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FULL TITLE: **NKG2D-dependent effector function of bronchial epithelium activated alloreactive T cells**

SHORT TITLE: alloreaction in the lung

AUTHORS: Kirsten Kraetzel[1], Benjamin Stoelcker[1], PhD; Guenther Eissner*, PhD, Gabriele Multhoff**, PhD, Michael Pfeifer, MD; Ernst Holler*, MD, Christian Schulz, MD.

AFFILIATIONS: Department of Internal Medicine II, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

* Department of Hematology and Oncology, University of Regensburg, Germany. Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

** Department of Radiotherapy and Radiooncology, Klinkum rechts der Isar, Technische Universität München, Germany. Ismaningerstr. 22, 81675 Munich, Germany

[1] contributed equally

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CORRESPONDENCE: Christian Schulz, MD
Department of Internal Medicine II
University of Regensburg
Franz-Josef-Strauss-Allee 11
93053 Regensburg
Germany
Tel: +49-941-9440-7281
Fax: +49-941-9440-7282
E-mail: christian.schulz@klinik.uni-regensburg.de

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Abstract

Allogeneic hematopoietic stem cell transplantation (SCT) has emerged as a curative therapeutic option. However, The role of graft-versus-host disease in lung injury after SCT has still to be determined.

In the present study primary bronchial epithelial cells and the bronchial epithelial cell line BEAS-2B were used to investigate immune responses of allogeneic $CD8^+$ T-cells directed against respiratory epithelial cells.

Following stimulation with irradiated bronchial epithelial cells, $CD8^+$ T-cells produced significant amounts of IFN-γ, upregulated alloantigen activation markers and proliferated highly compared to T-cells stimulated with IL-2 alone. Furthermore, cytotoxicity assays demonstrated that bronchial epithelial cell specific and Granzyme B-mediated cytolytic activity was induced in $CD8^+$ T cells. Generation of NK-, NK-like T cells (NKT cells), cytokine-induced killer (CIK) or lymphokine activated killer (LAK) cells could be excluded by phenotyping, culture conditions and neglectable lytic activity following stimulation with IL-2 alone. Inhibition experiments showed that lysis of bronchial epithelial cells was not MHC I restricted but depended on natural killer group 2, member D (NKG2D) signaling, a stimulatory receptor initially shown to be expressed on NK cells.

Our data imply that respiratory epithelium has antigen presenting function and directly alloactivates cytotoxic $CD8^+$ T cells which show nonclassical effector function.

KEY WORDS: alloreaction, CD8 T cells, lung, MHC I, NKG2D.

Introduction

Diffuse lung injury is a major complication of allogeneic hematopoietic stem cell transplantation (SCT) and can account for up to 50% of transplant-related mortality [1]. Bronchiolar inflammation with pronounced epithelial damage and lymphocytic bronchitis can be found besides idiopathic pneumonia syndrome (IPS), a widespread and mainly alveolar and interstitial lung injury within the first 100 days after SCT [2]. The epithelial damage is discussed to be an alloreactive immune response targeting bronchial epithelial cells [2].

Nevertheless, the role of graft-versus-host disease (GvHD) and alloreactive T cells in pathogenesis of acute and chronic lung injury following SCT still remains controversial [3]. However, an association between lung injury and acute or chronic GvHD has been reported in clinical investigations as well as animal models [4-6]. Alloactivation depends on T cell receptor (TCR) stimulation and CD28 costimulation via CD80/CD86. Additional nonclassical pathways for alloactivation with the consequence of acute and chronic graft damage have also to be considered [7]. Inhibition of CD70 prolongs allograft survival independently from CD28 and [8] and $CD8^+$ T cells have been shown to be directly activated by the tissue of the grafted organ, irrespective of antigen-presenting cells (APC) [9].

Initially demonstrated to be a stimulatory receptor on natural killer (NK) cells, NKG2D (natural killer group 2, member D)-costimulation induces proliferation, survival, activation marker expression, cytokine production and cytotoxicity in $CD8^+$

T cells [10-13]. MHC class I-related chain A and B (MIC A/B) are known ligands for NKG2D [14-16].

We investigated if bronchial epithelial cells can function as nonclassical APC inducing epithelium directed allospecific cytotoxicity in CD8+ T cells through triggering NKG2D in a bronchial epithelial cells line (BEAS-2B) as well as in primary bronchial epithelial cells (PBEC).

