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Alloreactive Effector T Cells Require the Local Formation of a Proinflammatory Environment to Allow Crosstalk and High Avidity Interaction with Nonhematopoietic Tissues to Induce GVHD Reactivity

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Based on clinical observations that donor T cells specific for minor histocompatibility antigens (MiHA) ubiquitously expressed on both hematopoietic and nonhematopoietic cells were detected in patients showing evident graft-versus-leukemia/lymphoma (GVL) reactivity with no or limited coinciding graft-versus-host disease (GVHD), we hypothesized that nonhematopoietic tissues may be relatively unsusceptible to the cytotoxic effect of MiHA-specific T cells under normal, noninflammatory conditions. To test this hypothesis, we investigated the reactivity of alloreactive T cells specific for ubiquitously expressed MiHA against skin-derived primary human fibroblasts. We demonstrated that this reactivity was not merely determined by their antigen-specificity, but was highly dependent on adhesion molecule expression. ICAM-1 expression on the fibroblasts upregulated under proinflammatory conditions and induced during cross-talk with the T cells was demonstrated to be a crucial factor facilitating formation of high avidity interactions with the T cells and subsequent efficient target cell destruction. Furthermore, we provide supporting evidence for the role of ICAM-1 in vivo by demonstrating that ICAM-1 expression on nonhematopoietic target cells was dependent on the presence of infiltrating activated T cells, as was illustrated by restricted ICAM-1 expression at the sites of T cell infiltration in skin biopsies of patients with acute GVHD (aGVHD), by the absence of ICAM-1 expression in the same biopsies in areas without T cell infiltration and by the absence of ICAM-1 expression in biopsies of patients without GVHD independent of the presence of infiltrating nonactivated T cells. In conclusion, under noninflammatory conditions, nonhematopoietic tissues are unsusceptible to the GVHD reactivity of alloreactive T cells due to their inability to establish high avidity interactions.

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INTRODUCTION

Allogeneic stem cell transplantation (alloSCT) followed by cellular immunotherapy with donor-derived T cells can be a curative therapy for patients with hematological cancers [1,2]. After HLA-matched allo SCT, donor T cells can recognize patient cells as foreign, due to the expression of specific minor histocompatibility antigens (MiHA) [3-5]. Donor T cells recognizing MiHA specifically expressed on recipient hematopoietic cells, including the malignant cells, can mediate a therapeutic graft-versus-leukemia/lymphoma (GVL) effect [2,3,6-9]. However, donor T cells recognizing polymorphic peptides presented on normal nonhematopoietic tissues from the recipient may also mediate graft-versus-host disease (GVHD), which is the main cause of morbidity and mortality after alloSCT [10-12].

Surprisingly, high frequencies of circulating T cells expressing specific T cell receptors with high affinity for MiHA with ubiquitous expression in both hematopoietic and nonhematopoietic tissues have been detected in patients showing a strong GVL reaction in the

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absence of GVHD or with only limited GVHD [13-15]. These clinical data suggest that nonhematopoietic tissues are not always targeted by high affinity cytotoxic MiHA-specific T cells despite expression of the antigen. In contrast, in other studies, a clear correlation between the presence of T cells directed against ubiquitously expressed MiHA and the occurrence of GVHD has been suggested [16-18]. Intriguingly, Warren et al. [19] demonstrated that adoptive transfer of MiHA-specific donor T cells, even after in vitro depletion for reactivity against patient-derived fibroblasts, resulted in significant GVHD induction in vivo. Apparently, other factors besides expression of the MiHA also determine the susceptibility to T cell attack of nonhematopoietic target cells.

Efficient T cell-mediated cytolysis of (nonhematopoietic) target cells in vitro requires the formation of high avidity interactions between the immune effector cells and the target cells [20,21]. This avidity is determined by multiple factors, including the affinity of the T cell receptor of the effector cell, the level of expression of relevant peptide/MHC complexes on the target cell surface, and the expression of adhesion molecules, such as ICAM-1 [22-29]. In vivo, the local cytokine milieu most likely dictates the expression of molecules involved in the formation of a high avidity interaction between immune effector cells and nonhematopoietic target cells [24,30]. Investigations in mouse models demonstrated that conditioning and underlying diseases may damage host tissues resulting in production of so-called danger signals, including proinflammatory cytokines, chemokines, and amplified expression of adhesion molecules, MHC antigens, and costimulatory molecules on host tissue, thereby rendering these tissues more sensitive to T cell attack [11,31,32].

We hypothesized that in vivo the formation of a local proinflammatory cytokine milieu by locally activated T cells and subsequent sensitization of the surrounding nonhematopoietic cells to T cell attack determines whether or not GVHD is induced by T cells directed against ubiquitously expressed MiHA. In the current study, we demonstrate that the reactivity against skin-derived primary human fibroblasts of these T cells under noninflammatory, steady state conditions is low due to their inability to form a high avidity interaction. However, under proinflammatory circumstances, high avidity interactions are formed resulting in efficient targeting of the fibroblasts by the MiHA-specific T cells. ICAM-1 is demonstrated to be a key molecule mediating the high avidity interaction between T cells and nonhematopoietic target cells necessary for execution of their effector function, which is supported by the demonstration of co-localization of massive T cell infiltrates and upregulated ICAM-1 expression on the epithelial cells of the basal layer in skin biopsies of patients with acute GVHD (aGVHD). This study illustrates that in vivo reactivity of MiHA-specific T cells leading to GVHD cannot solely be predicted based on their antigen specificity. Under circumstances in which the magnitude of the immune response does not cause a local proinflammatory environment in the GVHD target tissues, targeting of MiHA with broad expression profiles may result in specific GVL reactivity without GVHD. On the other hand, absence of in vitro reactivity against patient nonhematopoietic tissues, as measured under noninflammatory conditions, does not guarantee protection against GVHD induction under clinical circumstances resulting in a local proinflammatory environment, which increases the susceptibility of cells in GVHD target tissues.

MATERIALS AND METHODS

Target Cells

After informed consent, primary human fibroblasts were generated from skin biopsies from patients or healthy donors. The skin biopsies were washed with PBS, minced, and transferred to 6-well culture plates containing low-glucose Dulbecco's modified Eagle medium (Lonza, Verviers, Belgium) supplemented with 10% FBS (Invitrogen, Breda, The Netherlands). Fibroblasts were cultured up to 90% confluency and then harvested using trypsin (Lonza, Verviers, Belgium) for 7 minutes at 37°C, followed by 2 washing steps. Stock samples were cryopreserved in liquid nitrogen. After thawing, the samples were reseeded at a concentration of 5000 cells/cm^2 and cultured again to 90% confluency, harvested, and reseeded. Experiments were performed using fibroblasts cultured for 5 to 20 passages. In specific experiments, fibroblasts were pretreated for 2 days with IFNgamma (IFNg; 200 IU/mL; Boehringer Ingelheim, Alkmaar, The Netherlands). In specific experiments, transduced fibroblasts were used. These fibroblasts were retrovirally transduced with pLZRS-constructs encoding HLA-A*0201, ICAM-1 (kindly provided by Dr. E. Hooijberg, VUMC, Amsterdam), or empty vector (mock), linked to the truncated human nerve growth factor receptor (NGFR) selection marker gene via an internal ribosome entry site sequence [33-35]. The identity of all constructs was verified by sequencing. Retroviral supernatants were generated with phoenix packaging (Φ-NX-A) cells, as previously described, and used for transduction of fibroblasts using recombinant human fibronectin fragments CH-296 (Lonza, Verviers, Belgium) [33].

Mock, ICAM-1, and HLA-A*0201-transduced fibroblasts were stained with NGFR-PE and purified by magnetic bead separation using anti-PE beads according to manufacturer's instructions (Milteny Biotec, Bergish Gladbach, Germany). Stable

Epstein-Barr virus (EBV)-transformed B cell lines (EBV-LCL) were cultured in Iscove's modified Dulbecco's medium (IMDM; Lonza, Verviers, Belgium) supplemented with 10% FBS. Adherent solid tumor cell lines from colorectal adenocarcinoma cell line (CCL-228; American Type Culture Collection [ATCC], Manassas, VA) and 2 renal cell carcinoma (kindly provided by Dr. B. Seliger, Department of Internal Medicine, Mainz, Germany) were cultured in RPMI supplemented with 10% FBS.

