Upregulated expression in non-hematopoietic-tissues of the BCL2A1-derived minor histocompatibility antigens in response to inflammatory cytokines: relevance for allogeneic immunotherapy of leukemia

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Editorial note
The author and co-authors contributed in the following way: Freke M. Kloosterboer designed research, performed research, analyzed data and wrote the paper. Simone A.P. van Luxemburg-Heijs, Ronald A. van Soest and H.M. Esther van Egmond performed research and analyzed data. Roel Willemze designed research and wrote the paper. J.H. Frederik Falkenburg designed research, analyzed data and wrote the paper.
Abstract

T cells directed against hematopoietic-restricted minor histocompatibility antigens (mHags) may mediate graft-versus-leukemia (GVL) reactivity without graft-versus-host-disease. Recently, the HLA-A24-restricted mHag ACC-1 and the HLA-B44-restricted mHag ACC-2 encoded by separate polymorphisms within the BCL2A1 gene were characterized. Hematopoietic-restricted expression was suggested for these mHags. We demonstrate BCL2A1 expression in mesenchymal stromal cells (MSC), that was upregulated by inflammatory cytokines TNFα and/or IFNγ. Analysis of cytotoxicity and IFNγ production illustrated that ACC-2 specific T cells did not recognize untreated MSC or IFNγ-treated MSC, but showed specific recognition and killing of TNFα+IFNγ-treated MSC. We hypothesize that under steady-state circumstances BCL2A1 specific T cells may exhibit relative specificity for hematopoietic tissue but reactivity against non-hematopoietic cells may occur when inflammatory infiltrates are present. Thus, the role of BCL2A1-specific T cells in differential induction of GVL reactivity and graft-versus-host-disease may depend on the presence of inflammatory responses that may occur during graft-versus-host-disease.
Introduction

Allogeneic stem cell transplantation (SCT) and donor lymphocyte infusion (DLI) are effective treatments for patients with hematological malignancies, but can be complicated by graft-versus-host-disease (GVHD). Following HLA-identical SCT both the graft-versus-leukemia (GVL) effect and GVHD can be mediated by donor-derived T cells recognizing polymorphic minor histocompatibility antigens (mHags) on recipient cells. Immunotherapy directed against mHags with hematopoietic-tissue restricted expression may eradicate the leukemic cells without inducing GVHD. Recently, the HLA-A24-restricted mHag ACC-1 and the HLA-B44-restricted mHag ACC-2, encoded by separate polymorphisms within the BCL2A1 gene, were identified, and hematopoietic-restricted expression was suggested for these mHags. However, in a retrospective analysis GVHD, relapse and disease-free survival were not statistically different between patients receiving ACC-1 compatible or incompatible transplantation. Previous studies have indicated expression of the human BCL2A1 gene not only in hematopoietic cells, but also in non-hematopoietic tissues, including lung, small intestine and testis, and expression could be induced in endothelial cells by inflammatory cytokines TNFα and IL1β. Induction of BCL2A1 expression in non-hematopoietic tissues by inflammatory cytokines may have important consequences for the role of T cells recognizing ACC-1 and ACC-2 in differential induction of GVL and GVHD. Local inflammatory cytokine production can be triggered by T cells recognizing host APC in non-hematopoietic tissues.

In this study, we characterized the recognition pattern of mHag ACC-2-specific T cells isolated from a patient during a combined GVHD and GVL response after DLI. We show that ACC-2-specific T cells recognized not only leukemic cells but also non-hematopoietic cells under inflammatory conditions, and therefore could play a role in the etiology of GVHD.
Study Design

Isolation of T cell clones and cell culture

Cytotoxic T lymphocyte (CTL) clones were isolated from an HLA-A2 A3 B7 B44 positive male patient with relapsed chronic myeloid leukemia (CML) during a clinical response to DLI from his HLA-identical female donor. EBV-transformed B cell lines (EBV-LCL) and phytohemagglutinin (PHA)-activated T cells (PHA blasts) were generated as described. Mesenchymal stromal cells (MSC) were derived from bone marrow of healthy donors or CML patients as previously described. To induce gene expression in response to inflammatory cytokines, MSC were cultured for 48 hours with 10 ng/ml TNFα and/or 100 IU/ml IFNγ (Boehringer Ingelheim, Ingelheim, Germany). Prior to use as targets or stimulators, cells were thoroughly washed to remove all added cytokines. Colon tumor cell line CCL-228 was obtained from the ATCC (Rockville, USA). The primary airway-smooth-muscle (ASM) cells treated with or without 20 ng/ml TNFα, IFNγ and IL-1β (each cytokine 20 ng/ml) were generated by Sylvia Lazeroms (Department of Pulmonary Disease, LUMC). Approval was obtained from the LUMC institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki.