Material and Methods

Cell culture

The bronchial epithelial cell line BEAS-2B, immortalized by transfection of bronchial epithelial cells of a healthy human individual with adenovirus 12-SV40 hybridvirus, was obtained from ATCC (CRL-9609) [17]. Cells were cultured in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% heat-inactivated FBS (PAA, Cölbe, Germany), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2. Prior to use, BEAS-2B were harvested with Accutase (PAA).

K562 cells, a chronic myeloid leukemia cell line without MHC I expression, was obtained from ATCC (CCL-243), kept in suspension culture under the same conditions and used as NK-sensitive control targets.

Culture of Primary Bronchial Epithelial Cells

Primary bronchial epithelial cells (PBEC) were isolated and cultured as previously described [18]. In brief, human PBEC cells from healthy volunteers were obtained during bronchoscopy and cultured in serum-free bronchial epithelial cell growth medium (Promocell, Heidelberg, Germany) containing penicillin G (100 U/ml), streptomycin (100 µg/ml), and amphotericin-B (0.25 µg/ml) (Invitrogen, Karlsruhe, Germany).

Isolation, stimulation and activation of $CD8^+$ lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood or apharesis products of healthy human volunteers (donors) according to a standard protocol using Ficoll-Hypaque density gradient centrifugation (Pharmacia, Freiburg, Germany). Subsequently, $CD8^+$ T cells were negatively selected from PBMCs using magnetically labeled beads following the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Interindividual differences between T cell donors did not exceed interexperimental differences.

Irradiated (30 Gy) BEAS-2B or PBEC served as stimulator cells in the following experiments. For alloactivation, $CD8^+$ T cells from a given donor were mixed at a concentration of 0.5×10^6 cells/ml with stimulators cells at a ratio of 1:1 and incubated in RPMI 1640 in the presence of low-dose interleukin 2 (IL-2, 100 U/ml). $CD8^+$ T cells were stimulated for 7 days or subsequently restimulated with fresh stimulator cells under the same conditions for an additional 3, 7 or 14 days, respectively. $CD8^+$ T cells

cultured in the presence of IL-2 alone served as control. Supernatants of these cultures were collected for analysis by ELISA.

Cell surface expression analysis

Expression of cell surface molecules was assessed by flow cytometry with FACSCalibur and CellQuest analysis software (BD Biosciences, Heidelberg, Germany).

Purity of isolated $CD8^+$ T cells was determined flowcytometrically before and after 7 days of stimulation with the Simultest IMK-Lymphocyte kit from BD Biosciences.

To determine expression of activation markers, both freshly isolated and activated T cells were stained with the following antibodies (BD Biosciences, if not noted otherwise): CD25 (M-A251), CD30 (BerH8), CD54 (HA58), CD69 (L78), CD 70 (Ki-24), CD71 (YDJ1.2.2, immunotech), CD80 (3771.11, R&D Systems), CD86 (37301.111, R&D Systems), CD95 (ZB4, immunotech), NKG2D (1D11, ebioscience) and HLA-DR (B8.12.2, immunotech) and the appropriate isotype controls.

Expression of MIC A and B on BEAS-2B and PBEC was determined by indirect immunofluorescence staining with anti-MIC A/B (clone 6D4, BD Biosciences). Cells were counterstained with propidium iodide (PI, Sigma-Aldrich, Deisenhofen, Germany) at a final concentration of 200 ng/ml, with only PI-negative cells being subjected to analysis.

Enzyme-linked immunosorbent assays

Colorimetric enzyme-linked immunosorbent assays (ELISA) were performed according to the manufacturer's protocol for detection of interferon γ (IFN-γ) (Pierce Endogen, Rockford, USA) in coculture supernatants after activation and for detection of granzyme B (Hoelzel Diagnostics, Cologne, Germany) in the supernatants of cytotoxicity assays.

CFSE proliferation assay

Freshly isolated $CD8^+$ T cells were labeled with CFSE (succinimidyl ester of carboxyfluorscein; Invitrogen) at a final concentration of 2 μM, washed and incubated with IL-2 alone, with stimulator cells in the presence of IL-2 or with staphylococcal enterotoxin B (SEB; Sigma-Aldrich) at a final concentration of 100 ng/ml as positive control. After 7 days of activation, fluorescence intensity of $CD8^+$ T cells was analyzed by FACS and the percentage of proliferating cells was determined.