Cytotoxic T Cell Clone Generation and Culture

The cytotoxic CD8 positive T cell clone (CTL) RDR2, specific for the HLA-A*0201-restricted MiHA LB-ADIR-1F, was previously isolated from peripheral blood of a patient at the time of clinical response to donor lymphocyte infusion (DLI) as treatment for relapsed multiple myeloma after alloSCT [14,36]. The HLA-A*0201-restricted $SMCY_{311-319}$-HY-specific CTL was isolated from the peripheral blood of a female patient after rejection of a male stem cell graft [37]. The allo-HLA-A*0201-specific CTL MBM13 was isolated in vitro from an HLA-A*0201 mismatched mixed lymphocyte reaction. CTL clones were expanded in expansion-medium consisting of IMDM, supplemented with 5% FBS, 5% pre-screened pooled human serum, and 100 IU/mL IL-2 (Chiron, Amsterdam, The Netherlands). CTLs were restimulated using a feeder mixture containing expansion-medium supplemented with 5× irradiated (50 Gy) allogeneic mononuclear cells and 0.2× irradiated (50 Gy) allogeneic EBV-LCL, and 0.8 μg/mL phytohemagglutinin-HA16 (Remel, Dartford, United Kingdom) every 3 weeks. T cells were used for functional tests at 2 to 3 weeks after restimulation.

Abs and Flowcytometric Analysis

The FITC-labeled mAbs against CD2, CD4, CD11a, CD15 CD19, CD57, CD58, CD107a, and HLA-A*0201, the PE-labeled mAbs against CD19, CD90, CD102, CD103, HLA-A*02, Perforin, and NGFR, the cyanin-dye-7-coupled R-phycoerythrin (PE-Cy7)-labeled mAbs against CD3, and the allophycocyanin-labeled mAbs against CD137 and CD8 were obtained from Becton Dickinson (San Jose, CA). FITC-labeled mAbs against CD49D, CD49E, CD49F, and CD90, and panTCR-PE-Cy7 were obtained from Beckman Coulter (Fullerton, CA). CD11b-FITC, CD43-FITC, CD54-FITC, and granzyme-B-PE mAbs were purchased from CLB/Sanquin (Amsterdam, The Netherlands). FITC-labeled mAbs against CD18, CD166, and HLA-ABC were purchased from Serotec (Dusseldorf, Germany). FITC-labeled mAbs against CD31, CD62L, and CD62P were purchased from Bender MedSystem (Vienna, Austria). CD50-PE was purchased from ITK/Biolegend (San Diego, CA), CD62E-FITC from Bio-Connect (Huissen, The Netherlands), and CD106 from R&D System (Minneapolis, MN). allophycocyanin-labeled or PE-labeled tetramers for MiHA LB-ADIR-1F and SMCY-HY were constructed as described [38]. For surface molecule staining, cells were labeled with mAbs for 30 minutes at 4°C in PBS supplemented with 2% pasteurized plasma protein solution (Sanquin, Amsterdam, The Netherlands). For intracellular staining, cells were washed after surface staining, fixated with paraformaldehyde (1%), and permeabilized for 30 minutes at 4°C using PBS with 0.1% Saponine (Sigma-Aldrich, Steinheim, Germany). After permeabilization, cells were stained with mAbs for intracellular staining for 30 minutes at 4°C in PBS-Saponine. Flowcytometric analysis was performed on a BD flowcytometer using CellquestPro software and FlowJo software (Treestar, Ashland, OR). For quantitative fluorescent activated cell sorter (FACS) analysis, cells were taken up in 75 μL PBS/2% pasteurized plasma protein solution after surface staining, and short before performing FACS analysis 0.2 mg/mL propidium iodide and 10,000 flowcount fluorosphere beads (Beckman-Coulter, Woerden, The Netherlands) were added. For each sample, 2000 beads were acquired. The numbers of viable fibroblasts were calculated by correcting the analyzed numbers of CD90 positive, propidium iodide negative cells for the amount of beads.

Functional T Cell Analysis

Analysis of T cell activation and degranulation upon target cell encounter was performed by exposing 10,000 CTLs to fibroblasts or EBV-LCLs at various responder to stimulator (R/S) ratios (1/0.1-1/10). Supernatants were harvested, and IFNg release was measured by standard ELISA (Sanquin, Amsterdam, The Netherlands). In the same samples, the percentages of degranulating T cells were determined using flowcytometric analysis of CD107a, granzyme-B, and perforin expression. Analysis of cytotoxicity was performed by quantitative-FACS analysis or by conventional ^{51}Cr-release assay, in which target cells were labeled with 50 μCi $Na_2{}^{51}CrO_4$ (NEZ030005MC 185 MBQ, Perkin Elmer) for 1 hour at 37°C. In specific experiments, after labeling target cells were exogenously loaded with different concentrations (1 pM-1 μM) of the relevant MiHA peptides (SVAPALALFPA [LB-ADIR-1F] or FIDSYICQV [SMCY-HY]) for 1 hour at 37°C. Target cells (fibroblasts 1000 cells/well and EBV-LCL 10,000 cells/well) were seeded in 96-well plates together with MiHA-specific T cells at different effector/target (E/T)-ratios (100/1-0.01/1) in the presence or absence of blocking anti-ICAM-1 (CD54) (5 ng/mL) MoAbs (ITK/Biolegend). After 4, 8, and 20 hours of incubation, cytotoxicity was measured.

Confocal Microscopy

Primary human fibroblasts were seeded at 50,000 cells per glass bottom dish (P35G-1.5-14-C, MatTek Corporation) and cultured in Dulbecco's modified Eagle medium without phenolred (Invitrogen, Breda, The Netherlands) supplemented with 10% FCS. After 3 days, medium was removed and the adherent cells were washed 3 times with 2 mL IMDM w/o phenolred (Invitrogen) supplemented with 10% FCS. CTLs were labeled with 5 μM PKH26 according to the manufacturer's instructions (Sigma-Aldrich) and added to the fibroblasts at a 3/1 CTL/fibroblast ratio in IMDM without phenolred supplemented with 10% FCS and IL-2 (30 IU/mL). The dishes were incubated for 1 to 2 hours at 37°C, followed by analysis of cellular interactions by confocal microscopy using a 488 laser (Leica TCS SP2 inverted stand DM IRE2 microscope with PL APO 40× 1.25-0.75 with oil, Leica Microsystems Heidelberg, Germany). Series of differential-interference-contrast images and fluorescent (PKH26) images were acquired at intervals of 0.5 μm in z-direction. For each individual sample, 10 positions in the dish were examined (10 zstacks). Images and videos were analyzed using Leica Confocal Software/Image J.

Immunohistochemistry

After informed consent, biopsies of involved skin were obtained from healthy control individuals and from patients with clinically diagnosed acute skin GVHD (Lerner 1-2) after HLA-matched alloSCT as treatment for several hematological malignancies after myeloablative conditioning (n = 5). Tissues were fixed in 10% v/v formalin and embedded in paraffin. Immunohistochemical analysis was performed on 4 μm paraffin sections. Sections were deparaffinized, rehydrated, and antigen retrieval was performed using 0.01M citrate, pH 6.0, and boiling of the slides for 10 minutes in a microwave. Triple stainings were performed at room temperature with mouse monoclonal anti-human CD3 (clone PS1, IgG2a, 1:50; Abcam, Cambridge, MA), rabbit monoclonal anti-human CD54 (clone EP1442Y, 1:100; Abcam), or mouse monoclonal anti-human HLA-class II (clone CR3/43, IgG1, 1:50; Dako Netherlands BV, Heverlee, Belgium), and mouse or rabbit monoclonal anti-human cytokeratin (clone AE1/AE3, IgG1, 1:500; Dako) diluted in PBS/1% BSA (100 μL per sample). The next day, the slides were washed with PBS and incubated for 1 hour at room temperature with goat anti-mouse IgG2a ALEXA 546 (red), goat-anti-rabbit ALEXA 488 (green), and goat-anti-mouse IgG1 ALEXA 647 (blue) (Alexa, Invitrogen, Breda, The Netherlands). Next, the slides were washed with PBS. Images were captured with a confocal laser scanning microscope (LSM510; Zeiss, Sliedrecht, The Netherlands) in a multitrack setting (Department of Cell Biology, LUMC).

