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IDENTIFICATION OF A COORDINATED CD8 AND CD4 T-CELL RESPONSE DIRECTED AGAINST MISMATCHED HLA CLASS I CAUSING SEVERE ACUTE GVHD

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ABSTRACT

After HLA class I mismatched stem cell transplantation allo-HLA directed CD8 T-cell responses can be activated without the help of CD4 T-cells if memory CD8 T-cells crossreactive against the allo-HLA class I are present, or if naïve CD8 T-cells are administered during inflammatory conditions. However, in the absence of inflammatory conditions, cooperation between CD4 and CD8 T-cells is likely to be required for an effective primary CD8 T-cell response directed against allo-HLA class I. In this study we investigated whether a coordinated response of CD8 and CD4 T-cells could be demonstrated in an HLA class I directed immune response in a patient who developed severe graft versus host disease (GVHD) after the administration HLA-A2 mismatched donor lymphocyte infusion (DLI) in the absence of inflammatory conditions. A previously administered DLI from the same donor did not lead to an immune response, excluding the presence of a substantial pool of CD8 T-cells crossreactive against HLA-A2 within the memory T-cell compartment of the donor. Analysis of isolated donor CD8 and CD4 T-cell clones which were activated during the GVHD in the patient illustrated a polyclonal CD8 T-cell response directed against the mismatched HLA-A2 and a polyclonal CD4 T-cell response recognizing HLA-A2 derived peptides presented in HLA class II. In addition, we demonstrated that patient leukemic blasts present at the time of the emergence of GVHD expressed HLA-A2 and HLA class II and could activate both the CD4 and CD8 alloreactive T-cells. The results demonstrate that the GVHD was mediated by a cooperative CD4 and CD8 response directed against the mismatched HLA-A2 and suggest that leukemic blasts possibly activated both the CD8 and CD4 T-cell response.

INTRODUCTION

In vivo, naive CD8 T-cells require priming by activated antigen presenting cells (APCs) to be able to proliferate and differentiate to effector T-cells. APCs can be activated by the inflammatory signals from pathogens[1;2]. Alternatively, in the absence of inflammatory signals, CD4 T-cells can activate APCs by CD40-CD40L interaction and cytokine production[3-5]. T-cell responses to non-inflammatory immunogens therefore require dual recognition of antigen or antigen expressing cells by CD8 as well as the CD4 T-cells, which is thought to serve as a safeguard against autoimmunity[6]. In contrast to naïve CD8 T-cells, memory CD8 T-cells do not need priming by activated APCs[7;8], indicating that CD4 T-cell help is not required for the effective activation of memory CD8 T-cell responses.

Allo-HLA reactive T-cells involved in the generation of graft versus host disease (GVHD) and graft rejection after HLA mismatched stem cell transplantation (SCT) can be derived from the naïve as well as the memory T-cell pool[9]. As we have previously shown, allo-HLA reactivity exerted by virus specific T-cells is common, suggesting that many memory T-cells are able to exert allo-HLA reactivity[10]. Based on the lower activation threshold of memory CD8 T-cells, an allo-HLA class I directed immune response can easily be activated without the presence of activated APCs and CD4 T-cell help, if T-cells cross reactive against the mismatched HLA class I molecule are present within the memory CD8 T-cell compartment. If these CD8 T-cells are not present in the memory T-cell pool, allo-HLA directed immune response has to develop from the naïve CD8 T-cell compartment, probably requiring activated APCs. Prior to SCT patients are treated with conditioning regimens including irradiation and/or chemotherapy which cause tissue damage leading to production of pro-inflammatory cytokines and thereby activation of APCs[11]. In addition, SCT is often complicated by infections which can lead to systemic inflammatory signals[12]. In the presence of these inflammatory conditions, cooperation between CD8 and CD4 T-cells may not be required for effective activation of primary allo-immune responses, as was demonstrated by Korngold et al. who showed that in heavily irradiated MHC class I mismatched mice purified CD8 T-cells were able to initiate GVHD without the help of CD4 T-cells[13;14].

However, in the absence of tissue damage or infection, allogeneic cells are non-inflammatory immunogens. Under these circumstances cooperation between CD4 and CD8 T-cells is likely to be required for an effective primary CD8 T-cell response. Chakraverty et al. demonstrated that in delayed MHC class I mismatched donor lymphocyte infusion (DLI), not preceded by irradiation, CD4 T-cells were necessary for the expansion of GVHD inducing CD8 T-cells[15]. Since for a coordinated response CD4 and CD8 T-cells need to be activated by the same antigen expressing cells, allogeneic cells expressing both HLA class I as well as HLA class II would be required.

In this study we investigated whether a coordinated response of CD8 and CD4 T-cells could be demonstrated in a HLA class I directed immune response in a patient with acute myeloid

leukemia (AML), who received a T-cell depleted SCT and two DLIs from the same HLA-A2 mismatched donor. The first DLI did not lead to an immune response, indicating that there was not a substantial pool of CD8 T-cells crossreactive against HLA-A2 within the memory T-cell compartment of the donor. The second DLI administered in the presence of HLA class II positive leukemic cells, led to a coordinated response of CD8 and CD4 T-cells resulting in a severe acute GVHD. Characterization of the allo-immune response leading to the GVHD showed that the CD8 T-cell response was directed against the mismatched HLA-A2 molecule and that the CD4 T-cell response recognized HLA-A2 derived peptides presented in the context of the HLA-DR1 molecule shared between patient and donor. Patient leukemic cells present at the time of the emergence of GVHD expressed HLA-A2 and HLA-DR, and were able to activate the CD8 as well as the CD4 alloreactive T-cells, suggesting that the leukemic blasts may have activated both the CD8 and the CD4 T-cell response.

DESIGN AND METHODS

Patient

The patient was a 55 year old male with acute myeloid leukemia (AML) secondary to myelodysplastic syndrome in complete remission. After a non-myeloablative conditioning regimen consisting of ATG, Fludarabine and Busulfan[16], he received a T-cell depleted SCT from a sibling donor. No immune suppression was administered post transplantation. Six months after the SCT, a DLI of $2.5x10^6$ T-cells/kg was given for mixed chimerism and 12 months after the SCT, AML for which a second DLI containing 7.5*10e6 T-cells/kg was given. Five weeks after the second DLI the patient died of grade IV GVHD.

