ fractions (median d21: 12.9×10^7 and 9.7×10^7 cells), respectively (*Online Supplementary Figure S2A,B*). In contrast, the $CD62L^{neg}$ and $CCR7^{neg}$ counterparts showed significantly lower cell expansion (median d21: 3.6×10^7 and 1.7×10^7 cells; P=0.015 and P=0.037). Similar differences in alloproliferation were not found for CD45RA and CD45RO subsets. We also analyzed the expression of T-cell differentiation markers in all MLR populations on d14 of culture by flow cytometry. As expected, subsets enriched for naïve CD8 T cells retained expression of early differentiation markers in a higher proportion of MLR responders compared to memory counterparts (*data not shown*).

Stronger leukemia reactivity in $CCR7^{pos}$ compared to $CCR7^{neg}$ CD8 T cells

Because the $CCR7^{pos}$ CD8 T-cell subset of healthy individuals contained most allo-HLA-reactive effector cells (Figure 2), depletion of this compartment from entire donor lymphocytes would potentially reduce graft-*versus*-host-reactivity of HLA-mismatched allografts. However, elimination of donor $CCR7^{pos}$ CD8 T cells may also influence T-cell reactivity to leukemia cells restricted by HLA alleles matched between the donor and leukemia patient. We, therefore, investigated sorted $CCR7^{pos}$ and $CCR7^{neg}$ CD8 T cells of three healthy donors for reactivity to primary AML blasts in donor-patient pairs with complete HLA match by allogeneic MLLC. In parallel experiments, reactivity of both subsets to K562 cells expressing a single HLA class I mismatch allele was also analyzed by MLR. We observed high numbers of AML-reactive CD8 T cells in $CCR7^{pos}$-derived MLLC, both in HLA-matched sibling

and unrelated donors (Figure 3A). Responding CD8 T cells showed cytolytic activity against AML blasts, but did not lyse natural killer target K562. A lower level of lysis was observed against patient-derived B-lymphoblastoid cell lines. In contrast, we did not detect AML-reactive CD8 T cells in CCR7^{neg}-derived MLLC. As expected from our previous data, allo-HLA reactivity mainly resided in CCR7^{pos} fractions (Figure 3B). Similar MLLC results were obtained if CD45RO was used for sorting naïve and memory CD8 T cells (*data not shown*).

Separation of allogeneic HLA reactivity in subsets enriched for naïve and memory CD4 T cells

CD4 T cells of six healthy donors were sorted into subsets enriched for naïve or memory T cells using a strategy analogous to that described for CD8 T cells (Figure 1), with a median purity of 99.8 (82.8-100) % for all fractions (n=48). The subsets were stimulated weekly in MLR with K562 cells expressing single HLA-DR/-DQ mismatch alleles in nine donor/allo-HLA combinations (*Online Supplementary Table S1*). Again, allorecognition was measured by IFN-γ ELISpot assay in the second week of cultures (d12). We observed that the numbers of allo-HLA class II reactive T cells were usually higher in subsets enriched for naïve T cells than in those enriched for the memory counterparts (Figure 4B-C). Statistical significance was found for the CD62L (P=0.008) and CD45RA (P=0.011) subsets. The median level of mismatch reactivity was clearly higher for the used HLA-DR than for the HLA-DQ allele (601 *versus* 93 SFC per 10^4 MLR responders for all naïve CD4 subsets). In all experiments, allo-HLA class II reactivity could be blocked by monoclonal antibodies binding to the class II molecule used for primary *in vitro* stimulation (Figure 4A). Furthermore, significant alloresponses to parental K562 cells as well as to transfectants carrying third-party HLA alleles were not observed, again demonstrating the specificity of the MLR/K562-HLA screening approach. As for the CD8 experiments, alloreactivity of MLR cultures derived from memory-enriched CD4 T-cell subsets was substantially lower than that of total CD4 T cells. The median numbers of SFC per 10^4 MLR/allo-HLA II responder cells were 25 (range, 2-1237) for memory CD45RA^{neg} CD4 T cells, 46 (range, 6-644) for memory CD62L^{neg} CD4 T cells, and 287 (range, 4-1204) for