RESULTS

Primary Human Fibroblasts are Inefficiently Targeted by Minor Histocompatibility Antigen-Specific T Cells

To investigate whether nonhematopoietic tissues are susceptible to the cytotoxic effect of alloreactive MiHA-specific T cells, we exposed HLA-A*0201 positive, MiHA-positive primary human fibroblasts to high avidity cytotoxic CD8 positive CTL clones specific for the ubiquitously expressed HLA-A*0201-binding MiHA LB-ADIR-1F, SMCY-HY, or allo-HLA-A*0201, and examined cytotoxicity using conventional ^{51}Cr release assays and quantitative-FACS analysis. As shown in Figure 1A, only 5% to 30% of the fibroblasts were lysed within 4 hours by the T cell clones. In contrast, the same CTLs showed efficient lysis of 60% to 80% of MiHA-positive EBV-LCL, used as relevant substitutes for hematopoietic tumor targets. To investigate whether the inability of the MiHA-specific CTLs to efficiently attack MiHA-positive primary human fibroblasts was due to suboptimal presentation of the relevant antigens on the cell surface of the fibroblasts, we artificially increased the numbers of peptide-MHC complexes available on the HLA-A*0201 positive, LB-ADIR-1F negative, or positive fibroblasts by retroviral transduction with HLA-A*0201 expression constructs and exogenous loading with increasing concentrations of the relevant LB-ADIR-1F-peptide. Transduction with HLA-A*0201 expression constructs resulted in a 3-fold increase in the level of HLA-A*0201 expression compared to transduction with an empty vector (mock) (HLA-A*0201 mean fluorescence intensity [MFI] from 35 to 188). Whereas exogenous loading with up to 1 μM of LB-ADIR-1F peptide of MiHA-negative EBV-LCL resulted in efficient T cell-mediated cell death, exogenous peptide loading of MiHA-negative, or MiHA-positive fibroblasts did not increase the efficiency of T cell-mediated cell death induction within 4 hours, even after HLA-A*0201 transduction (Figure 1B). Similar results were obtained using SMCY-HY-specific CTL clones (data not shown). These experiments illustrate that the cytotoxic activity of CTLs against primary human fibroblasts is not merely limited by the number of peptide-MHC complexes on the target cell surface. Because fibroblasts are large adherent cells, we hypothesized that fibroblasts may be less efficiently killed by T cells because of a shortage of T cell effector molecules (granzymes, perforins, and death receptor ligands) available. However, increasing the amount of effector T cells available for engaging the fibroblasts by increasing E/T ratios up to 100/1 did not result in increased lysis (Figure 1C, black line). Furthermore, other large adherent HLA-A*0201 positive, LB-ADIR-1F positive cell lines, including 2 renal cell

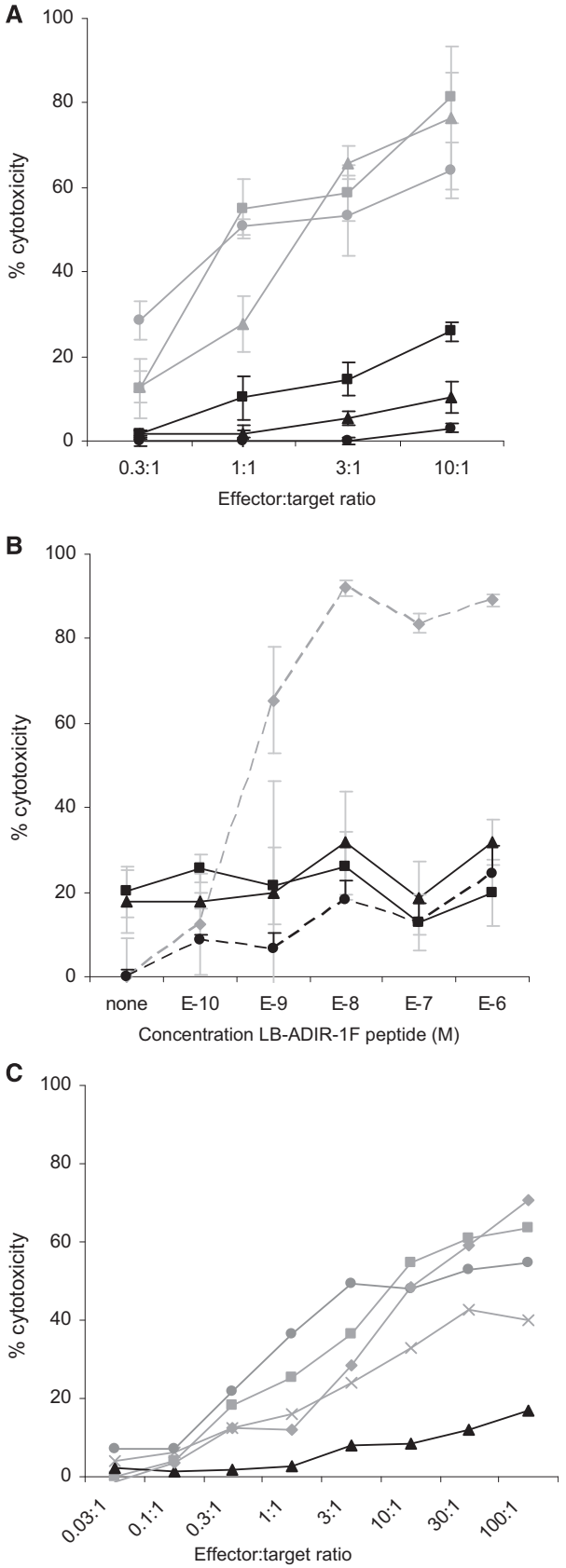

Figure 1. Primary human fibroblasts are inefficiently targeted by minor histocompatibility antigen-specific T cells cytotoxicity against primary human fibroblasts of different minor histocompatibility antigen (MiHA)-specific and allogeneic (allo)-HLA-specific T cell clones (CTLs) was analyzed in 4-hour ^{51}Cr release assays or by quantitative-fluorescent activated cell sorter (FACS) analysis. Epstein-Barr virus-transformed B cell lines (EBV-LCL) were used as hematopoietic control cells. (A) Cytotoxicity against primary human fibroblasts (black lines) and EBV-LCL (grey lines) expressing the relevant antigens of 3 CTL clones specific for the MiHA LB-ADIR-1F (■), allo-HLA-A*0201 (●), or the male-specific antigen SMCY-HY (▲) was analyzed at different effector/target (E/T) ratios. Primary human fibroblasts were less efficiently attacked than EBV-LCL (mean ± SD of 3 experiments, $P < .01$). (B) Cytotoxicity of LB-ADIR-1F-specific CTLs against HLA-A*0201-positive, LB-ADIR-1F-negative EBV-LCL (--◆--), HLA-A*0201-positive, LB-ADIR-1F-negative fibroblasts (--●--), and HLA-A*0201-positive, LB-ADIR-1F-positive fibroblasts, transduced with MOCK-nerve growth factor receptor (NGFR) (▲) or HLA-A*0201-NGFR (■), exogenously loaded with increasing concentrations of the LB-ADIR-1F peptide, was analyzed (E/T ratio 10/1). Increment of HLA-A*0201 expression and exogenous loading of the fibroblasts with the relevant LB-ADIR-1F peptide did not result in increased cytotoxicity. Data shown are means ± SD of 3 replicates from a representative of 3 independent experiments. (C) Cytotoxicity of increasing numbers of LB-ADIR-1F-specific CTLs against HLA-A*02 positive, LB-ADIR-1F positive primary human fibroblasts (▲) and other target cells, including EBV-LCL (■), colon carcinoma cell line (×), and 2 renal cell carcinoma cell lines (● and ◆) was analyzed. All target cells except the primary human fibroblasts were properly killed within 4 hours. Data shown are a representative example from 3 independent experiments.

carcinoma cell lines and a colon carcinoma cell line, could be effectively killed by LB-ADIR-1F-positive CTL clones within 4 hours (Figure 1C, grey lines). These data illustrate that the inferior killing of fibroblasts was not caused by the size of the fibroblasts, a shortage of available peptide-MHC complexes, or shortage of effector T cells.

Inferior Kill of Fibroblasts is not Caused by Lack of T Cell Activation or Lack of Degranulation of Cytotoxic Granules

Because the same CTLs that induced only marginal lysis of the primary human fibroblasts were able to exert profound cytotoxicity against EBV-LCL, we investigated whether the LB-ADIR-1F-specific CTLs were activated upon HLA-A*0201-positive, LB-ADIR-1F fibroblast encounter at an E/T-ratio of 1/1. Although the percentages of activated T cells were higher upon EBV-LCL encounter, still more than 50% of the T cells were antigen-specifically activated within 4 hours upon fibroblasts encounter, as shown by the induction of expression of the activation marker CD137 (Figure 2A) and a decrease in tetramer expression (Figure 2C). Furthermore, CTLs produced IFNg upon MiHA-positive fibroblast encounter within 4 hours, although the concentrations were lower compared to EBV-LCL encounter (Figure 2B). Next, we examined whether this T cell activation by the fibroblasts resulted in release of their cytotoxic granules. As shown in Figure 2C, stimulation with MiHA-positive fibroblasts resulted in degranulation of 50% of the LB-ADIR-1F-specific CTLs after 4 hours, as visualized by the decrease in intracellular