HLA typing. Patient: *A0201*, A0301, B0702, B3501, C0401, C0701, DRB1-0101, DRB1-1501, DQB1-0501, DQB1-0602, DPB1-0402. Donor: *A2601*, A0301, B0702, B3501, C0401, C0701, DRB1-0101, DRB1-1501, DQB1-0501, DQB1-0602, DPB1-0402

Cell collection and preparation

After informed consent, peripheral blood and bone marrow samples were obtained from the patient and donor as well as from other patients and healthy donors. Mononuclear cells were isolated by Ficoll-Isopaque separation and cryopreserved. Stable Epstein–Barr virus (EBV)-transformed B-cell lines (EBV-LCLs) were generated using standard procedures. HLA-A2+ donor EBV-LCLs were generated by transduction of donor derived EBV-LCLs with a retroviral vector encoding for HLA-A*0201[17]. Fibroblasts were cultured from skin biopsies in Dulbecco's modified Eagle's medium with 1g/l glucose (BioWhittaker, Verviers, Belgium) and 10% fetal bovine serum (FBS, BioWhittaker). Keratinocytes were cultured from skin biopsies in keratinocyte serum free medium supplemented with 30 µg/ml bovine pituitary extract and 2 ng/ml epithelial growth factor (Invitrogen, Carlsbad, CA, USA).

Flow cytometry

The monoclonal antibodies (mAbs) anti-HLA-A2-FITC, anti-HLA-DR-APC, anti-CD40-PE and anti-CD86-PE, were obtained from Pharmingen (San Jose, CA, USA). Anti-CD45-PerCp, anti-CD3-APC, anti-CD33-APC, anti-CD80-PE, anti-CD54-PE and anti-CD11c-PE were derived from Becton Dickinson (BD, San Jose, CA, USA). Anti-CD4-PE, anti-CD8-PE, anti-CD19-PE and anti-CD14-PE were purchased from Caltag (Carlsbad, CA, USA). Anti-BDCA1-PE and anti-BDCA2-FITC antibodies were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). For determination of TCR Vβ usage of the CD4 T-cell clones, the TCR Vβ kit (Beckman Coulter, Fullerton, CA) was used. Flow cytometric analysis was performed on a BD flow cytometer.

Generation of alloreactive T-cell clones

Patient peripheral blood mononuclear cells (PBMCs) were collected during the start of the GVHD, three weeks after the second DLI, in the absence of administration of immune suppressive drugs. The PBMCs were stained with anti-HLA-A2, anti-HLA-DR, anti-CD4 and anti-CD8 mAbs at 4°C for 30 min and washed once. HLA-DR positive (activated), HLA-A2 negative (donor) CD4+ or CD8+ T-cells were sorted single cell per well into U-bottom microtiter plates containing 100 µl of feeder mixture consisting of Iscove's Modified Dulbecco's Medium (IMDM, Cambrex, Rutherford, NJ, USA), 5% FBS, 5% human serum (HS), IL-2 (120 IU/ml, Chiron, Novartis, Emeryville, CA, USA), phytohemagglutinin (PHA, 0.8 µg/ml, Murex Biotec Limited, Dartford, UK), and 50 Gy irradiated allogeneic third-party PBMCs ($0.5x10^6$ /ml). Proliferating T-cell clones were selected and further expanded using nonspecific stimulation and third-party feeder cells.

Characterization of T-cell clones

To analyze the alloreactivity of the expanded CD8 T-cell clones, cytotoxicity assays and cytokine production assays were performed. In the cytotoxicity assays the CD8 T-cells clones were tested in a standard 4 hour ^{51}Cr-release assay against patient EBV-LCLs, donor EBV-LCLs, and third party HLA-A2+ and HLA-A2- EBV-LCLs in an effector to target ratio of 10:1. In the cytokine production assays IFNγ production of the CD8 T-cells clones in response to patient EBV-LCLs, donor EBV-LCLs and third party HLA-A2+ and HLA-A2- EBV-LCLs was tested. To determine IFNγ production, 5,000 T-cells were cultured with 20,000 stimulator cells in a final volume of 150µl IMDM culture medium supplemented with 60 IU/ml IL-2. After 18 hours of incubation, supernatants were harvested, and IFNγ production was measured by standard ELISA (CLB, Amsterdam, the Netherlands). To determine the HLA-restriction of the CD8 T-cell clones, blocking studies were performed using BB7.2 (anti-HLA-A2), W6.32 (anti-HLA class I) or B1.23.2 (anti-HLA-B and C) mAbs. Patient EBV-LCLs, donor EBV-LCLs, donor EBV-LCLs transduced with HLA-A2 and HLA-A2+ EBV-LCLs were preincubated with saturating concentrations of mAbs for 1 hour at 20°C before addition of T-cells.

To analyze the alloreactivity of the expanded CD4 T-cell clones, IFNγ production in response to patient EBV-LCLs and donor EBV-LCLs was tested. To determine the HLA-restriction of the alloreactive CD4 T-cell clones, blocking studies were performed using W6.32 (anti-HLA class I), PdV5.2 (anti-HLA class II), B8.11.2 (anti-HLA-DR), SPV-L3 (anti-HLA-DQ) or B7.21 (anti-HLA-DP) mAbs. Patient EBV-LCLs, donor EBV-LCLs and donor EBV-LCLs transduced with HLA-A2 were preincubated with saturating concentrations of mAbs for 1 hour at RT before addition of T-cells. In addition, the IFNγ production in response to a panel of HLA-DR*0101+ HLA-A*0201+, HLA-DR*0101+ HLA- A*0201-, HLA-DR*1501+ HLA- A*0201+ and HLA-DR*1501+ HLA- A*0201- EBV-LCLs was tested. To identify the recognized HLA-A2 derived peptides and the minimal recognized epitope of the HLA-A2 derived peptides, IFNγ production of the CD4 T-cells clones in response to donor EBV-LCLs loaded overnight with the different HLA-A2 derived peptides was measured after 18 hours of co-culture.