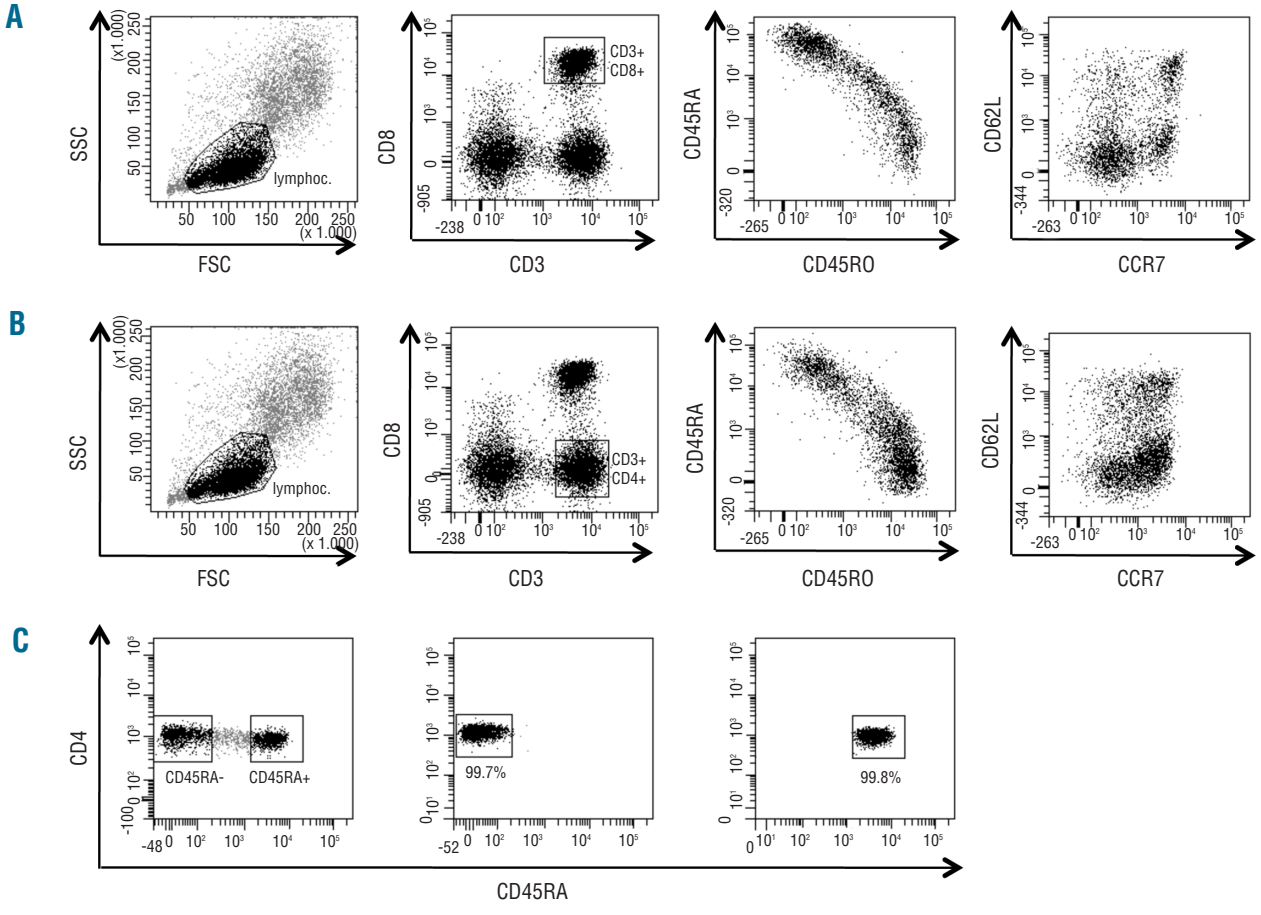

Figure 1. Flow cytometric staining and sorting of T-cell subsets. PBMC from healthy donors were stained with monoclonal antibodies for CD3 and CD8 together with monoclonal antibodies for T-cell differentiation markers (i.e. CD45RA, CD45RO, CD62L, CCR7). (A, B) Gating of CD3 CD8 T cells (A) and CD3 CD4 T cells (B) with subsequent analysis of CD45RA and CD45RO as well as CD62L and CCR7 co-expression. Note the reciprocal expression of CD45RA and CD45RO, and the unequal distribution of CD62L and CCR7 expression, indicating that CD62L and CCR7 identify different T-cell subsets. Representative results with PBMC of donor SIB 369 are shown. (C) Gating strategy for cell sorting. After gating of CD3 CD8 T cells or CD3 CD4 T cells, the gates for a single T-cell differentiation marker were set according to strong or absent expression of this marker (>0.5 log difference in fluorescence intensity). The sorting gates and resulting fractions for CD45RA in CD4 T cells of donor 372 are shown here. For sorting gates of CD45RO, CD62L and CCR7 see *Online Supplementary Figure S1*.