Genotyping of BCL2A1 polymorphisms and quantitative real-time RT-PCR

RNA isolation and cDNA synthesis were performed as described. A 311 bp amplification product containing all known BCL2A1 polymorphisms was generated using forward primer 5’-ttggatatatttacaggctgc-3’ and reverse primer 5’-gggcaattttgctgtc-3’. PCR-products were sequenced as described. Quantitative real-time RT-PCR for BCL2A1 was performed as described using primers 5’-gtcccgtagacactgc-3’ and 5’-ccattttccc-3’ and probe 5’-TET-ttctacagc-3’.
**Analysis of cytotoxicity and IFNγ production**

To determine cytotoxicity, $^{51}$Cr-release-assays were performed.\textsuperscript{22} As effector cells, CTL clone 65 and the HLA-A2-specific control CTL clone MBM13 were used. To analyze cytotoxicity against ACC-2 peptide KEFEDDIINW or ACC-2 control peptide KEFEDGIINW, donor-derived EBV-LCL were loaded with 1 ug/ml peptide during $^{51}$Cr-labeling. IFNγ production was measured as described.\textsuperscript{16}
Results and discussion

Recently, we isolated mHag-specific CTL clones from a male patient with relapsed CML during the clinical response to DLI from his HLA-identical female donor.\textsuperscript{16,23} The DLI induced a complete remission but was complicated by GVHD resulting in organ damage. Not only CTL clones specific for the mHags HA-1 and HY-B7 were isolated. CTL clone 65 appeared to be specific for ACC-2 because it lysed donor-derived EBV-LCL labeled with ACC-2 peptide KEFEDDIINW (Figure 1A). The ACC-2-specific T cells lysed patient-derived EBV-LCL and CML cells, and leukemic cells from unrelated HLA-B44\textsuperscript{+} mHag ACC-2\textsuperscript{+} individuals but not HLA-B44\textsuperscript{+} mHag ACC-2\textsuperscript{-} leukemic cells (Figure 1). These data are in line with the reactivity reported by Akatsuka \textit{et al}.\textsuperscript{8}

To investigate potential cytolytic activity against non-hematopoietic cells, first BCL2A1 expression levels in non-hematopoietic and hematopoietic cells were measured by quantitative real-time RT-PCR. A screen of commercially available tissue samples indicated BCL2A1 expression in normal and leukemic hematopoietic cells, lung and heart (data not shown). We analyzed in more detail hematopoietic cells, ASM cells and MSC. High BCL2A1 expression was found in patient-derived EBV-LCL and PHA blasts. In primary ASM cells expression was 15-40 times lower but treatment with TNF\textsubscript{α}+IFN\textsubscript{γ}+IL1β-resulted in expression levels even exceeding that of EBV-LCL. MSC showed a 300-1000 fold lower expression as compared to EBV-LCL but expression was five fold upregulated in IFN\textsubscript{γ}- or TNF\textsubscript{α}+IFN\textsubscript{γ}-treated MSC.