HLA-A2 derived peptides

To investigate epitopes of HLA-A*0201 which could be recognized in the context of HLA class II by the CD4 T-cell clones, synthetic 20-mer peptides covering the whole sequence of the HLA-A*0201 molecule, with an overlap of 7 amino acids between each two subsequent peptides, were made by solid phase peptide synthesis. To further analyze the minimal recognized epitope, truncated peptides of the recognized region of amino acids 99-122 of the HLA-A2 molecule were generated. All peptides were checked for purity by analytic reversed phase HPLC and amino acid analysis.

Stimulatory capacity of patient hematopoietic cells and non-hematopoietic cells

Bone marrow cells (BMCs) and PBMCs collected from the patient prior to the first and second DLI were stained with anti-HLA-A2 mAb, and the HLA-A2+ cells from the two time points were selected by FACS sorting. In addition, BMCs collected from the patient prior to the second DLI were stained with HLA-A2, CD33 and CD3 mAbs, and patient T-cells (HLA-A2+, CD3+) and leukemic cells (HLA-A2+, CD33+) were selected by FACS sort. IFNγ production of the CD4 and CD8 clones in response to the different T-cell subsets was measured by ELISA. Recognition of non-hematopoietic cells was analyzed using fibroblasts and keratinocytes as stimulator cells. Following cell culture of 3 days in the presence or absence of 200 U/ml IFNγ (Immukine, Boehringer Ingelheim, Alkmaar, the Netherlands), cells were thoroughly washed and 10,000 stimulator cells were co-cultured with 5,000 T-cells. IFNγ production was measured by ELISA after overnight incubation.

RESULTS

CD8 and CD4 T-cells activated during GVHD after single HLA class I mismatched DLI

In this study we characterized the allo-immune response in a patient experiencing GVHD after single HLA class I mismatched DLI. Based on a cross-over, patient and donor had a disparity in HLA-A2, whereas the other HLA class I and II molecules were completely matched. The patient received a T-cell depleted SCT and was treated 6 months after SCT with DLI for mixed chimerism. No change in chimerism was observed and in addition no GVHD developed. 12 months after the SCT, AML relapse occurred with 9% blasts in bone marrow, and 0.1% malignant cells in peripheral blood for which a second DLI was given. In contrast, the second DLI induced severe acute GVHD, and chimerism analyses on bone marrow derived mononuclear cells demonstrated a rapid change in patient chimerism from 41% before second DLI to 2% after the second DLI (data not shown).

To investigate whether the occurrence of GVHD correlated with activation of donor T-cells, the expression of the T-cell activation marker HLA-DR on donor T-cells before and after the

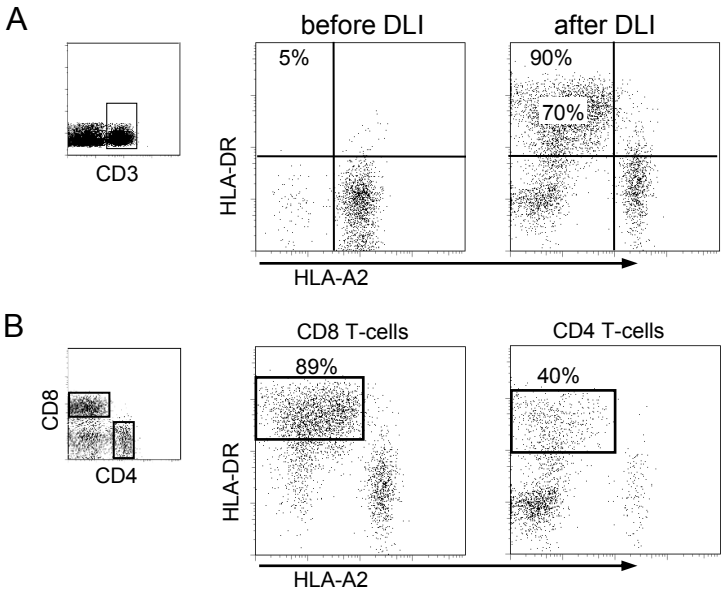

Figure 1. Correlation of GVHD and donor CD4 and CD8 T-cell activation.
Patient (HLA-A2+) and donor (HLA-A2-) cells could be discriminated by the expression of HLA-A2. HLA-DR expression marks the activated T-cells. **(A)** PBMCs collected three weeks before and after the DLI leading to GVHD were stained with mAbs against CD3, HLA-A2 and HLA-DR. The gated CD3+ cells are shown. After the DLI, the percentage of donor T-cells increased from 5% to 90% and 70% of donor T-cells were activated **(B)** PBMCs collected during the GVHD, were stained with anti-HLA-A2 and anti-HLA-DR mAbs in combination with anti-CD4 and anti-CD8. The gated CD4 and CD8 T-cells are shown. 89% of the CD8 donor T-cells and 40% of the CD4 donor T-cells were activated during the GVHD.

second DLI was determined by flow cytometric analysis. After the second DLI, the percentage of donor T-cells increased from 5% to 90%, indicating strong T-cell expansion. In addition, 70% of donor T-cells was activated, as shown by high HLA-DR expression (figure 1A). To investigate whether donor CD8 as well as donor CD4 T-cells were activated, the expression of HLA-DR on the CD8 and CD4 T-cells derived from the GVHD was determined by flow cytometry. As shown in figure 1B, 89% of donor CD8 T-cells and 40% of donor CD4 T-cells were activated during the GVHD.