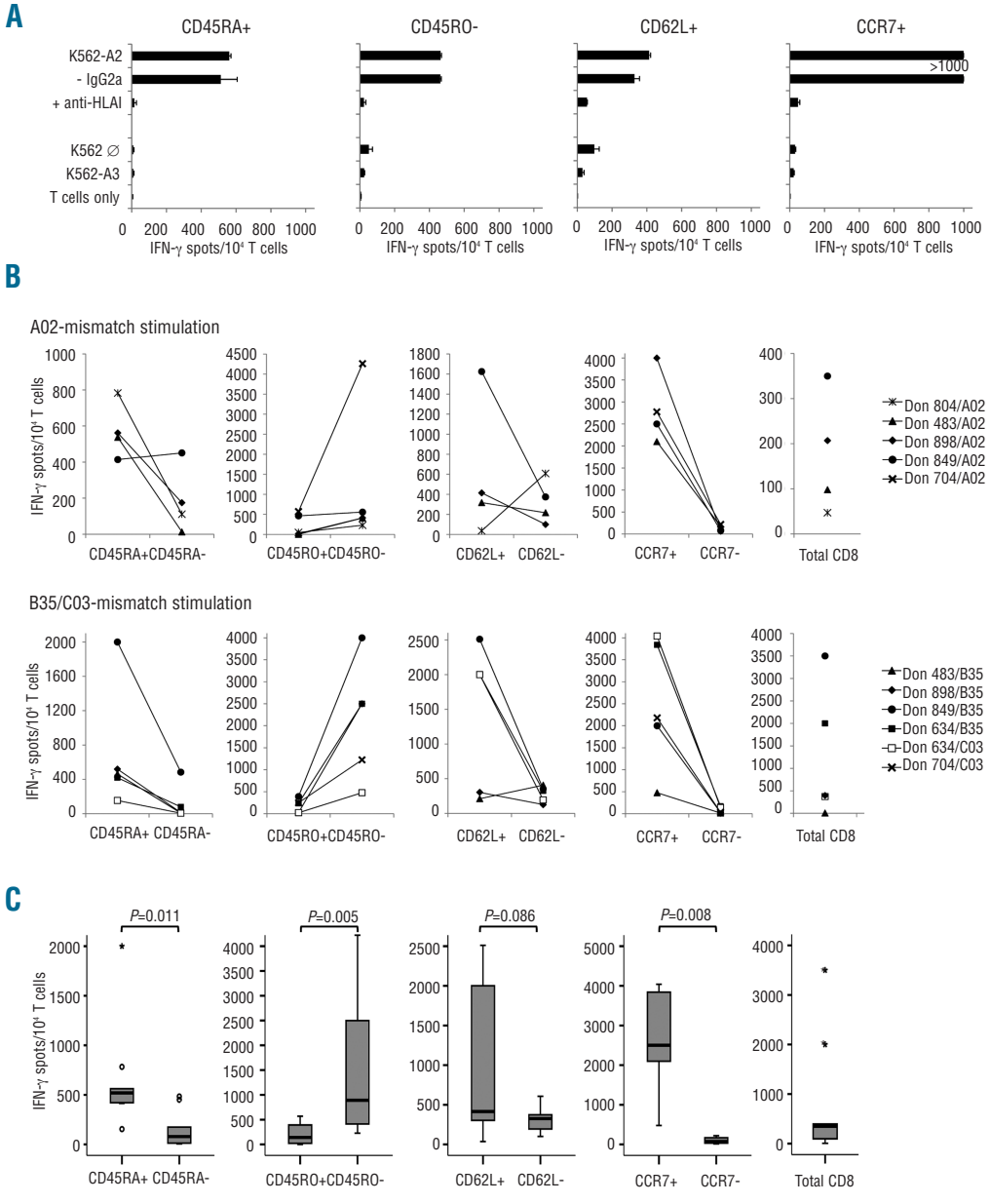

Figure 2. HLA class I allele-specific allorecognition of CD8 T-cell subsets. MLR cultures were tested for allo-HLA reactivity in an IFN-γ ELISpot assay on d12 (i.e. 5 days after first allo-HLA restimulation on d7). (A) Reactivity to original K562-HLA mismatch stimulator cells, as well as to parental K562 cells and K562 transfectant cells carrying an irrelevant HLA allele. To demonstrate HLA-restriction of detected reactivity, monoclonal antibodies blocking T-cell receptor-HLA interactions (and IgG isotype controls) were used. Representative results with naïve-enriched CD8 T-cell subsets of donor 898 for allo-HLA-A*02:01 are shown. (B) Numbers of spot-forming cells from d12 cultures initiated with sorted subsets (CD45RA, CD45RO, CD62L, CCR7) or entire CD8 T cells from six healthy donors. Allo-HLA mismatch alleles used for *in vitro* stimulation were HLA-A*02:01 (upper panels), HLA-B*35:03 and HLA-C*03:03 (lower panels). (C) Box plots and *P* values of data presented in (B). Median (line), 25th to 75th percentile (box), minimum and maximum values (error bars) are indicated. If PBMC numbers were limited, MLR stimulations were restricted to fewer allo-HLA alleles or T-cell subsets.

entire CD4 T cells. In addition to IFN-γ, CD4 MLR responder populations of individual donors secreted low levels of IL-4 and tumor necrosis factor-α, with a tendency of stronger production in MLR derived from naïve-enriched subsets (*data not shown*).