To determine whether this upregulation of BCL2A1 expression in MSC was sufficient for recognition of these non-hematopoietic cells, we measured lysis of untreated or inflammatory cytokine-treated MSC, and IFN\textsubscript{γ} production by the ACC-2-specific CTL clone (Figure 2). Similar to the results from Akatsuka \textit{et al}.,\textsuperscript{8} the ACC-2-specific CTL clone 65 did not recognize untreated or IFN\textsubscript{γ}-treated HLA-B44\textsuperscript{+}, mHag ACC-2\textsuperscript{+} MSC.
However, HLA-B44⁺, mHag ACC-2⁺ MSC treated with TNFα+IFNγ were lysed by the ACC-2-specific CTL clone, which also produced IFNγ at levels comparable to after stimulation with patient-derived EBV-LCL. Non-specific reactivity was excluded because ACC-2-specific CTL showed no IFNγ production in response to untreated or cytokine-treated HLA-B44⁺ ACC-2⁻. Untreated or cytokine-treated MSC were not lysed and did not induce IFNγ production by non-reactive control clones (data not shown). To determine whether the ACC-2-specific T cells may have contributed to the GVHD observed in the patient after DLI, we also analyzed the reactivity against patient-derived MSC. We found no recognition of untreated or IFNγ-treated MSC but low lysis of TNFα+IFNγ-treated MSC (14% at an ET ratio of 10:1).

In this report, we demonstrate that in non-hematopoietic tissue under steady-state circumstances expression of BCL2A1 is relatively low, but can be upregulated during inflammation. Although we could not determine whether the ACC-2-specific T cells were involved in the GVHD in the patient studied which may also have been due to the presence of several other mHag-specific T cells including male-specific T cells, we hypothesize that the concomitant disease state of a patient poses a riskfactor for GVHD and may therefore determine whether BCL2A1-specific T cells exclusively exhibit a GVL effect or also contribute to GVHD. Especially the presence of inflammatory cells such as monocytes or dendritic cells in non-hematopoietic tissues may pose a risk factor for inducing or enhancing GVHD. Recognition of the inflammatory cells by donor-derived T cells may provide the necessary cytokines leading to upregulated expression of the BCL2A1-derived mHags in damaged tissue. The subsequent recognition by ACC-1 or ACC-2 specific T cells may then enhance GVHD. Accordingly, the patient from which we isolated the ACC-2-specific CTL suffered from skin and pulmonary GVHD. A recent report on patients treated with mHag-specific T cell clones may also be indicative of inflammatory cytokine-induced expression in non-hematopoietic tissue of mHags that under steady-state
circumstances are preferentially expressed in hematopoietic tissue. In one of the patients treated for relapsed leukemia with donor T cells recognizing a mHag preferentially expressed in hematopoietic cells, pulmonary toxicity developed which was attributed to recognition of the mHag in lung microenvironment.\textsuperscript{24}

Our study does not necessarily exclude the use of BCL2A1-specific T cells in adoptive immunotherapy to eliminate leukemic cells. In the absence of inflammatory cells in non-hematopoietic tissues, ACC-1 or ACC-2 CTL may act as hematopoietic-specific T cells eradicating leukemia. However, when patients suffer from signs of GVHD the production of inflammatory cytokines may be triggered, resulting in the upregulated expression of BCL2A1 in target tissues of GVHD.
References


(16) Kloosterboer FM, Luxemburg-Heijs SA, Soest RA et al. Minor histocompatibility antigen specific T cells with multiple distinct specificities can be isolated by direct cloning of IFN secreting T cells from patients with


Figure 1  Reactivity of the ACC-2 specific CTL clone against hematopoietic cells. (A) Cytolytic activity of CTL clone 65 against patient-derived EBV-LCL and donor-derived EBV-LCL loaded with ACC-2 peptide KEFED\text{D}INW but not against donor-derived EBV-LCL loaded with ACC-2 control peptide KEFED\text{Q}INW. (B) Lysis of patient-derived CML cells and leukemic cells from unrelated HLA-B44\textsuperscript{+}, mHag ACC-2\textsuperscript{+} individuals but not of leukemic cells from an unrelated HLA-B44\textsuperscript{+}, mHag ACC-2\textsuperscript{-} individual by CTL clone 65. Cytotoxicity was measured at an ET ratio of 10:1.
**Figure 2**  Reactivity of the ACC-2 specific CTL clone against non-hematopoietic cells. Cytolytic activity and/or IFNγ production by CTL clone 65 against MSC treated with or without TNFα and/or IFNγ for 48 hours prior to labeling with 51Cr. MSC were derived from an unrelated HLA-A2−, HLA-B44+ ACC-2+ individual or an HLA-A2− HLA-B44+, ACC-2− individual. Cytotoxicity was measured at an ET ratio of 10:1. IFNγ production was measured by determining the concentration of IFNγ in the supernatant after 24 hours of coculture.
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