Isolation of alloreactive CD8 and CD4 T-cells

To characterize the CD8 and CD4 allo-immune responses, activated donor CD8 and CD4 T-cells were isolated from patient PBMCs collected during the GVHD by single cell sort based on the expression of HLA-DR and the absence of HLA-A2. Isolation and expansion of these T-cells resulted in 56 CD8 T-cell clones and 88 CD4 T-cell clones for further analysis

To investigate whether the CD8 T-cell clones were alloreactive, the clones were tested for reactivity against patient and donor EBV-LCLs. 50 of 56 isolated CD8 clones, were shown to be alloreactive, since these T-cells were cytotoxic against patient EBV-LCLs, but not against donor EBV-LCLs (data not shown). In addition to cytotoxicity, these 50 CD8 T-cell clones also produced IFNγ against patient EBV-LCLs, but not against donor EBV-LCLs (data not shown). To determine the alloreactivity of the CD4 T-cell clones, the 88 clones were tested against patient and donor EBV-LCLs in cytokine production assays. 21 of the 88 CD4 clones were alloreactive as shown by IFNγ production upon stimulation with patient EBV-LCLs but not with donor EBV-LCLs (data not shown).

Polyclonal CD8 response directed against allo-HLA-A2

To determine the diversity of the CD8 alloresponse, the TCR Vβ usage of the 50 alloreactive CD8 T-cell clones was analyzed by flow cytometric analysis with Vβ mAbs and sequencing of the CDR3 region of the TCR Vβ chains. The CD8 clones showed usage of at least 13 different TCR Vβs, and all TCR Vβ chains had a different CDR3 sequence (data not shown), demonstrating that all 50 CD8 T-cell clones were of different clonal origin, and that the CD8 response in the patient was polyclonal.

To define the HLA restriction, the CD8 T-cell clones were tested against patient EBV-LCLs, donor EBV-LCLs, donor EBV-LCLs transduced with HLA-A*0201 and a panel of 8 HLA-A*0201+ EBV-LCLs in combination with HLA blocking mAbs. In figure 2A recognition of one representative clone is shown. Donor EBV-LCLs transduced with HLA-A*0201 and the panel of HLA-A*0201+ EBV-LCLs, of which one is shown, were recognized by all CD8 T-cell clones. Recognition of patient EBV-LCLs, donor EBV-LCLs transduced with HLA-A*0201 and third party HLA-A*0201+ EBV LCLs was blocked by HLA-A2 and HLA class I mAbs, but not by HLA-B/C mAb. These results demonstrate that all 50 alloreactive CD8 clones were restricted to HLA-A*0201, the only mismatched HLA allele between patient and donor.

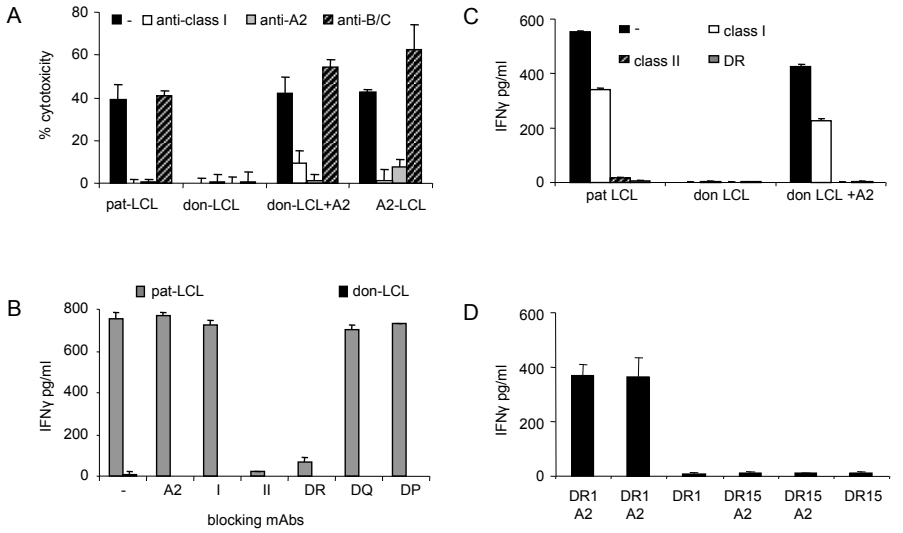

Figure 2. Characterization of the HLA restriction and specificity of donor CD8 and CD4 T-cell clones isolated from the GVHD.

(A) CD8 T-cell clones were tested for cytotoxicity against patient EBV-LCLs (pat-LCL), donor EBV-LCLs (don-LCL), donor EBV-LCLs transduced with HLA-A2 (don-LCL+A2) and a panel of 8 HLA-A2+ EBV-LCLs of which one is shown (A2-LCL). During the 4 hour incubation either no (-), anti-HLA class I (anti-class I), anti-HLA-A2 (anti-A2) or anti-HLA B/C (anti-B/C) mAbs were present. All clones showed HLA-A2 restricted reactivity. Cytotoxicity of one representative clone (8.26) is shown. **(B)** CD4 T-cell clones were stimulated with patient EBV-LCLs (pat-LCL) and donor EBV-LCLs (don-LCL) in the presence of either no (-), anti-HLA- anti-A2 (A2), anti-HLA class I (I), anti-HLA-class II (II), anti-HLA-DR (DR), anti-HLA-DQ (DQ) or anti-HLA-DP (DP) mAbs, and IFNγ production was measured by ELISA. 14 of the 21 CD4 T-cell clones were HLA-DR restricted. IFNγ production of one representative HLA-DR restricted clone (4.12) is shown. **(C)** The HLA-DR restricted alloreactive CD4 T-cell clones were tested against patient EBV-LCLs (pat-LCL), donor EBV-LCLs (don-LCL) or donor EBV-LCLs transduced with HLA-A2 (don-LCL+A2) in the presence of either no (-), anti-HLA class I (class I), anti-HLA-class II (class II) or anti-HLA-DR (DR) mAbs. 13 of the 14 HLA-DR restricted CD4 T-cell clones showed recognition of patient EBV-LCLs as well as of donor EBV-LCLs transduced with HLA-A2 and recognition could be blocked by anti-HLA-class II and anti-HLA-DR mAbs. IFNγ production of one representative clone (4.12) is shown. **(D)** CD4 T-cell clones were stimulated with a panel of HLA-DR1+HLA-A2+ (DR1 A2), HLA-DR1+HLA-A2- (DR1), HLA-DR15+HLA-A2+ (DR15 A2) and HLA-DR15+HLA-A2- (DR15) EBV-LCLs. IFNγ production of one representative clone (4.12) is shown. These results indicate that 13 of the 21 alloreactive CD4 clones recognized an HLA-A2 derived peptide presented in the context of HLA-DR1.