Functional cytotoxicity assay

T cell-mediated cytotoxicity was measured with a standard 4 hour chromium ^{51}Cr radioisotope assay. Target cells (BEAS-2B, PBEC or K562) were labeled with sodium [^{51}Cr]chromate (100 μCi; Hartmann Analytics, Braunschweig, Germany) for 1.5 h, washed twice and then coincubated with activated $CD8^+$ T cells as effectors (see above), at descending effector-to-target cell (E/T) ratios. After 4 h incubation at 37°C,

cell free supernatants were harvested, radioactivity was determined by γ-counting and the percentage of specific lysis was calculated as: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. The percentage of spontaneous release [(spontaneous release/maximal release) x 100%] was less than 15% for each target cell in all experiments.

To determine Granzyme B-release, supernatants were collected from a cytotoxicity assay performed in parallel to the described ^{51}Cr release assay in the absence of radioactivity.

Following blocking assays were performed: $CD8^+$ T cells were preincubated with functional grade blocking antibodies directed against NKG2D (1D11, Biolegend) or IgG1 (MG1-45, Biolegend) at a final concentration of 5 μg/ml, or labeled target cells were incubated with functional grade blocking antibody directed against CD95 (ZB4, immunotech), TNF (MAK195, Knoll AG, Germany) or MHC class I antigens (W6/32). The Cells were immediately used in standard ^{51}Cr release assays after a 30-minute incubation period at room temperature. Control assays without blocking antibodies were always performed in parallel.

For cold target inhibition experiments, unlabeled (=”cold”) targets as competitors were added to the labeled (=”hot”) targets into a standard ^{51}Cr release assay ranged with increasing cold/hot ratios from 1 to 40 at a constant E/T ratio of 10:1.

Statistical analysis

If not stated otherwise, at least 4 independent experiments were performed and means ± SEM are shown. Means between two groups were tested for differences using the Student's t-test or the nonparametric Mann-Whitney rank sum test, means of three groups were compared using ANOVA (and following Dunn's or Tukey's post hoc test, respectively).

Results

Characterization of the lymphocyte population

The purified population of $CD8^+$ T cells obtained from PBMCs was controlled for its purity prior to use in all experiments. The population consisted of 89.4 (± 5) % $CD3^+CD8^+$ T cells with only 1.8 (± 1.7) % $CD3^+CD4^+$ T cells and less than 1 % $CD3^-$ $CD16/56^+$ NK cells (mean values ± SD of all experiments). No difference was found in these phenotypic characteristics between freshly isolated and 7 day stimulated cells finally used in functional assays.

Allogeneic activation of purified $CD8^+$ T cells by bronchial epithelial cells

HLA-mismatched $CD8^+$ T cells were stimulated with irradiated BEAS-2B cells for 7 days in the presence of low dose IL-2 (100 U/ml). $CD8^+$ T cells from the same donor were cultured in IL-2 medium alone to determine levels of unspecific activation. IFN-γ release as a hallmark of $CD8^+$ T cell activation was measured by ELISA in the supernatant of both cell cultures. Irradiated BEAS-2B cells produced no IFN-γ by

themselves (data not shown), $CD8^+$ T cells cultured in IL-2 medium alone produced low levels of IFN-γ (figure 1). BEAS-2B stimulated $CD8^+$ T cells released significantly higher amounts of IFN-γ than control cells. Restimulation of activated $CD8^+$ T cells for another 3 or more days resulted in a further significant increase in IFN-γ release (figure 1).

In addition, the surface expression of the activation markers CD25, CD30, CD54, CD69, CD 70, CD71, CD80, CD86, CD95 and HLA-DR was analyzed on $CD8^+$ T cells before and after 7 days of stimulation with irradiated BEAS-2B. Freshly isolated $CD8^+$ T cells showed either no, or only neglectable expression of the analyzed markers (data not shown). Following IL-2 stimulation alone, the percentage of positive cells and the mean fluorescence intensity (MFI, reflecting the antigen density on the cells) of positive cells were slightly but significantly upregulated for all activation markers except for CD86 (figure 2). In contrast, stimulation with irradiated BEAS-2B induced the expression of the alloactivation markers CD30 and CD69, but also CD25, CD70, CD71 and CD80 significantly with respect to both the percentage of positive cells as well as the antigen density on the positive cells (figure 2). This demonstrates that BEAS-2B cells activated $CD8^+$ T cells stronger than IL-2 alone.