The proliferation of MLR cultures from CD4 T-cell subsets toward single HLA class II mismatch alleles was overall stronger (*Online Supplementary Figure S3*) than that of cultures from similar allo-HLA class I/CD8 experiments. This was particularly detectable for all memory fractions. Significant differences in alloproliferation between MLR cultures derived from subsets enriched for naïve or memory CD4 T cells were not observed. Furthermore, flow cytometric analysis of MLR on d14 showed that subsets enriched for naïve CD4 T cells retained the expression of early differentiation markers in a higher proportion of MLR responders compared to subsets enriched for memory counterparts (*data not shown*). In order to determine the distribution of naturally occurring Treg to memory *versus* naïve CD4 T-cell subsets, $CD45RA^{pos}$ and $CD45RA^{neg}$ fractions from five donors were co-stained for $CD25^{high+}$ $FOXP3^{pos}$ $CD127^{low}$. Of entire Treg, a median of 33.5 (19-41) % was found in $CD45RA^{pos}$ T cells and a median of 66.5 (59-81) % in $CD45RA^{neg}$ T cells. The median percentage of Treg was 3.4 (2.8-3.9) % in total CD4 T cells (*data not shown*).

Finally, we combined the allo-HLA recognition data of CD8 and CD4 T cells (Figures 2 and 4) to identify which subset marker would allow for most efficient separation of alloreactivity in entire T cells. As shown in *Online Supplementary Figure S4A*, sorting strategies based on all four markers resulted in stronger allorecognition in subsets enriched for naïve compared to memory T cells (CD45RA: P=0.000; CD45RO: P=0.000; CD62L: P=0.006; CCR7: P=0.002). The memory-enriched fractions showed significantly lower allorecognition than entire T cells (e.g. for