Polyclonal CD4 response directed against HLA-A2 derived peptide presented in HLA class II

To determine the diversity of the CD4 response, the TCR Vβ usage of the alloreactive CD4 T-cell clones was analyzed by flow cytometric analysis. This showed that the 21 alloreactive CD4 T-cell expressed at least 10 different TCR Vβ chains (data not shown), indicating that the CD4 response was also polyclonal. To define the HLA-restriction, the alloreactive CD4 T-cell clones were tested against patient EBV-LCLs blocked with different HLA mAbs. IFNγ production of

14 of the 21 clones could be blocked by HLA class II and HLA-DR mAbs, indicative for HLA-DR restriction. In figure 2B the recognition of patient EBV-LCLs by one representative CD4 T-cell clone is shown.

To analyze whether the CD4 T-cells recognized an HLA-A2 derived peptide presented in HLA class II, the CD4 clones were tested against donor EBV-LCLs transduced with HLA-A*0201. 13 of the 14 HLA-DR restricted alloreactive CD4 clones, of which one is shown in figure 2C, recognized donor EBV-LCLs transduced with HLA-A*0201, and this recognition could be blocked by HLA-class II and HLA-DR mAbs but not by HLA class I mAb. To further analyze the HLA-DR restriction, the CD4 T-cell clones were tested against a panel of HLA-DR*0101+ HLA-A*0201+, HLA-DR*0101+ HLA- A*0201-, HLA-DR*1501+ HLA- A*0201+ and HLA-DR*1501+ HLA- A*0201- EBV-LCLs. All 13 CD4 T-cell clones recognizing donor EBV transduced with HLA-A*0201 recognized the HLA-DR*0101+ HLA-A*0201+ EBV-LCLs. The recognition of one representative clone is shown in figure 2D. These results demonstrate that 13 of the 21 isolated CD4 clones recognized an HLA-A2 derived peptide presented in HLA-DR1.

Eight of the 21 alloreactive CD4 T-cell clones did not recognize donor EBV-LCLs transduced with HLA-A*0201 (data not shown). Since these clones produced only low amounts of IFNγ, further characterization of their specificity was not pursued.

To investigate which epitopes of HLA-A*0201 were recognized in the context of HLA-DR1, the CD4 T-cell clones were tested against donor EBV-LCLs loaded with overlapping 20 mer peptides covering the whole sequence of the HLA-A*0201 molecule. All 13 CD4 T-cell clones recognizing donor EBV transduced with HLA-A*0201, showed recognition of sequence 101-122 derived from a hypervariable region of the HLA-A*0201 molecule. Three representative clones are shown in figure 3A. To further analyze the minimal recognized epitope, the CD4 T-cell clones were tested against truncated peptides of the recognized region. Based on the recognition pattern, the CD4 clones could be subdivided into three groups (figure 3B). The first group represented by clone 4.12, recognized the 15-mer epitope of aa 106-120 and the 17-mer epitope of aa 103-119. This group covered 9 of the 13 HLA-DR1 restricted HLA-A2 specific CD4 clones, which showed usage of at least 5 different TCR Vβ chains. The second group, representing 3 clones with different TCR Vβ chains including clone 4.79, showed recognition of aa 105-117. Clone 4.44, recognized the 14-mer epitope 101-114. These results show that, although the majority of the CD4 T-cell clones all recognize a peptide derived from the same region of the HLA-A2 molecule, there was diversity between the clones in minimal epitope recognition.

The results of the characterization of the allo-immune response demonstrated a coordinated allo-immune response consisting of a polyclonal CD8 response directed against the mismatched HLA-A2, and an also polyclonal CD4 response directed against peptides derived from the mismatched HLA-A2 molecule presented in HLA class II.

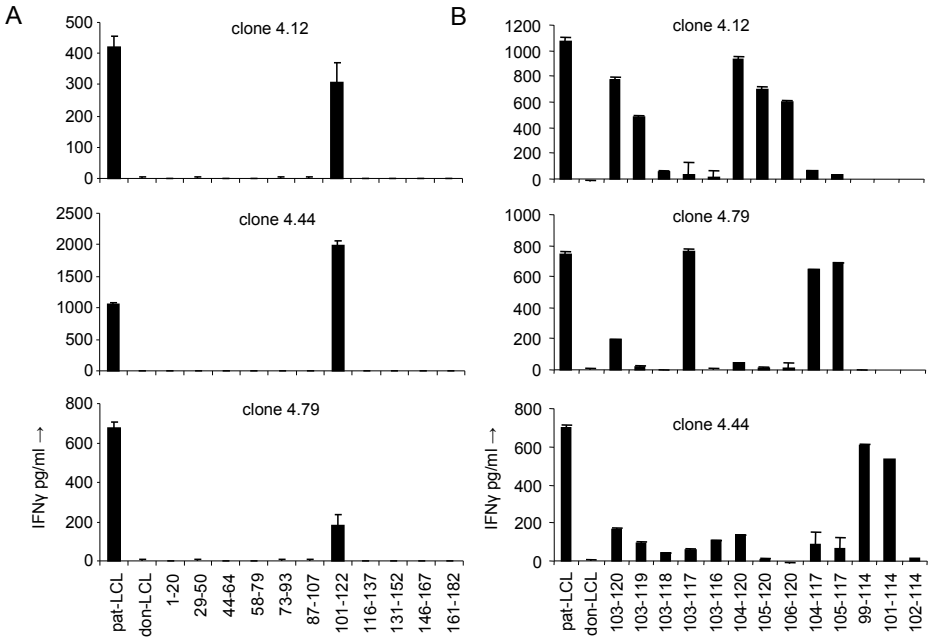

Figure 3. Identification of the HLA-A2 derived peptide and of the minimal epitopes recognized by the CD4 T-cell clones.