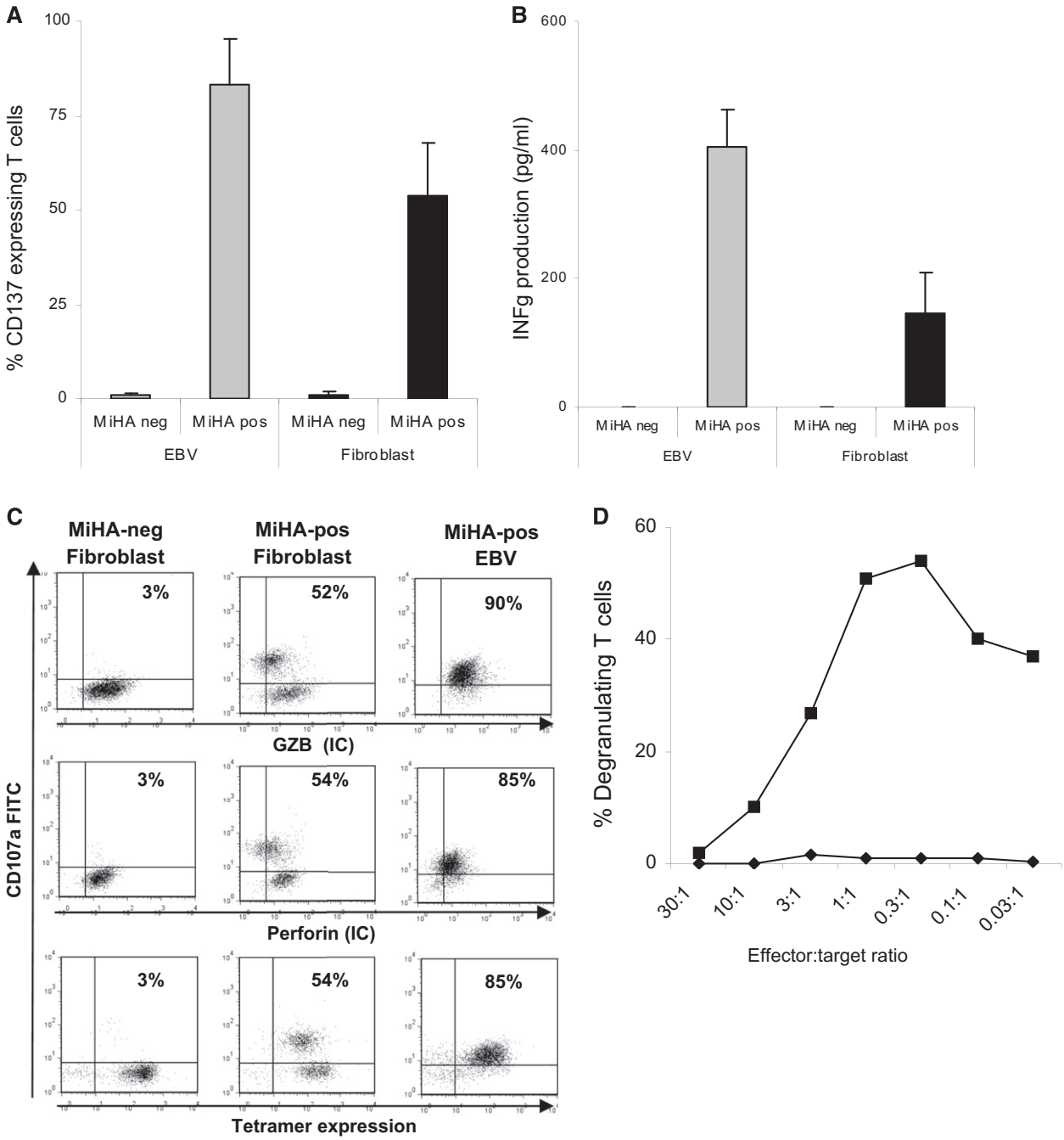

Figure 2. Inferior killing of fibroblasts is not caused by the absence of T cell activation or degranulation of cytotoxic granules. The activity of LB-ADIR-1F-specific T cell clones (CTLs) against primary human fibroblasts was analyzed after 4 hours using different read-out systems. Epstein-Barr virus-transformed B cell lines (EBV-LCL) were used as hematopoietic control cells. Activation of the CTLs in response to encounter of HLA*0201-positive, LB-ADIR-1F-negative or positive primary human fibroblasts (black bars), or EBV-LCL (grey bars) (responder to stimulator [R/S] ratio 1/1) was determined by (A) flowcytometric analysis of cell surface CD137 expression, and by (B) IFNgamma (IFNg) production using IFNg ELISA. (C) The percentages of activated and degranulating CTLs were determined by visualization of decreased intracellular expression of granzyme B (GZB) and perforin, or decreased tetramer staining, and induction of CD107a expression. A representative example of the flowcytometric analysis is shown. The dot plots shown are gated based on CD3 staining. T cell activation and degranulation of at least 50% of the CTLs was observed after encounter of the primary human fibroblasts. (D) The percentages of degranulating CTLs in response to increasing numbers of HLA*0201-positive, LB-ADIR-1F-negative (◆), or positive (■) primary human fibroblasts were examined. Increasing the amount of CTLs resulted in a decrease in the percentages of degranulating T cells.

granzyme B (GZB) and perforin levels and the induced expression of CD107a. In contrast, 85% to 90% of the CTLs released their cytotoxic granules upon EBV-LCL encounter. These observations also applied for the other CTLs tested (data not shown). As shown in Figure 2D, increasing the amount of CTLs added to the cultures resulted in a decrease in the percentages of degranulating T cells, indicating that there is a limiting amount of antigen and stimulator molecules available. This can explain why increasing the amount