Proliferation of $CD8^+$ T cells after alloactivation with bronchial epithelial cells

Following stimulation with BEAS-2B, 49.5 ± 12.4 % of the $CD8^+$ T cell population showed proliferation ($p<0.05$ vs. IL-2 alone, n=5, figure 3). Similar results were

obtained in the SEB control (50.3 ± 8.6 %), whereas IL-2 alone did not initiate relevant proliferation of cells (5.4 ± 2.6 %).

Cytolytic activity of alloactivated $CD8^+$ T cells against bronchial epithelial cells

Alloantigen-activated $CD8^+$ T cells showed high specific lysis of BEAS-2B target cells depending on the E/T ratio (figure 4). At the highest E/T ratio of 80:1, specific lysis of target cells amounted to a mean value of 50 % within 4 hours. IL-2 stimulated $CD8^+$ T cells showed only minor and significantly lower lytic activity against BEAS-2B, compared to BEAS-2B activated $CD8^+$ T cells ($p \leq 0.001$; data not shown). Freshly isolated, naive $CD8^+$ T cells were not able to kill BEAS-2B (figure 4). As BEAS-2B activated $CD8^+$ T cells showed low lytic activity against the MHC I negative NK cell target K562, we performed cold target inhibition assays to exclude residual NK cell or lymphokine activated killer (LAK) activity and to test whether BEAS-2B and K562 cells are damaged by the same or different effector cells. Both, BEAS-2B and K562 cells were simultaneously cocultured with the effectors, one target ^{51}Cr labeled (=hot target), the other added unlabeled (=cold) for competition. Lysis of BEAS-2B cells was not inhibited by the presence of K562 competitors at any cold/hot target ratio tested, whereas addition of cold BEAS-2B resulted in a concentration-dependent inhibition. On the other hand, addition of BEAS-2B competitors to hot K562 target cells inhibited K562 lysis, similarly effective as the cold K562 itself (figure 5).

Mechanism of the cytolytic activity of alloactivated $CD8^+$ T cells

To assess the mechanism of the lysis, the classical mediators of cytotoxicity (perforin/granzyme B, CD95 and TNF) were investigated. Granzyme B was detectable in significant amounts in the supernatants of the cocultures of cytotoxicity assays, increasing with higher E/T ratios. Alloactivated $CD8^+$ T cells did not produce granzyme B in the absence of BEAS-2B targets (figure 6 a). In blocking experiments, preincubation of targets with a neutralizing antibody against CD95 or TNF did not show any effect on lysis (figures 6 b and c). Interestingly, inhibition of MHC I did also not inhibit the lysis of BEAS-2B cells (figure 6 d).

FACS analysis demonstrated MIC A/B expression on BEAS-2B cells as well as NKG2D expression on $CD8^+$ T cells (figure 7 a), the latter being increased during activation (figure 7 b). Consequently, preincubation of $CD8^+$ T cells with anti-NKG2D antibody resulted in a significant inhibition of up to 25 % of BEAS-2B cell lysis in a standard ^{51}Cr release assay. An isotype-matched control antibody had no effect on lysis. The aforementioned low lysis levels of K562 without MHC I expression were inhibited to about 40% of controls with anti-NKG2D (figure 7 c).

To exclude cell type unspecific, artificial killing of BEAS-2B due to the viral immortalization, PBEC were used as activator and target cells in cytotoxicity assays. PBEC, which expressed MHC I (data not shown) and MIC A/B (figure 8a) were specifically lysed by PBEC alloactivated $CD8^+$ T cells. In line with the results seen in BEAS-2B, lysis was significantly inhibited by the anti-NKG2D antibody but blocking of MHC I did not reduce lysis of PBEC (figures 8 b and c).

Discussion

We could demonstrate that human bronchial epithelial cells are potent direct activators of cytotoxic $CD8^+$ T cells as well as targets for alloactivated $CD8^+$ T cells. This is of importance because T cells of donor origin have direct contact to lung epithelium, e.g. after cytokine-induced lymphocyte extravasation or because of TNF-induced endothelial apoptosis during the course of IPS [19]. The presence of donor T cells in bronchoalveolar fluids of patients after SCT furthermore underlines the relevance of the results of our study [20]. The findings support the hypothesis, that alloreactive $CD8^+$ T cells can account for a directly mediated lung injury seen after SCT.

BEAS-2B-activated $CD8^+$ T cells showed significant production of IFN-γ which was increased when the $CD8^+$ T cells were restimulated. Upregulation of the activation markers CD25, CD30, CD54, CD69, CD70, CD71, CD80, CD86, CD95 and HLA-DR on the BEAS-2B-activated $CD8^+$ T cells was significantly higher than after IL-2 stimulation alone which points towards an alloantigen-specific stimulation [21-23]. Particularly CD30 was reported to be present on alloreactive T cells, CD69 as well as HLA-DR were shown to be increased after alloantigen stimulation, and CD69 expression correlates with graft rejection [22, 24-26].