(A) The alloreactive CD4 T-cell clones which showed recognition of HLA-A2+ donor EBV-LCLs, were stimulated with donor EBV-LCLs loaded with different peptides covering the whole HLA-A2 sequence. All tested clones, of which one representative clone (4.12) is shown, recognized aa 101-122 of the HLA-A2 molecule. **(B)** To analyze the minimal recognized epitope, the CD4 T-cell clones were stimulated with donor EBV-LCLs loaded with truncated peptides of the recognized region. The clones can be divided into three groups based on their minimal epitope recognition. The first group, represented by clone 4.12, showed recognition of the 15-mer epitope of aa 106-120 and the 17-mer epitope of aa 103-119. The second group, represented by clone 4.79, showed recognition of aa 103-117. Clone 4.44 recognized the 14-mer epitope of aa 101-114.

Patient leukemic blasts possibly mediated the crosstalk between the CD4 and CD8 response

Since severe acute GVHD developed after administration of the second DLI, whereas no clinical signs of GVHD were observed after first DLI, we investigated whether a difference in the composition of the hematopoietic compartment in the patient at the time of the first and second DLI could explain the difference in clinical outcome. Patient and donor chimerism in different cell subsets was measured at the two time points by flow cytometric analysis using lineage specific mAbs in combination with HLA-A2 mAb. The only significant difference in the composition of the hematopoietic compartment in the patient at the time of the DLIs was the absence of leukemic blasts at the time of the first DLI and the presence of leukemic blasts at the time of the second DLI. In the bone marrow high numbers (figure 4A) and in the

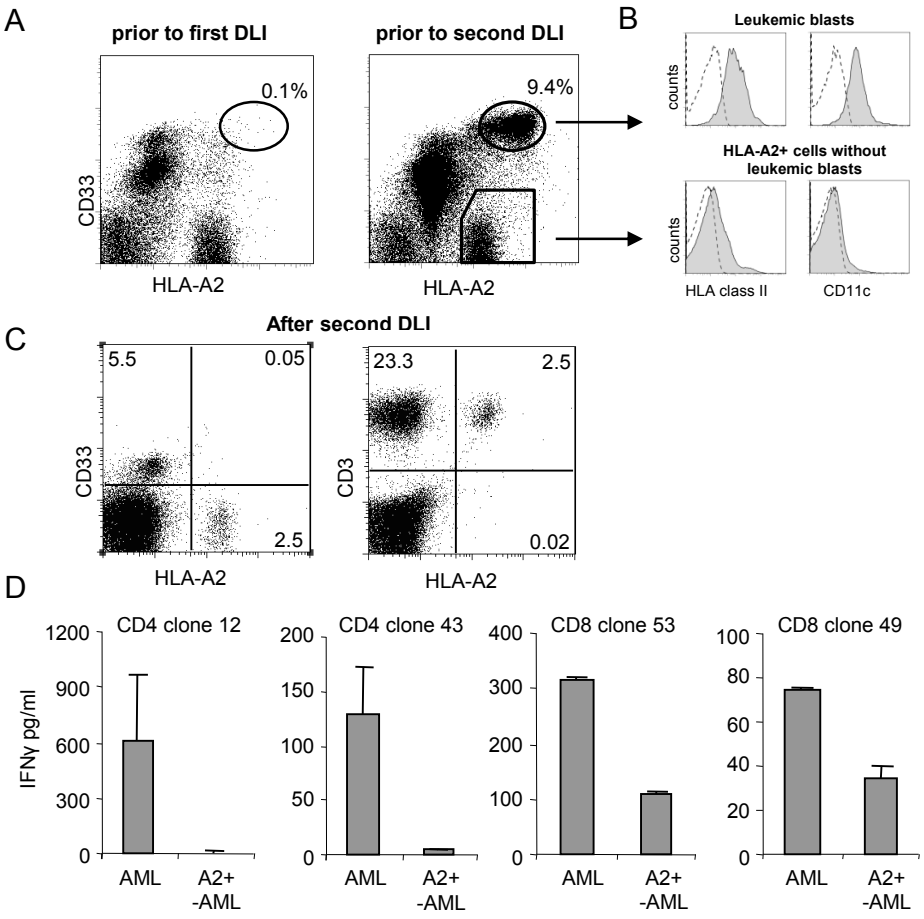

Figure 4. The presence, HLA class II and CD11c expression and stimulatory capacity of patient leukemic blasts.

(A) To visualize leukemic blasts, BMCs were stained with mAbs against HLA-A2 and CD33. At the time of the first DLI no leukemic blasts could be detected, whereas at the time of the second DLI 9% leukemic blasts were present in bone marrow. This percentage of leukemic blasts was confirmed by bone marrow morphology (data not shown). **(B)** Patient BMCs collected prior to the second DLI were stained with anti-HLA-A2, anti-CD33 mAbs, and either anti-HLA class II or anti-CD11c mAbs. Cells gated on HLA-A2 and CD33 positivity represent the leukemic blasts and cells gated on HLA-A2 positivity and absence of CD33 expression represent the HLA-A2+ cells without leukemic blasts. The dashed lines are the isotype controls and the grey lines represent the expression of HLA class II and CD11c on the gated cells. The leukemic blasts showed a high expression of HLA class II and CD11c. The remaining HLA-A2+ cells showed a marginal expression of HLA class II and no expression of CD11c. **(C)** To investigate whether at the time of GVHD also a GVL effect was seen, patient BMCs collected at 4 weeks after second DLI were stained with mAbs against HLA-A2, CD33, and CD3. **(D)** Two CD8 clones (8.53 and 8.49) and two CD4 clones (4.12 and 4.43) were stimulated with AML blasts (AML) or with HLA-A2+ PBMCs deprived of the leukemic blasts (A2+ -AML) from the time of the second DLI. The CD4 T-cell clones only recognized the leukemic blasts and the CD8 T-cell clones recognized all HLA-A2+ cells.

peripheral blood low numbers of leukemic blasts (data not shown) were present at the time of second DLI.