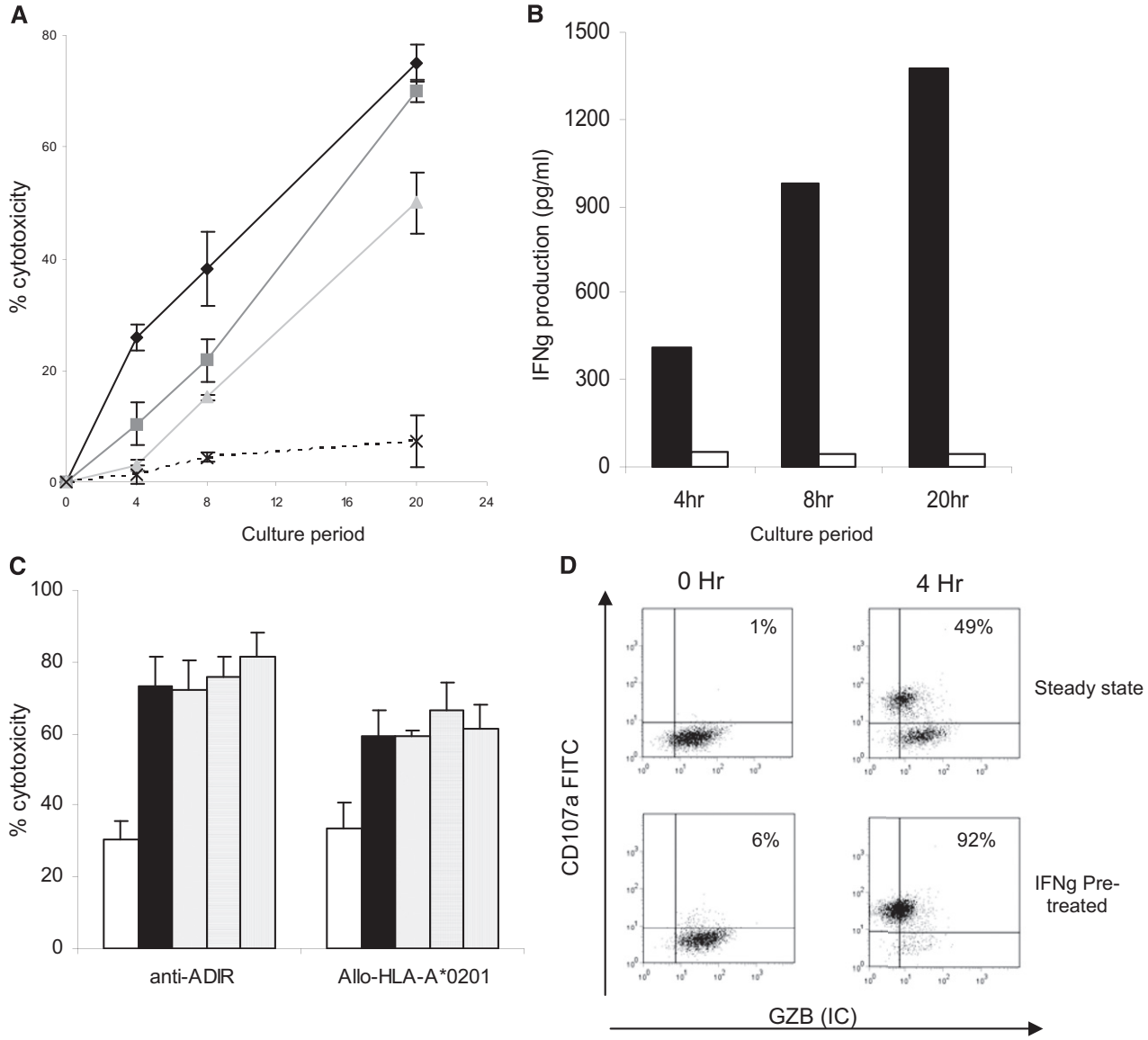

Figure 3. T cell clone (CTL)-mediated lysis of primary human fibroblasts is improved after prolonged co-culture due to IFNgamma (IFNg)-mediated enhanced crosstalk (A) The cytotoxic activity against HLA-A*0201-positive, minor histocompatibility antigen (MiHA)-positive primary human fibroblasts of CTLs specific for the MiHA LB-ADIR-1F (◆), HY-SMCY (■), or for allogeneic (allo)-HLA-A*0201 (▲) was analyzed using 4, 8, and 20 hour ^{51}Cr release assays (effector/target [E/T] ratio 10/1). A CTL clone specific for the hematopoiesis-restricted MiHA HA-1 was used as negative control (×, dashed line). Prolonged co-culture resulted in increased cytotoxicity. Values are means ± SD of 3 replicates from a representative of 3 independent experiments. (B) The cumulative levels of IFNg in the supernatant at different periods of co-culture of LB-ADIR-1F-specific T cells with HLA*0201-positive, LB-ADIR-1F-positive (black bars), and LB-ADIR-1F-negative (white bars) fibroblasts were determined using standard IFNg ELISA (responder to stimulator [R/S] ratio 1/3). (C) Cytotoxicity of LB-ADIR-1F-specific and allo-HLA-A*0201-specific T cells against non-pretreated (white bars), supernatant-pretreated (black bars), IFNg-pretreated (with 500 pg/mL [diagonal lines], 1000 pg/mL [horizontal lines], or 10,000 pg/mL [vertical lines] of IFNg) LB-ADIR-1F-positive, HLA*0201-positive primary human fibroblasts was analyzed in conventional 4 hour ^{51}Cr release assays (E/T ratio 10/1). Improved cytotoxicity of primary human fibroblasts was seen after pretreatment with pure IFNg or with IFNg-containing co-culture supernatants. (D) Degranulation of the LB-ADIR-1F-specific CTL in response to encounter of IFNg-pretreated (lower panel) and non-treated (upper panel) HLA*A0201-positive, LB-ADIR-1F-positive primary human fibroblasts (R/S ratio 1/3) was determined after 4 hours by flowcytometric analysis of intracellular expression of granzyme B (GZB), and degranulation visualized by CD107a expression. A representative example of the flowcytometric analysis is shown. Uniform degranulation was observed after IFNg-pretreatment of the fibroblasts.

of CTLs did not result in increased CTL-mediated lysis (Figure 1C). These data illustrate that the CTLs showed significant interplay with the MiHA-positive fibroblasts, resulting in their activation and subsequent degranulation. However, this crosstalk was not sufficient for efficient target cell lysis within 4 hours of co-culture.

CTL-Mediated Lysis of Primary Human Fibroblasts is Improved after Prolonged Co-culture due to IFNg-Mediated Enhanced Crosstalk

Because crosstalk between CTLs and fibroblasts did not lead to efficient target cell lysis within 4 hours, we investigated whether prolonged interaction could

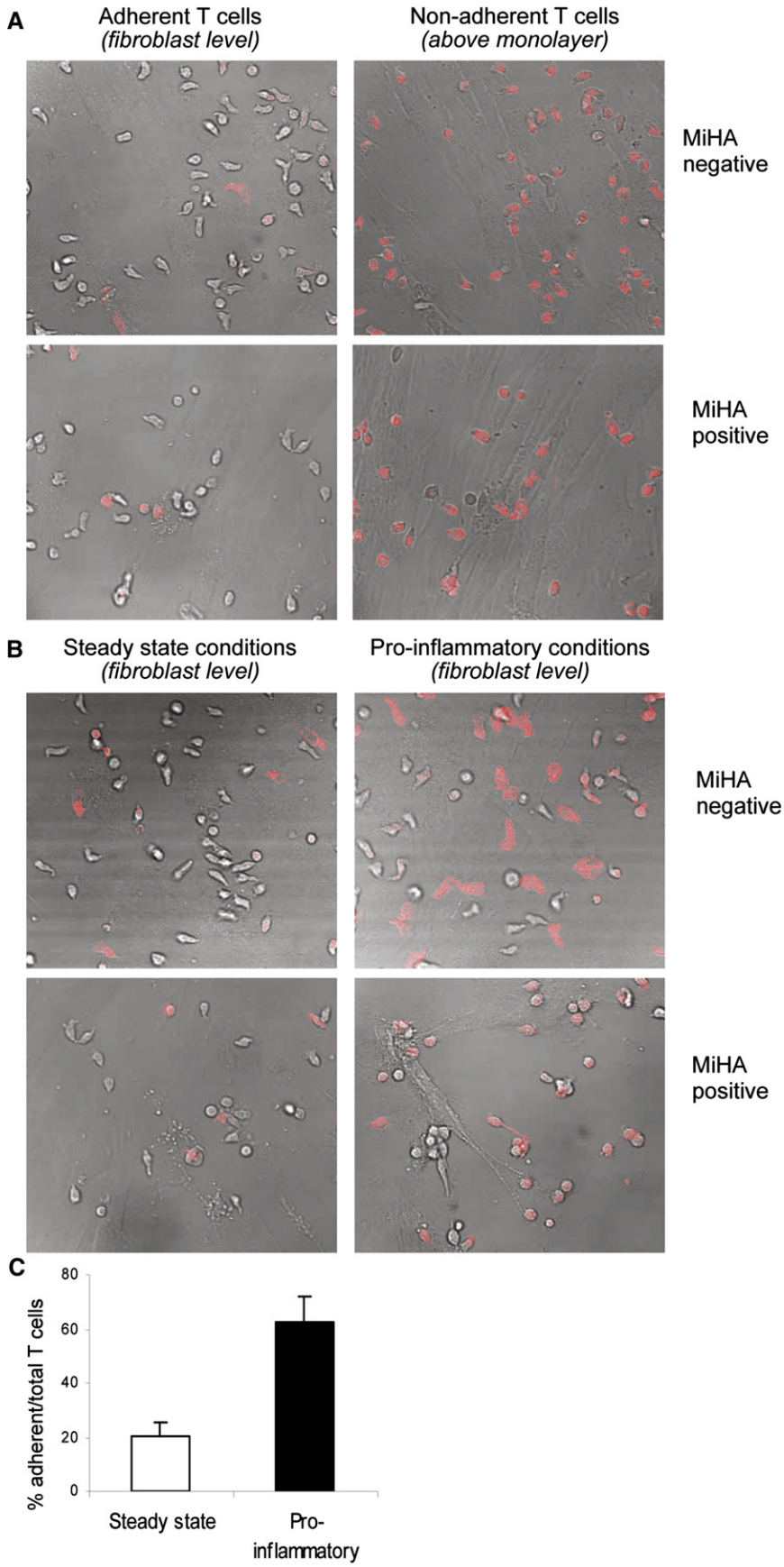

Figure 4. IFNgamma (IFNg) pretreatment of primary human fibroblasts allows strong antigen-independent adhesion of minor histocompatibility antigen (MiHA)-specific T cell clones (CTLs) to the fibroblasts. The percentages of adherent and non-adherent PKH26-labeled MiHA-specific CTLs to

increase the CTL-mediated lysis of primary human fibroblasts by performing 8 and 20 hours cytotoxicity assays. As shown in Figure 3A, prolonged co-culture of fibroblasts with either MiHA-specific or allo-HLA-A*0201-specific CTLs resulted in increased lysis of the HLA-A*0201-positive/MiHA-positive fibroblasts (solid lines), whereas no increase in a specific lysis by the negative HA-1-specific T cell clone was observed (dashed line). Next, we wondered whether this increased, Ag-specific lysis after prolonged co-culture was the result of the local formation of a more proinflammatory environment due to secreted cytokines. We already demonstrated that CTLs started to produce IFNg upon fibroblast encounter (Figure 2B), and analysis of longer culture periods showed increasing levels of IFNg up to 1400 pg/mL after 20 hours (Figure 3B). To examine the role of the secreted cytokines, we pretreated fibroblasts with supernatants harvested from 20 hours co-cultures of the MiHA-specific CTLs with MiHA-positive fibroblasts, and tested their susceptibility to CTL-induced lysis in 4 hours cytotoxicity assays. Pretreatment with these supernatants was sufficient to significantly increase 4 hours cytotoxicity (Figure 3C). To investigate the specific role of IFNg, we examined whether pretreatment of the fibroblasts solely with IFNg (500, 1000, and 10,000 pg/mL for 24 hours) resulted in similar sensitization of the fibroblasts to CTL-mediated lysis. As shown in Figure 3C, pretreatment of the fibroblasts with only 500 pg/mL IFNg was sufficient to increase their sensitivity to T cell-mediated cell death to the same extent as was observed when fibroblasts were pretreated with the supernatants. As shown in Figure 3D, IFNg pretreatment of LB-ADIR-1F-positive fibroblasts also increased their capacity to activate all LB-ADIR-1F-specific T cells as reflected by their uniform degranulation after 4 hours of co-culture. These data illustrate that, during prolonged co-culture, CTLs produced IFNg leading to increased interplay between the CTL and the fibroblast, which resulted in increased susceptibility to CTL-mediated lysis of the primary human fibroblasts.