The observed alloactivation is paralleled by the proliferative response of $CD8^+$ T cells during stimulation with irradiated BEAS-2B, which was as high as the SEB-induced positive control. In contrast, no proliferation was found after stimulation with IL-2

only. We also found, that irradiated stimulator cells are rendered metabolically inactive and thus are no longer able to secrete any T cell activating proteins. All these results suggest a need for physical contact, e.g. MHC-TCR allospecific interaction, between effector and stimulator cells for efficient induction of proliferation (and hence alloactivation) to occur.

It is unlikely that the presence of NK or NKT cells during BEAS-2B stimulation accounts for the observed low-level lysis of the classical NK-cell target K562. The phenotype of the population, i.e. $CD3^+$ $CD4^-$ $CD8^+$ cells lacking the expression of the NK and NKT markers CD16/CD56, did not change during stimulation. The low dose IL-2 used for stimulation also strongly argues against the generation of LAK or CIK cells capable to lyse any cell type in an equal efficient way [27-29]. The cold target inhibiton assays demonstrated that BEAS-2B inhibited the lysis of K562, but not *vice versa*, showing that BEAS-2B stimulated effector cells have a higher affinity for BEAS-2B than for K562 cells, which is in contradiction to a NK, NKT or LAK mediated killing. Interestingly, cold target inhibition also demonstrated that K562 are lysed by the same effector population killing the BEAS-2B cells, albeit clearly to a lesser extent, and the residual lysis of K562 is also inhibited when NKG2D is blocked.

Killing of BEAS-2B or PBEC by the $CD8^+$ T cell effector population was not impaired in the presence of the MHC I blocking antibody W6/32. We demonstrated by FACS that W6/32 binds to BEAS-2B and PBEC and we did confirm the blocking function of the used lots of W6/32 in parallel cytotoxicity assays with human vascular endothelial cells as targets ([30] and own data not shown).

The impact of NKG2D for the effector function is emphasized by significantly decreased cytotoxicity when blocking the NKG2D-receptor on $CD8^+$ T cells and the finding that BEAS-2B cells and PBEC express the NKG2D-ligands MIC A and B. This is paralleled by upregulation of NKG2D on $CD8^+$ T cells during the course of alloantigen-specific activation. Importantly, experiments done with PBEC gave the same results as with BEAS2B, which excludes cell type unspecific induced effector function due to an immune response against the virally immortalized cell line BEAS-2B.

Inhibition of NKG2D did not completely abolish lysis, therefore additional mechanisms have to be assumed for costimulation, target recognition and lysis. As CD70 expression is increased on alloactivated $CD8^+$ T cells, the CD70-CD27 interaction might contribute to the costimulation, as has already been shown [8]. In our study cell lysis was very likely caused by the granzyme B/perforin pathway. Interestingly, neither neutralizing CD95 nor TNF inhibited $CD8^+$ T cell cytotoxicity although both are well known for their cytotoxic activity.

In summary, the results of the present study affirm that respiratory epithelial cells can function as nonclassical APC and represent a target of cytotoxic T cell reactivity. This supports the hypothesis of a lung directed GvHD following allogeneic SCT. Interestingly, respiratory epithelial cell specific cytotoxic T cells show nonclassical partially NKG2D mediated T cell effector function but not MHC I restriction although they express classical activation markers upon allogeneic activation. This

might be of clinical relevance for understanding the course of GvHD and lung injury seen in patients following SCT.

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Figures:

Figure 1. IFN-γ production of CD8+ T lymphocytes during stimulation:

Stimulation with irradiated BEAS-2B in the presence of low dose IL-2 (100U/ml) activates CD8+ T cells to secrete IFN-γ in significantly higher amounts than control CD8+ T cells incubated with IL-2 alone. Additional restimulation of BEAS-2B-activated CD8+ T cells significantly enhances IFN-γ secretion compared to a stimulation of 7 days. Difference versus control (IL-2 only): ** $p \leq 0.001$, * $p \leq 0.01$; difference versus 7 day stimulation with irradiated BEAS-2B: ++ $p \leq 0.001$, + $p \leq 0.01$.