To investigate whether the leukemic blasts could have activated both the CD8 and CD4 alloimmune responses, we determined the expression of HLA class II on the patient leukemic blasts by flow cytometric analysis. In addition, we determined the expression of the costimulatory molecules CD80, CD86 and CD40 and the adhesion molecules CD54 and CD11c, also relevant for immune response initiation. No expression of CD40, CD80, CD86 or CD54 was found on leukemic cells (data not shown). However, a clear expression of HLA class II and CD11c was observed (figure 4B). The remaining HLA-A2+ cells present in patients blood and bone marrow at the time of the second DLI, consisting mostly of patient T-cells (data not shown), showed a marginal expression of HLA class II and no expression of CD11c (figure 4B). Since GVHD and graft versus leukemia (GVL) are often associated, we investigated if a decrease in the numbers of leukemic blasts could be measured at the time of severe GVHD. Results in Figure 4C demonstrate that the leukemic blasts completely disappeared from the bone marrow after the second DLI at the time of GVHD, leaving only some residual patient T-cells present in bone marrow, indicative for a GVL effect.

To investigate which cells were able to activate the donor T-cells, different CD8 and CD4 T-cell clones were tested against the leukemic blasts and against the HLA-A2+ PBMCs deprived of the leukemic blasts. The results show that the leukemic blasts were the only cells in the patient hematopoietic compartment able to activate both the CD8 and CD4 T-cell clones (figure 4D).

CD4 T-cells can recognize non-hematopoietic cells during inflammatory conditions, possibly enhancing the GVHD

Since at the time of administration of the second DLI the patient did not suffer from infections or GVHD, HLA class II expression of non-hematopoietic cells was unlikely. However, to investigate whether during the cytokine storm after development of GVHD, tissues could be targets for both alloreactive CD8 and CD4 T-cells, these T-cell clones were tested for recognition of fibroblasts and keratinocytes derived from HLA-A*0201+ HLA-DR*0101+ individuals. A three days pre-incubation with IFNγ of the fibroblasts and keratinocytes was used to mimic inflammatory conditions. As shown in figure 5A, after pre-treatment with IFNγ the fibroblasts and keratinocytes expressed HLA class II. In agreement with this, the CD4 clones recognized the fibroblasts and keratinocytes only after upregulation of HLA class II with IFNγ. Some of these CD4 clones clearly recognized the fibroblasts and keratinocytes after treatment with IFNγ, whereas other CD4 clones only showed minimal recognition of HLA class II expressing fibroblasts and keratinocytes (figure 5B). The CD8 clones, of which one representative clone is shown in figure 5B, recognized all HLA-A2+ target cells. These results indicate that once the GVHD was initiated, the non-hematopoietic cells were able to serve as target cells for the CD4 alloreactive T-cells, thereby possibly amplifying the GVH response.

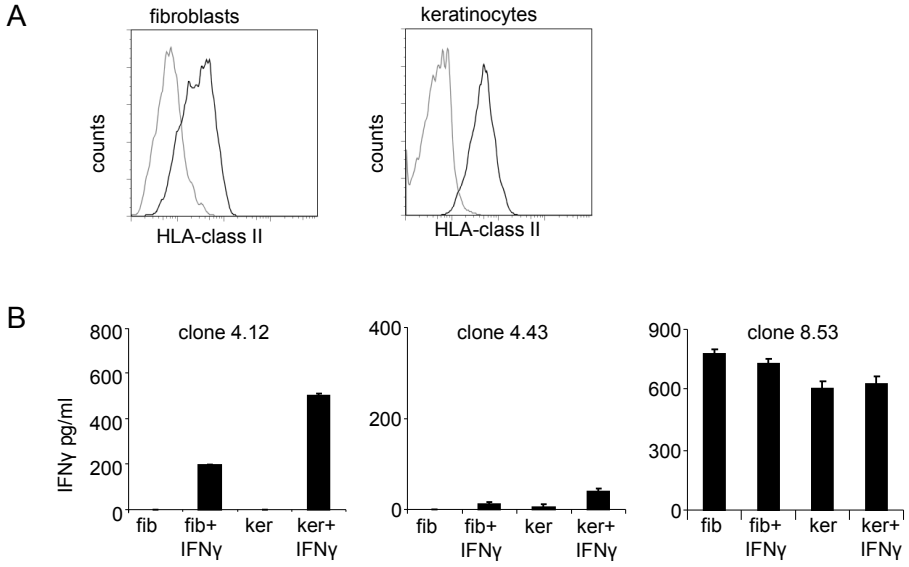

Figure 5. The stimulatory capacity of cells derived from the non-hematopoietic compartment on donor CD4 and CD8 T-cell clones.

(A) Fibroblasts and keratinocytes incubated for three days with or without IFNγ were stained with HLA-class II mAb. The black and grey lines represent the keratinocytes and fibroblasts with or without IFNγ pre-treatment, respectively. Only after the pre-treatment with IFNγ the fibroblast and keratinocytes expressed HLA class II. **(B)** CD4 and CD8 clones were stimulated with fibroblasts and keratinocytes derived from HLA-A2+ HLA-DR1+ individuals, which were pre-incubated for three days with or without IFNγ. The CD8 clones, as represented by clone 8.53, recognized all HLA-A2+ target cells. The CD4 clones only recognized fibroblasts and keratinocytes after upregulation of HLA class II with IFNγ. Some CD4 clones, as represented by clone 4.12, showed a strong recognition, whereas other CD4 clones, as represented by clone 4.43, showed a low recognition of the fibroblasts and keratinocytes which were pre-incubated with IFNγ.

DISCUSSION

In this study the allo-immune response in a patient suffering from GVHD after an HLA-A2 mismatched DLI was characterized. A polyclonal CD8 response, directed against the allo-HLA-A2 molecule was found in conjunction with a polyclonal CD4 response, of which a substantial part was directed against HLA-A2 derived peptides presented in HLA-DR1.