IFNg Pretreatment of Primary Human Fibroblasts Allows Formation of High Avidity Interactions with Minor Histocompatibility Antigen-Specific T Cells

Because IFNg pretreatment of fibroblasts resulted in increased interplay with the CTLs leading to their increased susceptibility to T cell-induced cell death, we analyzed in detail interactions between the 2 cell types using confocal fluorescent microscopy under different conditions. First, we examined under steady state conditions the interactions between HLA-A*0201-positive, MiHA-negative or MiHA-positive fibroblasts, and MiHA-specific CTLs, which were labeled with the fluorescent dye PKH26. After 1 to 2 hours of co-culture, the percentages of CTLs strongly adhering to the fibroblasts were assessed using fluorescent confocal microscopy. To assess this, we determined the number of T cells showing red fluorescence in focus at the level of the fibroblasts monolayer (adherent T cells) and above the monolayer (nonadherent T cells). As demonstrated in Figure 4A to C, only 20% of the CTLs strongly adhered to the fibroblasts layer under steady-state conditions, irrespective whether they were MiHA-negative or positive, indicating that only limited interactions occurred between the 2 cell types. Next, we examined interactions between CTLs and fibroblasts, which were pretreated with IFNg to mimic proinflammatory

Table 1. Surface Molecule Expression on Fibroblasts with or without IFNg Pretreatment

		Expression (MFI)	
CD-Number	Antigen Name	IFNg −	IFNg +
CD2	LFA-2	−	−
CD11a	LFA-1	−	−
CD11b	a-M-integrine	−	−
CD15	Leu-M1	−	−
CD18	b2-integrine	−	−
CD31	PECAM-1	−	−
CD43	Leukosialine	+ (106)	+ (112)
CD49d	VLA4a	+ (95)	+ (100)
CD49e	VLA5a	+ (192)	+ (220)
CD49f	VLA6a	+ (151)	+ (178)
CD50	ICAM-3	+ (98)	+ (88)
CD54	**ICAM-1**	**− (68)**	**++ (527)**
CD57	HNK-1	−	−
CD58	LFA-3	+ (116)	+ (116)
CD90	Thy-1	++ (7606)	++ (5993)
CD62E	E-selectine	−	−
CD62L	L-selectine	−	−
CD62P	P-selectine	−	−
CD102	ICAM-2	−	−
CD103	Integrine-a-E-b-7	−	−
CD106	VCAM-1	−	−
CD166	ALCAM	+ (103)	+ (101)

MFI indicates mean fluorescence intensity; IFNg, IFNgamma.
− = negative (MFI <70).
+ = positive (MFI ≥80).
++ = high expression (MFI ≥500).
The ICAM-1 result is emphasized in bold.

a monolayer of HLA-A*0201-positive, MiHA-negative, or MiHA-positive primary human fibroblasts was analyzed at the level of the fibroblasts monolayer (adherent T cells) and above the monolayer (non-adherent T cells) using fluorescent confocal microscopy after 1 to 2 hours of co-culture (effector/target [E/T] ratio 3/1). Ten to 12 regions per glass slide were analyzed. (A) Representative images are shown for both levels. The percentage of adherent T cells was limited. (B) The percentages of adherent and non-adherent PKH26-labeled MiHA-specific CTLs to a monolayer of IFNg pretreated (right pictures), or non-treated (left pictures) HLA-A*0201-positive, MiHA-negative, or MiHA-positive primary human fibroblasts were analyzed. Representative images for the number of adhering CTLs at the level of the fibroblast layer are shown. (C) Cumulative data of the percentages of adherent MiHA-specific CTLs to the IFNg pretreated (black bar), or non-treated (white bar) MiHA-negative fibroblasts of 3 independent experiments are shown (means ± SD). IFNg pretreatment of the fibroblasts significantly increased the percentages of CTLs adhering to the fibroblasts independent of antigen expression ($n = 3$, $P < .01$).

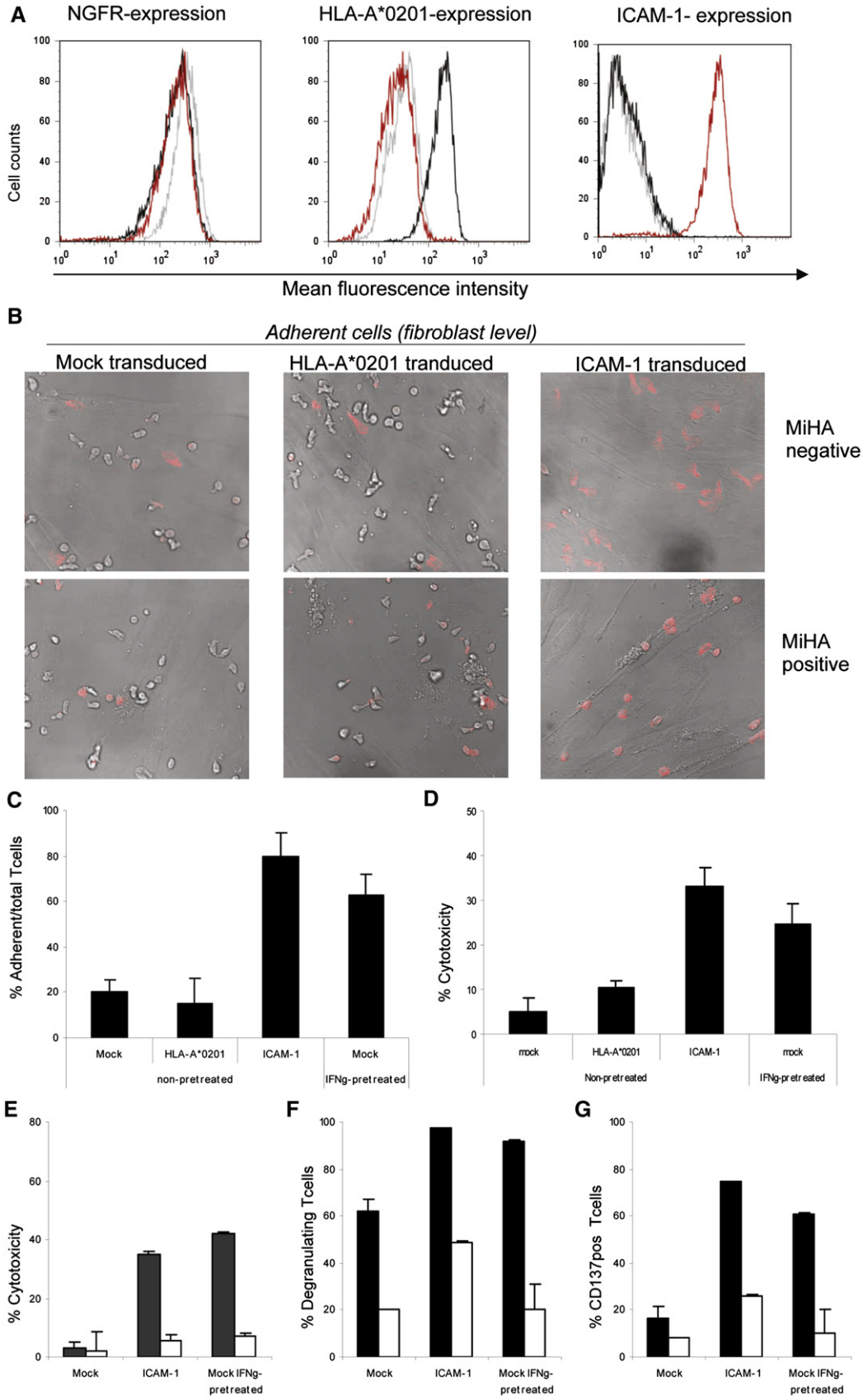

Figure 5. ICAM-1 is a key molecule in the interaction of minor histocompatibility antigen (MiHA)-specific T cells with fibroblasts mediating efficient T cell activation and target cell lysis. The activity of different MiHA-specific T cell clones (CTLs) against HLA-A*0201-positive primary human fibroblasts, which were retrovirally transduced with mock-nerve growth factor receptor (NGFR), HLA-A*0201-NGFR, or ICAM-1-NGFR expression constructs

conditions. As shown in Figures 4B and C, IFNg pretreatment of HLA-A*0201-positive MiHA-negative fibroblasts strongly increased the capacity of the CTLs to firmly adhere to them as indicated by the red fluorescence in focus at the fibroblast level, even in the absence of specific antigen recognition. Similarly, strong increases in the number of CTLs adhering to the MiHA-positive fibroblasts were observed, although quantitative analysis was hampered by the interference of T cell-mediated target cell lysis (Figure 4B, lower panel). These data illustrate that IFNg pretreatment of the fibroblasts allowed strong adherence of the CTLs to the fibroblasts, which enhanced their cross-talk leading to increased susceptibility for T cell-mediated lysis.