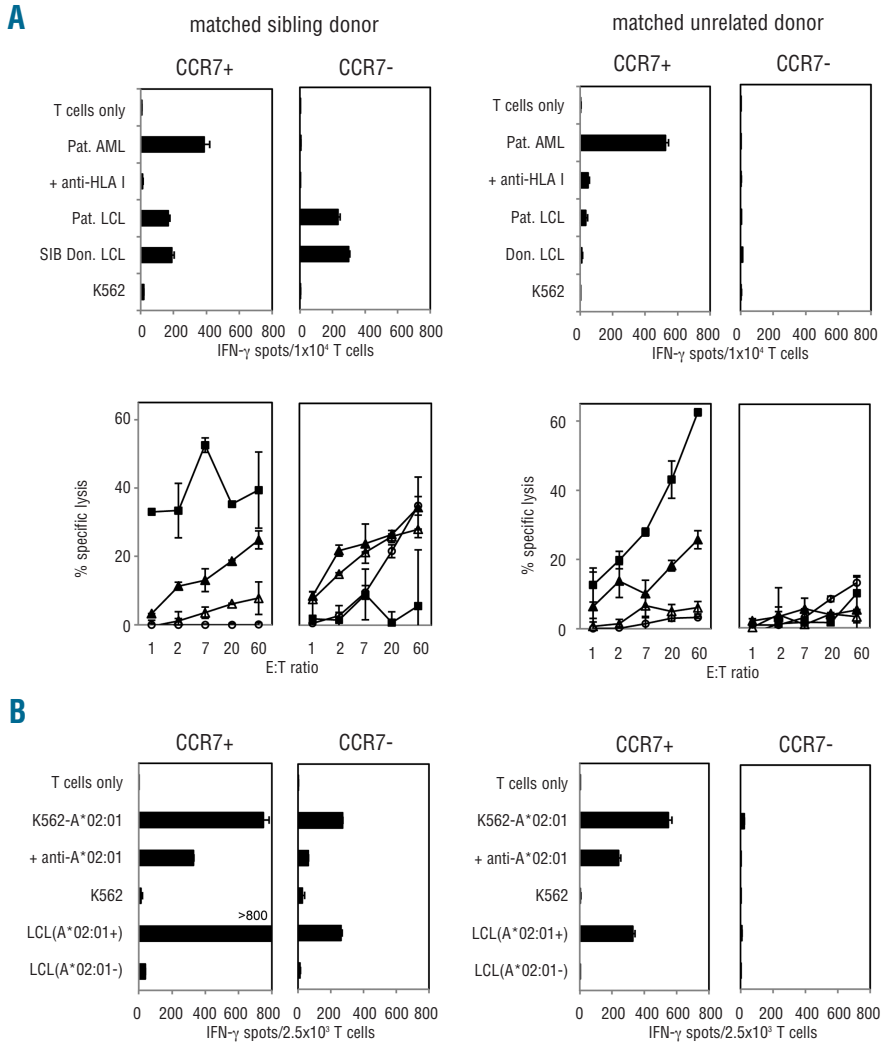

Figure 3. Parallel analysis of CD8 T-cell subsets for reactivity to HLA-matched AML blasts and to K562-HLA mismatch cells. MLLC and MLR were initiated with sorted CCR7^{pos} and CCR7^{neg} CD8 T-cell subsets from healthy individuals. Stimulator cells were primary AML blasts with complete HLA class I-match (4 digits) in MLLC and K562-HLA mismatch cells in MLR. (A) Representative functional data from MLLC of a sibling donor/patient pair SIB 369/MZ369-AML (left panels) and an unrelated donor/patient pair Don 069/MZ653-AML (right panels) are shown. Cultures were analyzed on d19 (i.e. 5 days after second AML restimulation on d14) in IFN-γ ELISpot (upper panel) and ^{51}Cr-cytotoxicity assays (lower panel). Targets of ^{51}Cr-assays were AML blasts (■), patient-derived LCL (▲), donor-derived LCL (△), and K562 cells (○). (B) Results from MLR of donors SIB 369 and Don 069, in which HLA-A*02:01 was used as the mismatch allele (for HLA types see *Online Supplementary Table S1*) are shown. Cultures were screened on d19 for allo-HLA-A*02:01-reactive CD8 T cells in IFN-γ ELISpot assays. LCL from HLA-A*02:01-positive and -negative third-party donors were included as control targets.

CD45RA^{neg} *versus* entire T cells, *P*=0.016; median reduction in IFN-γ spots, 7.4-fold). In contrast, similar differences between T-cell fractions were not observed, if alloproliferation data from CD4 and CD8 T cells (*Online Supplementary Figures S2 and S3*) were combined (*Online Supplementary Figure S4B*). The median reductions in alloproliferation between total CD4/CD8 T cells and memory fractions were 0.7-fold for CD45RA^{neg} (*P*=0.31), 1.1-fold for CD45RO^{pos} (*P*=0.46), 1.2-fold for CD62L^{neg} (*P*=0.29), and 1.3-fold for CCR7^{neg} (*P*=0.04).