Figure 2. Upregulation of activation markers on CD8+ T cells during stimulation:

Expression of the activation markers on CD8+ T cells was measured via FACS analysis immediately after isolation (naive) and after 7 days of stimulation with irradiated BEAS-2B in presence of IL-2 or with IL-2 alone (control). Means ± SEM of induction of mean fluorescence intensity (MFI) of positive cells (a) and of percentages of positively stained cells (b) out of 6 independent experiments are given as n-fold of naive: difference versus control: ** $p \leq 0.005$, * $p \leq 0.05$.

Figure 3. Proliferation of CD8+ T cells:

Proliferation was measured via CFSE-incorporation and FACS analysis in CD8+ T cells stimulated either with IL-2 alone as a control (a), with staphylococcus enterotoxin B (SEB) as positive control (b) or with irradiated BEAS-2B in the presence

of IL-2 (c) for 7 days. Histogram plots of one representative of 5 independent experiments are shown.

Figure 4. Cytolytic activity of $CD8^+$ T cells:

$CD8^+$ T cells were stimulated with irradiated BEAS-2B in the presence of IL-2 and subsequently subjected as effectors (E) to a ^{51}Cr release assay with BEAS-2B (black line) or K562 (dashed black line) as target cells (T) in increasing E/T ratios, or $CD8^+$ T cells were left naive and subjected to a ^{51}Cr release assay with BEAS-2B (dotted gray line) shortly after separation. Means ± SEM of percentage of specific lysis at varying E/T ratios of independent experiments are shown. Difference versus naive: ** $p \leq 0.001$; difference versus K562: ++ $p \leq 0.001$.

Figure 5. Cytolytic activity of $CD8^+$ T cells:

Standard ^{51}Cr release assay with activated $CD8^+$ T cells and ^{51}Cr-labeled BEAS-2B or K562, respectively, as targets (T) was performed at an E/T ratio of 10 as described (control). Additional unlabeled (=cold) BEAS-2B or K562, respectively (=competitors) were added to each labeled (=hot) target in increasing cold/hot target ratios. Percentage of specific lysis of the labeled targets is given as n-fold of control lysis at varying cold/hot ratios.

Figure 6. Mechanism of cytolytic activity of $CD8^+$ T cells against the respiratory target cell line BEAS-2B: (a) Activated $CD8^+$ T cells release Granzyme B during lysis

of BEAS-2B depending on E/T ratio in a cytotoxicity assay performed in parallel to the described ^{51}Cr release assay. Difference versus control ($CD8^+$ only): ** $p \leq 0.001$, * $p \leq 0.01$; difference versus E/T=20: ++ $p \leq 0.005$. Blocking experiments were performed by incubating BEAS-2B target cells with anti-CD95 (b), anti-TNF (c) or anti-MHC I (d) prior to use in a standard ^{51}Cr release assay with activated $CD8^+$ T cells as described above. Means ± SEM of independent experiments are shown.

Figure 7. Expression of NKG2D on $CD8^+$ T cells and two of its ligands MICA/B on BEAS-2B: (a) BEAS-2B or $CD8^+$ T cells were stained with anti-MIC A/B or anti-NKG2D specific antibody and analyzed by flow cytometry. Histogram plots show one representative out of 6 independent experiments with isotype control (thin line). (b) Means ± SEM of induction of NKG2D on positive $CD8^+$ T cells during stimulation out of 5 independent experiments are given; difference versus control: * $p \leq 0.01$. **Inhibition of the cytolytic activity of $CD8^+$ T cells with anti-NKG2D**: (c) Activated $CD8^+$ T cells were incubated with anti-NKG2D antibody (1D11) or an unspecific isotype control antibody or BEAS-2B were incubated with anti-MHC class I (W6/32) for 30 min prior to use in a standard ^{51}Cr release assay. Means ± SEM of percentage of specific lysis of independent experiments are shown for varying E/T ratios and given as n-fold of control at a constant E/T=20. Differences versus unspecific isotype control antibody: ** $p \leq 0.001$, * $p \leq 0.01$.

Figure 8. Expression of MICA/B on PBEC: (a) PBEC were stained with anti-MIC A/B specific antibody and analyzed by flow cytometry. Histogram plot of one representative out of 5 independent experiments with isotype control (thin line) is shown. **Cytolytic activity of $CD8^+$ T cells against the PBEC:** $CD8^+$ T cells were stimulated with irradiated PBEC in the presence of IL-2 and subsequently subjected to a ^{51}Cr release assay as effectors (E) in increasing E/T ratios: (b) activated $CD8^+$ T cells were left untreated or incubated with anti-NKG2D antibody (1D11) prior to use in a standard ^{51}Cr release assay with PBEC as targets. (c) PBEC were left untreated, or incubated with anti-MHC I (W6/32; dashed line) prior to use as target cells in a standard ^{51}Cr release assay.