Efficient T Cell Activation and Target Cell Lysis is Primarily Dependent on ICAM-1 Expression on MiHA-Positive Fibroblasts

Next, we examined differential surface expression of a panel of adhesion and co-stimulatory molecules known to be involved in T cell-target cell interactions on fibroblasts before and after IFNg pretreatment. In addition to increased expression of MHC class I and II, only ICAM-1 (CD54) was strongly upregulated after IFNg pretreatment (Table 1). Although we already demonstrated that solely increasing the amount of the specific HLA molecules presenting the relevant CTL epitopes (HLA-A*0201) did not increase susceptibility to CTL-mediated lysis (Figure 1B), we investigated the individual role of ICAM-1 and HLA-A*0201 in CTL-fibroblast interaction, by inducing expression of ICAM-1 and HLA-A*0201 in MiHA-negative and positive fibroblasts by retroviral transduction. T cell clones showed profound expression of the ICAM-1-ligand lymphocyte function-associated antigen-1 (LFA-1/CD11a) (data not shown). Figure 5A shows the increased expression of NGFR, HLA-A*0201 (from 35 to 188) and ICAM-1 (from 6 to 297) after retroviral transduction. As shown in Figure 5B and C, solely, the induction of ICAM-1 expression on the surface of MiHA-negative or MiHA-positive fibroblasts was sufficient to allow firm adherence of the T cells to the fibroblasts to a similar extent as seen after IFNg pretreatment, whereas increased expression of HLA-A*0201 did not affect the numbers of adherent CTLs. In concordance with the previous observations, introduction of ICAM-1 expression on MiHA-positive human fibroblasts resulted in increased susceptibility to CTL-mediated cytotoxicity after 4 hours (Figure 5D). To investigate whether the increased levels of CTL-mediated cytotoxicity after IFNg pretreatment could be attributed to the ICAM-1 upregulation, we blocked the ICAM-1-LFA-1 interactions using anti-ICAM-1 mAbs. As shown in Figure 5E, blockade of ICAM-1 significantly diminished the CTL-mediated lysis of IFNg-pretreated and ICAM-1 transduced fibroblasts. Moreover, blockade of ICAM-1 also diminished activation of LB-ADIR-1F-specific T cells as reflected by reduction of the numbers of degranulating CTLs (Figure 5F) and the numbers of CD137 expressing CTLs (Figure 5G). Altogether, these data show that ICAM-1 expression on primary human fibroblasts is a key factor in the interaction with MiHA-specific T cells determining the efficiency of T cell activation and target cell lysis.

Co-Localization of T Cell Infiltration and Increased ICAM-1 Expression on Epithelial Basal Layer Keratinocytes in Skin Biopsies of Patients with aGVHD

To investigate whether ICAM-1 expression is associated with T cell-mediated target cell damage in vivo, we performed multicolor fluorescent confocal microscopy in skin biopsies of healthy controls and patients with acute skin GVHD after alloSCT (n = 5). In Figure 6, fluorescent images of these skin biopsies are shown stained for T cell infiltration (red) and ICAM-1 expression (green) in the dermis and epidermis regions. In normal skin biopsies taken from healthy individuals, T cells residing in the dermal layer of the skin are frequently detected. In these regions, as well as in regions without T cell infiltration, no ICAM-1 expression on the surrounding nonhematopoietic cells

was analyzed using different read-out systems. (A) Mean fluorescence intensity (MFI) of expression levels of NGFR, HLA-A*0201, and ICAM-1 in mock-NGFR (grey lines), HLA-A*0201-NGFR (black lines), or ICAM-1-NGFR (red lines) transduced fibroblasts was analyzed by flowcytometry. Increased MFI expression levels of NGFR, HLA-A*0201, and ICAM-1 were observed. Data shown are representative examples of 3 independent experiments. (B-C) The percentages of adherent and non-adherent PKH26-labeled MiHA-specific CTLs to a monolayer of HLA-A*0201-positive, MiHA-negative, or MiHA-positive primary human fibroblasts were analyzed at the level of the fibroblasts monolayer using confocal fluorescent microscopy after 1 to 2 hours of co-culture (effector/target [E/T] ratio 3/1). Ten to 12 regions per glass slide were analyzed. (B) Representative images are shown of CTLs adhering to the monolayers of mock transduced (left pictures), HLA-A*0201 transduced (middle pictures), or ICAM-1 transduced (right pictures) HLA-A*0201-positive, MiHA-negative (upper panel), or MiHA-positive (lower panel) primary human fibroblasts. (C) Cumulative data of the percentages of MiHA-specific CTL clones adhering to monolayers of HLA-A*0201-positive, MiHA-negative fibroblasts of 3 independent experiments are shown. IFNgamma (IFNg) pretreatment of the fibroblasts or induction of ICAM-1 expression significantly increased the percentages of CTLs adhering to the fibroblasts independent of antigen expression ($n = 3$, $P < .01$). (D-G) Cytotoxicity of LB-ADIR-1F-specific CTL against mock, HLA-A*0201, or ICAM-1-transduced HLA*0201-positive, LB-ADIR-1F-positive fibroblasts, which were non-pretreated or pretreated with IFNg (200 U/mL for 48 hours) was analyzed in conventional 4-hour ^{51}Cr release assays (E/T ratio 10/1). Improved cytotoxicity of primary human fibroblasts was seen after induction of ICAM-1 expression, comparable with the level of cytotoxicity seen after IFNg-pretreatment of the fibroblasts (D) ($n = 3$, $P < .01$). Improved CTL-mediated cytotoxicity (E), T cell degranulation (F) and activation (G) against both ICAM-1 transduced fibroblasts and Mock transduced IFNg-pretreated fibroblasts (black bars) could be blocked by the addition of ICAM-1-blocking mAbs (white bars). Values are means ± SD of 3 replicate wells from a representative of 3 independent experiments.