The first DLI that the patient received did not lead to an immune response, whereas the second DLI with a 3-fold higher dose led to severe GVHD. A higher dose of administered lymphocytes could explain the difference in emergence of GVHD, since the occurrence of GVHD has been previously correlated with the dosage of lymphocytes in DLI. Testing donor PBMCs against patient PBMCs in mixed lymphocyte reaction (MLR) demonstrated a very low frequency of alloreactivity (<1:100.000). We therefore assume that the difference in DLI dose was too small to explain the difference between complete absence of GVHD with the first DLI

dose and development of lethal grade 4 GVHD with the second DLI dose. We can, however, not completely rule out that higher T-cell dose contributed to the emergence of GVHD after the second DLI.

Based on the absence of an immune response after the first DLI and the low frequency of alloreactive donor cells measured by MLR, we additionally presume that there was not a substantial pool of CD8 T-cells crossreactive against HLA-A2 present within the memory repertoire of the donor. The DLIs were administered a long time after the conditioning regimen and in the absence of active infections, indicating that no significant inflammatory conditions were apparent around the time of initiation of the GVHD. We hypothesize that due to the absence of allo-HLA-A2 reactive memory CD8 T-cells and inflammatory conditions, a coordinated response of CD8 and CD4 T-cells directed against the allogeneic HLA class I was required for the development of GVHD.

In the immune response analyzed in this study, all alloreactive CD8 T-cells were directed against the mismatched HLA-A2, and the majority of the CD4 T-cells recognized HLA-A2 derived peptides presented in HLA class II, indicating that these HLA-A2 derived peptides were abundantly expressed by the antigen presenting cells responsible for the initiation of the response. The other CD4 T-cell clones most likely recognized peptides polymorphic between the patient and the donor based on single nucleotide polymorphisms, termed minor histocompatibility antigens (MiHAs), presented in HLA class II. HLA derived peptides are frequently presented in both HLA class I and HLA class II[18;19], indicating that after HLA mismatched transplantation, the mismatched HLA molecules will also be presented as peptides in the context of self-HLA. Therefore, the common direction of the CD8 and CD4 T-cell responses against allo-HLA-A2 suggests initiation of the two responses by the same HLA-A2 and HLA class II expressing cells.

The leukemic blasts were the only cells in patient blood and bone marrow which highly expressed HLA class II and HLA-A2 and were able to activate the alloreactive CD8 as well as the CD4 T-cells, suggesting a role for the leukemic blasts in the initiation of the allo-immune response. Conversely, no expression of costimulatory molecules, previously shown to be relevant for initiation of a primary immune response, could be detected on the AML blasts. However, upon activation AML cells express costimulatory molecules[20;21], and these activated AML-DCs can induce autologous anti-leukemic reactive T-cells. In addition, it was recently shown that cross-talk between CD4+ T-cells and leukemic cells in vivo can change leukemic cells into an APC phenotype[22]. We therefore postulate that a small part of the allo-HLA reactive T-cells exhibiting high avidity allo-HLA reactivity can become activated in the absence of costimulatory molecules, and subsequently induce expression of costimulatory molecule on the AML cells, which on their turn more broadly activate the allo-HLA reactive T-cells.

It cannot be excluded that patient dendritic cells (DCs) were still present in other tissues during the second DLI. Merad et al.[23] demonstrated that host Langerhans cells can self renew and thereby remain present in the skin for long periods of time after allogeneic SCT. It is possible

that Langerhans cells and other tissue-resident DCs of the patient remained in the patient post-transplant and thus represented a major priming population for the immune response leading to the GVHD. However, this would indicate that patient DCs were also present at the time of the first DLI, which did not lead to GVHD. Alternatively, donor DCs may have cross presented HLA-A2 peptides. Lechler and colleagues described cross presentation of HLA class I derived peptide in HLA class II by host APCs which migrated to the donor kidney, thereby causing chronic graft rejection[24;25]. However, since donor DCs were HLA-A2 negative, the CD8 alloresponse could not have been initiated by these cells. Although it has been demonstrated in vitro that DCs are able to cross present intact HLA molecules, a phenomenon termed semi-direct presentation[26], this phenomenon has not been demonstrated in vivo and is far less likely to occur than direct presentation by patient APCs.

GVHD and GVL effects are often closely associated. In this study we demonstrate a complete elimination of the AML blasts, indicative for a GVL effect at the time of GVHD. The elimination of the leukemia can be mediated by allo-HLA reactive T-cells that recognize both the malignant as well as healthy tissue of the patient and thereby mediate both GVL and GVHD effects. T-cells selectively directed against the malignancy may also have contributed to the GVL effect. Separation of the GVHD/GVL and solely GVL inducing T-cells may lead to the identification of T-cells directed against tumor specific antigens, useful for adoptive T-cell therapy. Although non-hematopoietic cells, which under normal conditions do not express HLA class II, were unlikely to have initiated the coordinated CD4 and CD8 T-cell response, upregulation of HLA class II molecules under inflammatory conditions during GVHD may have amplified the effector phase of the immune response. As shown in figure 5, some of the alloreactive CD4 T-cells could be activated by non-hematopoietic cells forced to express HLA class II by culturing under conditions mimicking inflammation. We hypothesize that, once GVHD was initiated, activated T-cells may have generated a cytokine storm leading to upregulation of HLA class II on non-hematopoietic cells, and thereby increasing the destruction of these cells. Based on the results in this study we conclude that the GVHD following a delayed HLA-A2 mismatched DLI was mediated by a cooperative CD4 and CD8 response directed against the mismatched HLA-A2. We postulate that the CD8 T-cells directed against the allo-HLA-A2 molecule acted as the primary effector cells and thereby caused the majority of tissue damage. We hypothesize that the CD4 T-cell response, mostly directed against HLA-A2 derived peptide presented in HLA class II, was essential for the initiation and possibly also the amplification of the response, and we speculate that the leukemic blasts expressing HLA-A2 and HLA class II may have activated both the CD8 and CD4 responses.

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