Figure 1

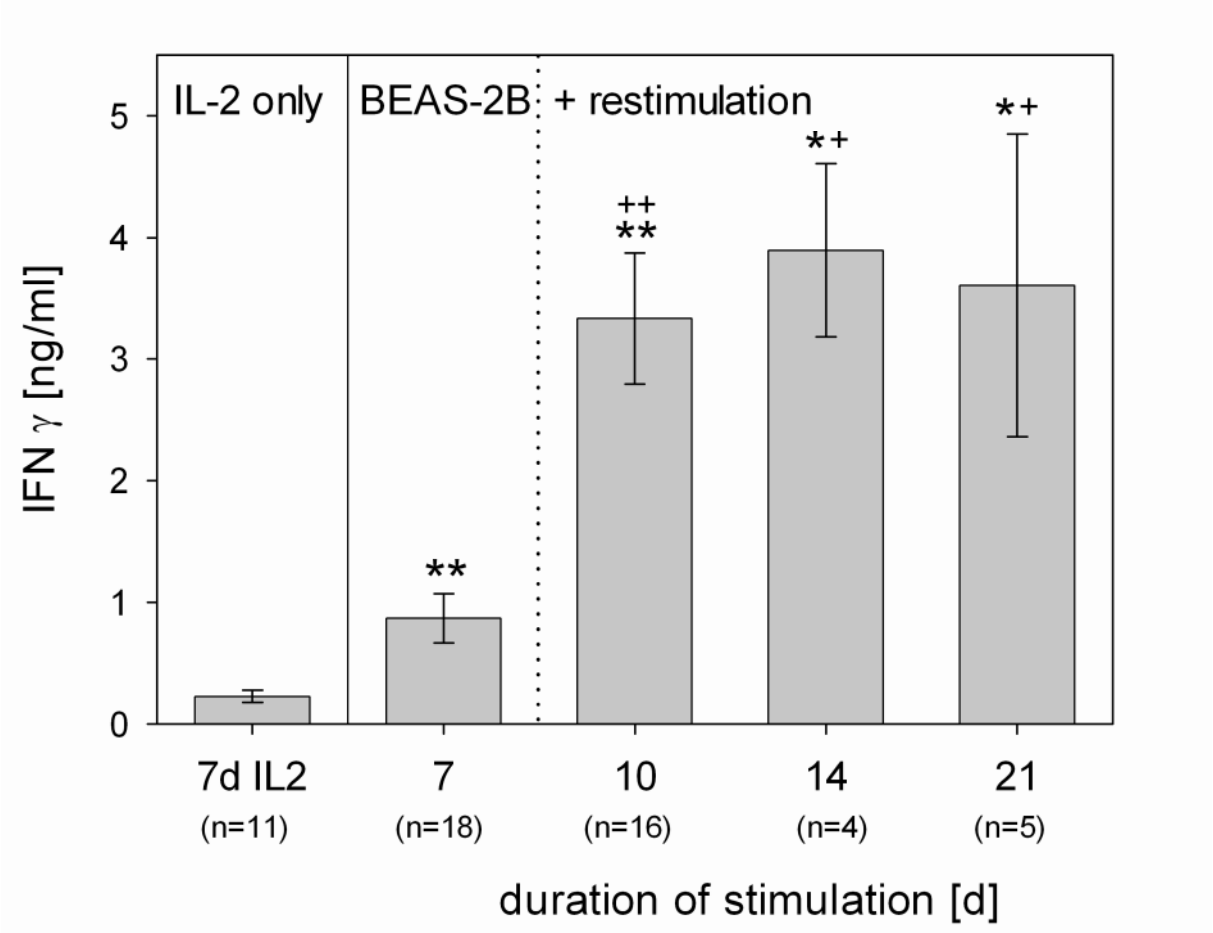

IL-2 only
BEAS-2B
+ restimulation
IFN γ [ng/ml]
0
1
2
3
4
5
**
++
**
*+
*+
7d IL2
(n=11)
7
(n=18)
10
(n=16)
14
(n=4)
21
(n=5)
duration of stimulation [d]