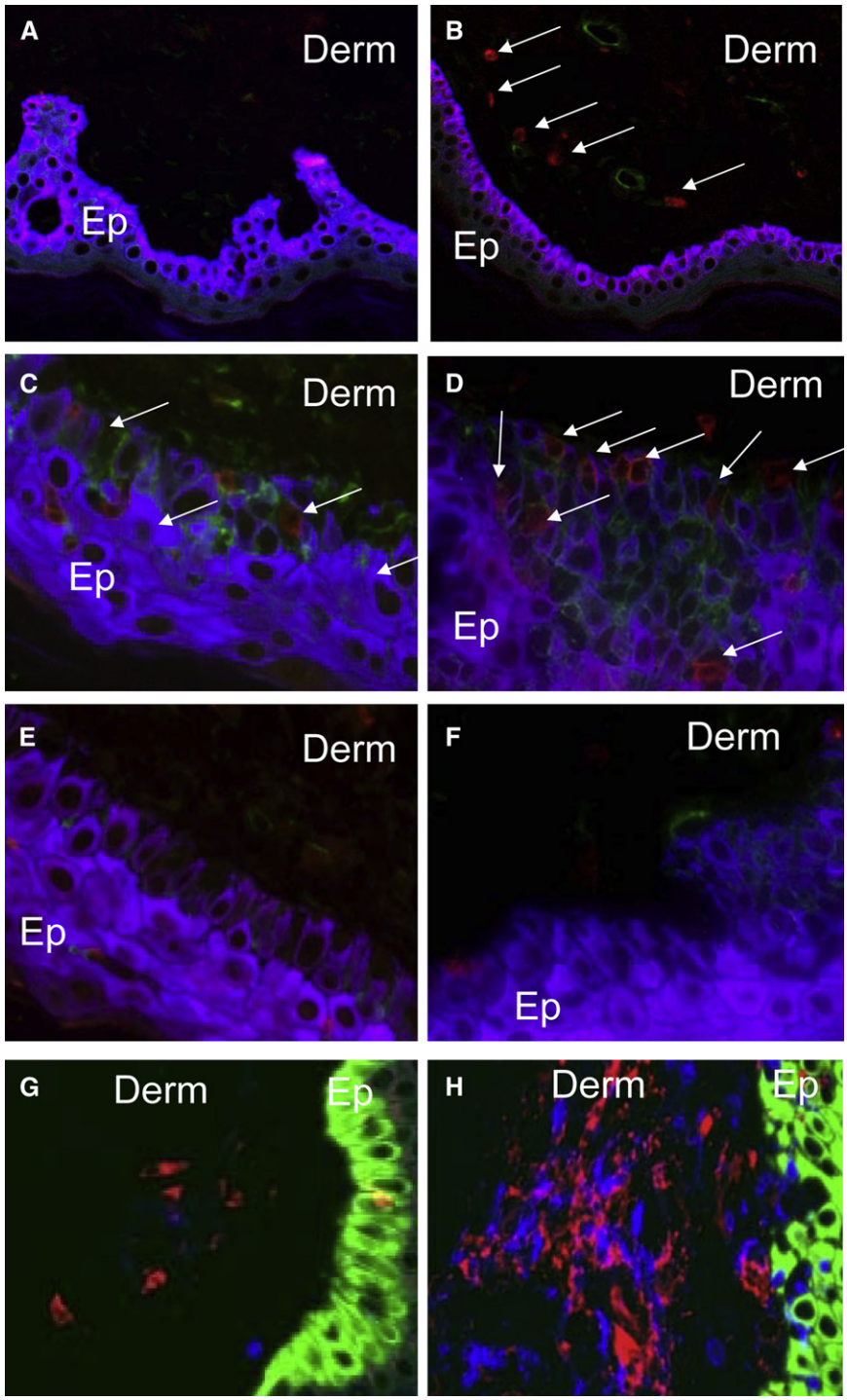

Figure 6. Co-localization of T cell infiltration and increased ICAM-1 expression on nonhematopoietic tissue in skin biopsies of patients with acute graft-versus-host disease (aGVHD). The expression and localization of ICAM-1 (green), CD3 (T cells, red), and cytokeratin (keratinocytes, blue) expression in normal skin biopsies (A-B) and in skin biopsies of patients suffering from acute skin GVHD (C-F) was determined using fluorescent immunohistochemistry. (A-B) Representative images (×250 magnification) of the epithelial (Ep) and dermal layer (Derm) of normal human skin are shown for regions with (A) or without (B) residing T cells (arrows) show no ICAM-1 expression on the surrounding nonhematopoietic cells. Circular green staining in the Derm represents vascular structures. Representative images (×250 magnification) of the epithelial (Ep) and dermal layer (Derm) of human skin at the site of aGVHD of 2 patients are shown for regions of the epithelial basal layer where T cell infiltration was present (C-D) and regions of the same corresponding biopsies in which no T cell infiltration was observed (E-F). In the regions in which infiltration of T cells and damage to the epithelial layers was observed (C-D), ICAM-1 expression was upregulated on the surrounding nonhematopoietic cells in the basal layer. In contrast, regions in the same biopsies in which no T cell infiltration was observed showed only minimal to absent ICAM-1 expression on the cells in the basal layer (E-F). To analyze the activation status of the residing/infiltrating T cells we performed a CD3 (blue), HLA-class-II (red), and cytokeratin (green) triple staining. Whereas T cells residing in normal skin showed no co-expression of HLA-class-II (G), T cells infiltrating in the skin GVHD lesion (H) were activated as indicated by their co-expression of HLA-class II.

was observed (Figure 6A, B). In the biopsies taken from skin GVHD lesions of patients who underwent alloSCT, regions could be identified in which massive T cell infiltrates were present (Figure 6C, D) as well as regions of the same biopsies showing no T cell infiltration (Figure 6E, F). In the regions with large amounts of T cells infiltrating both the dermis and epidermis and inducing damage to the epithelial layers, ICAM-1 expression was clearly upregulated on the surrounding nonhematopoietic cells in the basal layer (Figure 6C, D). In contrast, in surrounding parts in which no or only limited numbers of T cells were present, no significant ICAM-1 expression on the nonhematopoietic cells in the basal layer was detected (Figure 6E, F). To analyze the activation state of the T cells in biopsies from normal skin and in biopsies taken from skin GVHD lesions, we determined the HLA-class II expression on CD3 positive T cells. As shown in Figure 6G, T cells residing in normal skin were not activated as indicated by the absence of HLA-class II expression, whereas the majority of T cells in the dermal infiltrate in the GVHD biopsies showed clear co-expression of CD3 and HLA-class II reflected by the pink color (Figure 6H). These data illustrate that there is an association between the magnitude of the local T cell response, the coinciding formation of a local proinflammatory environment by the activated T cells, and ICAM-1 expression on nonhematopoietic tissues in the human skin during aGVHD.

DISCUSSION

The aim of the current study was to investigate in detail the reactivity of alloreactive T cells specific for ubiquitously expressed MiHA against nonhematopoietic patient-derived target cells. We demonstrated that in vitro reactivity of MiHA-specific T cells against nonhematopoietic targets cannot solely be predicted by their antigen-specificity and confirmed that adhesion molecule expression on the target cells was pivotal to facilitate the formation of high avidity interactions necessary for proper target cell destruction [20-27]. ICAM-1 expression on primary human fibroblasts was demonstrated to be a key molecule mediating these high avidity interactions. Because fibroblasts do not express ICAM-1 under noninflammatory steady-state conditions, only limited numbers of T cells adhered to the fibroblasts and, although these interactions did initialize crosstalk between the cells, this was not enough for efficient target cell destruction. During extended co-culture, crosstalk was intensified fueled by IFNg that was locally produced by the activated T cells leading to ICAM-1 expression on the fibroblasts and strong adherence to the T cells, which in the end rendered the fibroblasts sensitive to T cell-mediated lysis. Moreover, we provided supporting evidence for the role of ICAM-1 expression on nonhematopoietic tissues during ongoing GVHD responses in vivo by demonstrating co-localization of infiltration of activated T cells and increased ICAM-1 expression on the epithelial cells of the basal layer in skin biopsies of patients with aGVHD.

This study illustrates that, in the absence of local danger signals, low expression of ICAM-1 on nonhematopoietic GVHD target tissues prevents the occurrence of high avidity interactions with potent alloreactive T cells, thereby sparing these normal tissues. This can explain the clinical observations that high frequencies of T cells expressing specific T cell receptors with high affinity for MiHA with ubiquitous expression in both hematopoietic and nonhematopoietic tissues can circulate in patients showing GVL reactions in the absence of GVHD or with only limited GVHD [13-15]. When danger signals are present and ICAM-1 expression on the target cells is induced, strong adherence to the T cells is facilitated allowing target cell destruction. In our in vitro model, as well as in previous studies, the importance of the local proinflammatory cytokine milieu produced by the activated T cells in the induction of ICAM-1 expression is demonstrated [11,24,29,32,39,40].

Recent studies have shown the importance of the leukocyte function-associated antigen 1 (LFA-1)-ICAM-1 interaction between T cells and their targets cells for the execution of a proper cytotoxic effect [22-25,41-43]. There is strong evidence that the engagement of LFA-1 on the CTL by ICAM-1 on the target cells is essential for T cell activation and especially for directing the release of cytolytic granules toward the antigen-bearing tumor cells and the subsequent effective destruction of these cells by the CTLs [7,9,10,25,41]. Moreover, the ability of tumor cells to escape T cell immunity by ICAM-1 downregulation illustrates the importance of ICAM-1 expression on target cells in their susceptibility CTL attack [44-46].

In the current study, we demonstrated the absence of ICAM-1 expression on nonhematopoietic cells in skin biopsies of healthy control individuals, even at the regions in the dermis in which low numbers of T cells were residing, whereas profound ICAM-1 expression on the nonhematopoietic cells in the epidermis was demonstrated in biopsies taken from focal skin GVHD lesions at regions with profound infiltration of activated T cells. At the time of alloSCT, danger signals may be abundant due to the conditioning regimen preceding the transplantation, infections with pathogens, and concurrent inflammatory responses [31,47,48]. Moreover, malignant cells or professional antigen presenting cells (APCs) of the patient present in the skin can locally interact with T cells that reside in these tissues leading to T cell activation, proinflammatory cytokine production, upregulation of adhesion molecule expression, and

subsequent local amplification of the T cell response and tissue damage (GVHD). This may explain why infusion of mature donor T cells with or early after alloSCT often results in the induction of GVHD [11]. On the other hand, delayed application of DLI at 3 to 6 months after T cell-depleted alloSCT is associated with less severe GVHD, most likely because the tissue destruction by conditioning regimen has been repaired and antigen-presenting cells (APCs) of patient origin in the tissues are gradually replaced by donor cells [49,50]. In the absence of local danger signals, potent alloreactive donor T cells will most likely only briefly interact with the nonhematopoietic target cells and the high avidity interactions necessary for target cell destruction and local amplification of the T cell response will not be formed. Thus, after application of delayed DLI under appropriate circumstances, targeting of MiHA with a broad expression profile may result in specific GVL reactivity without GVHD.

Another possibility to achieve specific GVL reactivity without GVHD induction is adoptive transfer of donor T cells preferentially directed against hematopoietic tissues of the patient [19,51,52]. One possible strategy that has been exploited is the negative selection of potent GVHD-reactive donor-derived T cells by depleting T cells that show reactivity against patient-derived fibroblasts [19]. However, despite the lack of in vitro reactivity against patient-derived fibroblasts, these selected donor-derived T cells gave rise to significant GVHD after adoptive transfer. Based on our data showing that the presence of proper peptide-MHC complexes and also ICAM-1 expression is necessary for proper T cell reactivity upon fibroblast encounter, pretreatment with IFNg and longer assay periods could increase the sensitivity of this negative selection strategy using patient-derived fibroblasts as stimulator cells.

In conclusion, this study demonstrates that in vivo reactivity of MiHA-specific T cells leading to GVHD cannot solely be predicted by their antigen-specificity, but is also determined by adhesion molecule expression on the GVHD target tissues, which is dependent on the local proinflammatory environment of the host target tissues. Based on these findings, we hypothesize that in the situation of minimal residual disease under noninflammatory circumstances, application of unmodified DLI containing a broad repertoire of T cell specificities may mediate a specific GVL response in the absence of GVHD.

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