Discussion

Here we introduce a rapid screening approach to detect T-cell reactivity of donors to single HLA mismatch alleles. It uses a combination of short-term MLR with a sensitive IFN-γ ELISpot readout system. The main components are HLA-deficient K562 cells that are transfected with 'off-the-shelf' HLA class I and II molecules, resulting in stable or transient HLA expression depending on the favored transfection method. By *in vitro* stimulation of T cells of healthy individuals with single HLA mismatch alleles, we observed that the number of allo-HLA-reactive IFN-γ-producing T cells is usually higher in subsets enriched for naïve cells compared to memory T cells. This finding was very consistent, regardless of whether CD8 or CD4 T cells were analyzed. We hereby confirm a previous report showing stronger alloreactivity in human CD62L^{pos} compared to CD62L^{neg} T-cell subsets *in vitro*.[13] This earlier study used a mixture of several HLA-mismatched B-lymphoblastoid cell lines as MLR stimulator cells, which has the limitation compared to the K562-HLA test system that detected alloreactivity cannot be attributed to single HLA alleles. Furthermore, if residual HLA molecules are matched between donor T cells and recipient B-lymphoblastoid cell lines, alloreactivity interferes with T-cell responses against recipient antigens presented by shared HLA. Such responses (e.g. against Epstein Barr virus) are most likely not equally distributed between donor-derived naïve and memory T cells.

It has long been debated which combination of differentiation markers spans the naïve T-cell pool most precisely.[15] We used the common definition of naïve T cells by CD45 isoforms (i.e. RA^{pos}, RO^{neg}) and also included CCR7 and CD62L comprising naïve and central memory T cells in the analysis.[14] We found that depletion of central memory T cells additionally to naïve precursors (as represented by CD62L^{neg} or CCR7^{neg} subsets) did not lead to significantly decreased allo-HLA responses compared to central

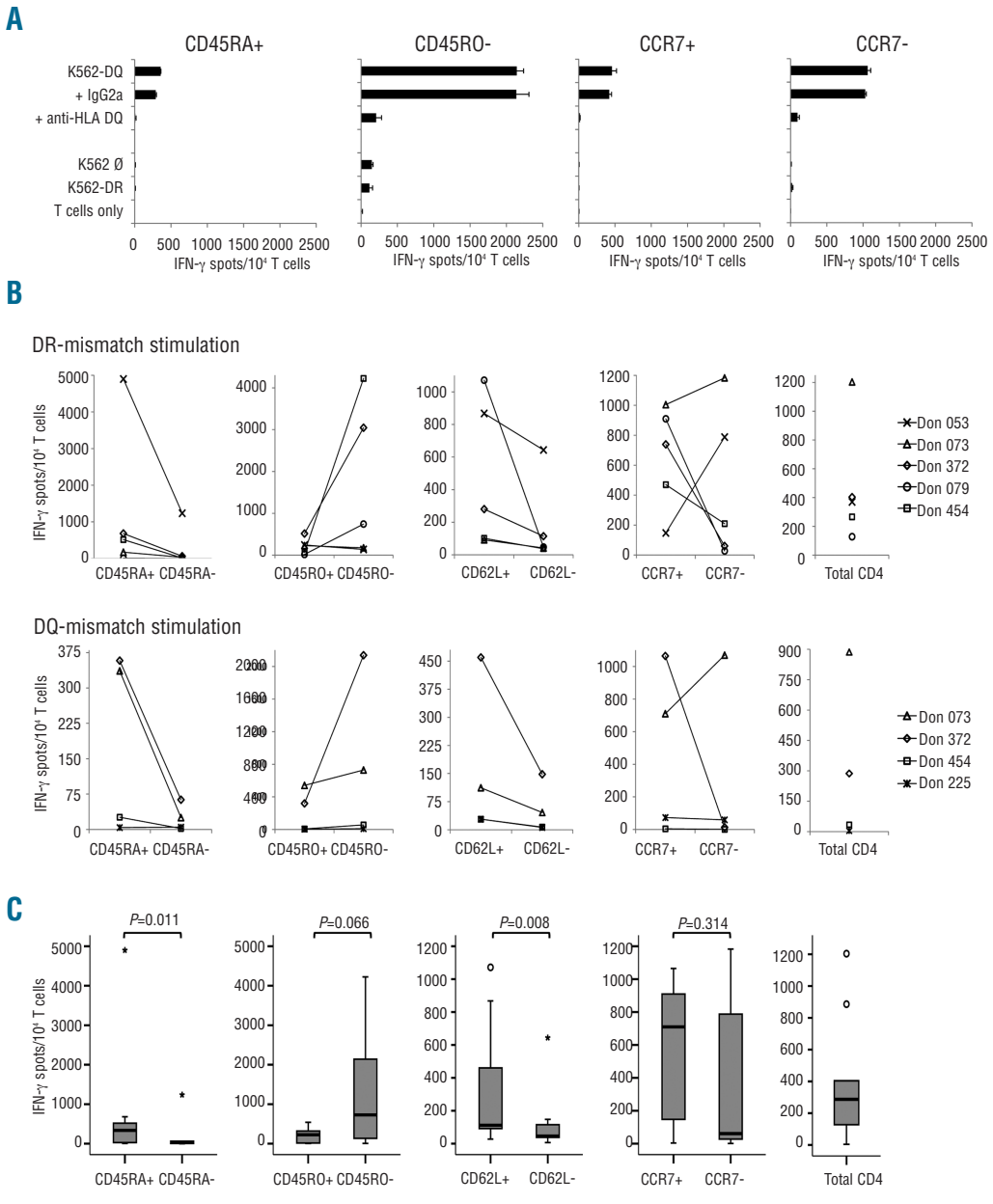

Figure 4. HLA class II allele-specific allorecognition of CD4 T-cell subsets. MLR cultures were tested for allo-HLA reactivity in IFN-γ ELISpot assays on d12 (i.e. 5 days after first allo-HLA restimulation on d7). (A) Reactivity to original K562-HLA mismatch stimulator cells, as well as to parental K562 cells and K562 transfectant cells carrying an irrelevant HLA allele. To demonstrate HLA-restriction of detected reactivity, monoclonal antibodies blocking T-cell receptor-HLA interactions (and IgG isotype controls) were used. Representative results with naïve-enriched CD4 T-cell subsets of donor 372 for allo-HLA-DQB1*06:02 are shown (B) Numbers of IFN-γ spot-forming cells from d12 cultures of MLR started with sorted subsets (CD45RA, CD45RO, CD62L, CCR7) or total CD4 T cells from six healthy donors. HLA class II mismatch alleles used for allostimulation were HLA-DRB1*07:01 (upper panel) and HLA-DQB1*06:02 (lower panel). (C) Box plots and *P* values of data presented in (B); for explanation see legend to Figure 2C. If PBMC numbers were limited, MLR stimulations were restricted to fewer allo-HLA alleles.

memory containing CD45RA^{neg} (or CD45RO^{pos}) T cells. On the other hand, we observed overall stronger allo-HLA IFN-γ responses in naïve and central memory containing CCR7^{pos} T-cell fractions compared to naïve-enriched CD45RA^{pos} T-cell subsets devoid of central memory cells. The latter finding pointed at least in part to significant alloreactivity residing in the central memory compartment.

Of all subsets, only data for CD45RA-sorted naïve and memory populations were significantly different ($P<0.05$) for both CD8 and CD4 T cells. In addition, the number of allo-HLA-reactive effector cells was reduced in median by 7.2-fold and 16.6-fold in CD45RA^{neg} memory CD8 and CD4 T cells compared to in entire CD8 and CD4 T cells, respectively. We also observed that a higher fraction of naturally occurring Treg resided in the CD45RA^{neg} CD4 subset than in the CD45RA^{pos} one and might have contributed to lower alloreactivity mediated by memory CD4 T-cell fractions.

The proliferative response of sorted memory T-cell subsets to allo-HLA was more variably reduced (CD8) or equivalent (CD4) than the IFN-γ response when compared to naïve T-cell subsets. This finding requires further consideration, since proliferation is a much more complex feature of alloreactivity than IFN-γ secretion. We, therefore, investigated whether the lack of correlation between d21 alloproliferation and d12 allorecognition (IFN-γ ELISpot) data might be a result of the prolonged culture period that promoted a switch from naïve to memory T cells in MLR. However, we did not find significant differences in alloproliferation between naïve and memory T-cell subsets during the first week of MLR when using cell counting and CFSE staining as readout assays (*data not shown*).

Our data also suggest that depletion of naïve T cells will

likely impair the generation of donor-derived CD8 cytotoxic T lymphocytes recognizing leukemia antigens in the context of shared HLA molecules. This was demonstrated in HLA-matched sibling and unrelated donor-patient pairs by primary MLLC with naïve CD8 subsets either including (i.e. CCR7^{pos}) or devoid of (i.e. CD45RO^{neg}) central memory T cells.[14,15] We and others have reported that cytotoxic T lymphocyte-mediated reactivity to leukemia cells mainly derives from naïve CD8 T cells in healthy unprimed individuals[19,21-23] and that such cytotoxic T lymphocytes target polymorphic minor histocompatibility antigens (mHag) and non-polymorphic leukemia-associated antigens (LAA).[21,22,24] These findings are further supported by HLA-peptide multimer data in *ex vivo* PBMC showing most precursors of LAA-reactive CD8 T cells in the naïve subset of healthy individuals.[25] In contrast, other groups detected HLA-peptide multimer binding leukemia-reactive CD8 T cells also in the antigen-experienced memory pool. This was demonstrated for LAA specificities in healthy donors[26] as well as for mHag specificities in donors with a history of alloantigen priming *in vivo* (e.g. multiparous females,[27] cord blood grafts[28]). Ideally, this would suggest memory T cells from donors previously primed against hematopoietically expressed mHag and LAA by natural immunization or vaccination should be used for transplantation.

Although the concept of 'memory T-cell therapy' is promising in terms of the expected lower GvHD potential, we detected single donors who had strong alloreactivity in individual memory subsets. Because memory T cells have the capability of rapid expansion upon secondary antigen challenge,[29] the formation of alloreactive memory T cells may have serious consequences in terms of GvHD. Presensitization would be a particular problem if pathogen-specific T cells with cross-reactivity to alloantigens are triggered by an encounter with infectious agents.[30-32]

We propose the use of the K562-HLA-based MLR approach for the screening of donor T-cell reactivity to individual HLA class I and II mismatch alleles in situations in which a donor with full HLA match is lacking. Such donor-patient HLA disparities, particularly at the A/B/C/DR loci, increase the risk of graft rejection and severe GvHD, and may even decrease survival after allogeneic HSCT.[33-36] The approach might help to select a HLA-mismatched donor (e.g. in haploidentical HSCT) with the lowest level of reactivity against non-shared recipient HLA antigens. With the test system, we detected much stronger allorecognition of HLA-A/-B/-C/-DR *versus* HLA-DQ alleles, which is consistent with the clinical observation that HLA-DQ mismatches are better tolerated and do not adversely affect the outcome of allogeneic HSCT.[35,36]

In conclusion, allo-HLA mismatch reactivity was preferentially derived from subsets enriched for naïve compared to memory T cells in most healthy individuals. This was demonstrated by using single alleles from all HLA-A/-B/-C/-DR/-DQ loci currently included in donor-patient typing for allogeneic HSCT. We also observed that *in vitro* strategies for depleting naïve T cells could use several major differentiation markers expressed by naïve T cells (i.e. CD45RA^{pos}, CD45RO^{neg}, CD62L^{pos}, CCR7^{pos}) to develop a suitable sorting strategy. In our hands, the most efficient results for entire T cells were obtained with CD45RA. We also demonstrated the major limitation of the approach, which is the concurrent impairment of T-cell responses against leukemia. This problem might be addressed by using donor allografts which contain leukemia-reactive T cells in the memory compartment due to previous priming against mHag and LAA during natural immunity or vaccination. Alternatively, memory T cells could be replenished with leukemia-reactive T cells previously isolated from the naïve T-cell subset of donors by primary *in vitro* stimulation.[19,22,23] The establishment of a Good Manufacturing Practice procedure for depleting naïve T cells (or selecting memory T cells) *in vitro* appears feasible. This would pave the way for clinical trials that investigate whether depletion of naïve T cells from donor-derived stem-cell grafts or lymphocyte products can indeed reduce GvHD in HSCT patients. Most suitable study populations are HLA-mismatched patients at high risk of GvHD and low risk of leukemia relapse, as well as patients with diseases other than leukemia.

Authorship and Disclosures

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