Figure 2

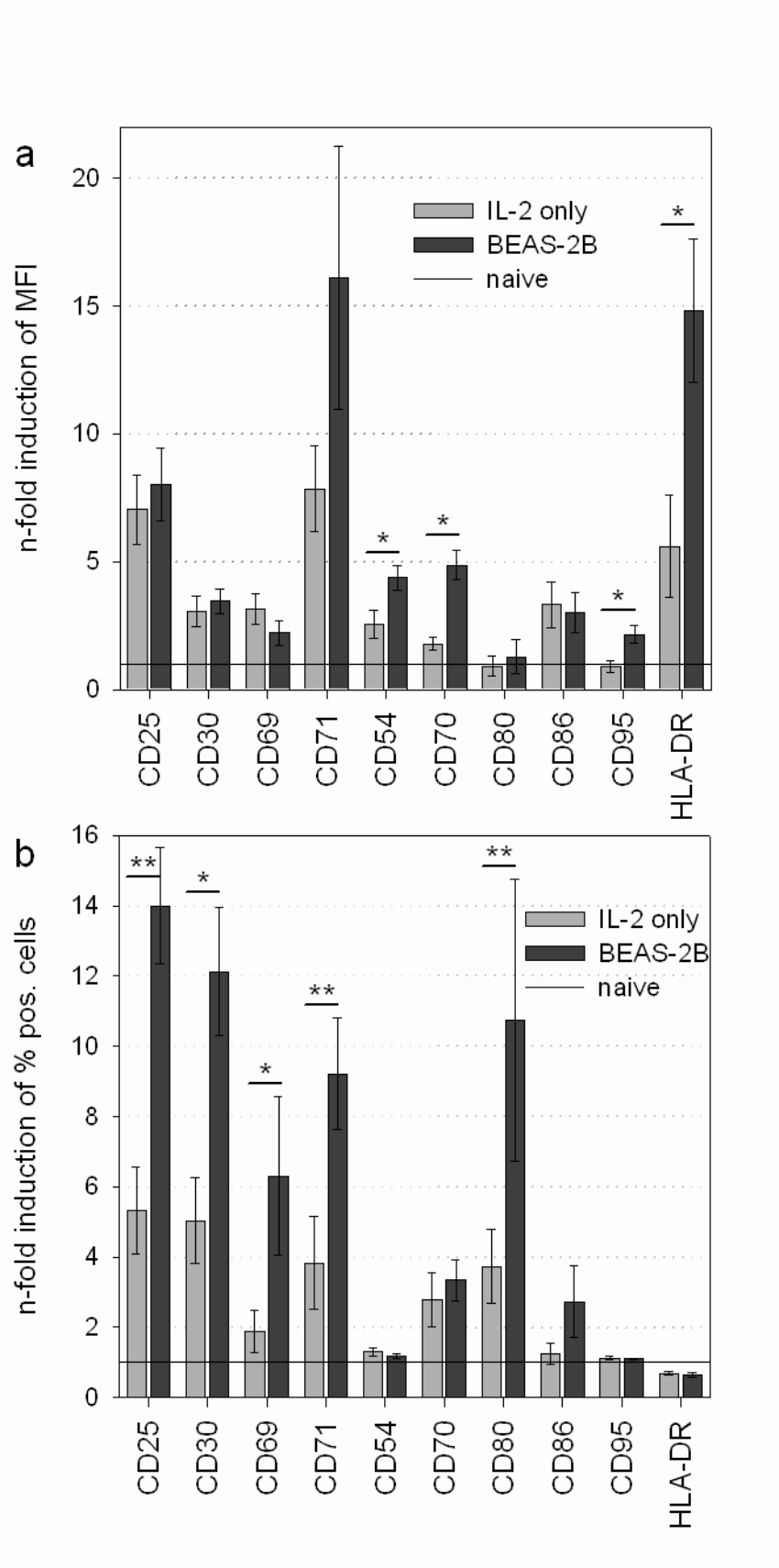

a
n-fold induction of MFI
20
15
10
5
0
IL-2 only
BEAS-2B
naive
*
*
*
*
CD25
CD30
CD69
CD71
CD54
CD70
CD80
CD86
CD95
HLA-DR
b
n-fold induction of % pos. cells
16
14
12
10
8
6
4
2
0
**
*
*
**
**
IL-2 only
BEAS-2B
naive
CD25
CD30
CD69
CD71
CD54
CD70
CD80
CD86
CD95
HLA-DR

Figure 3

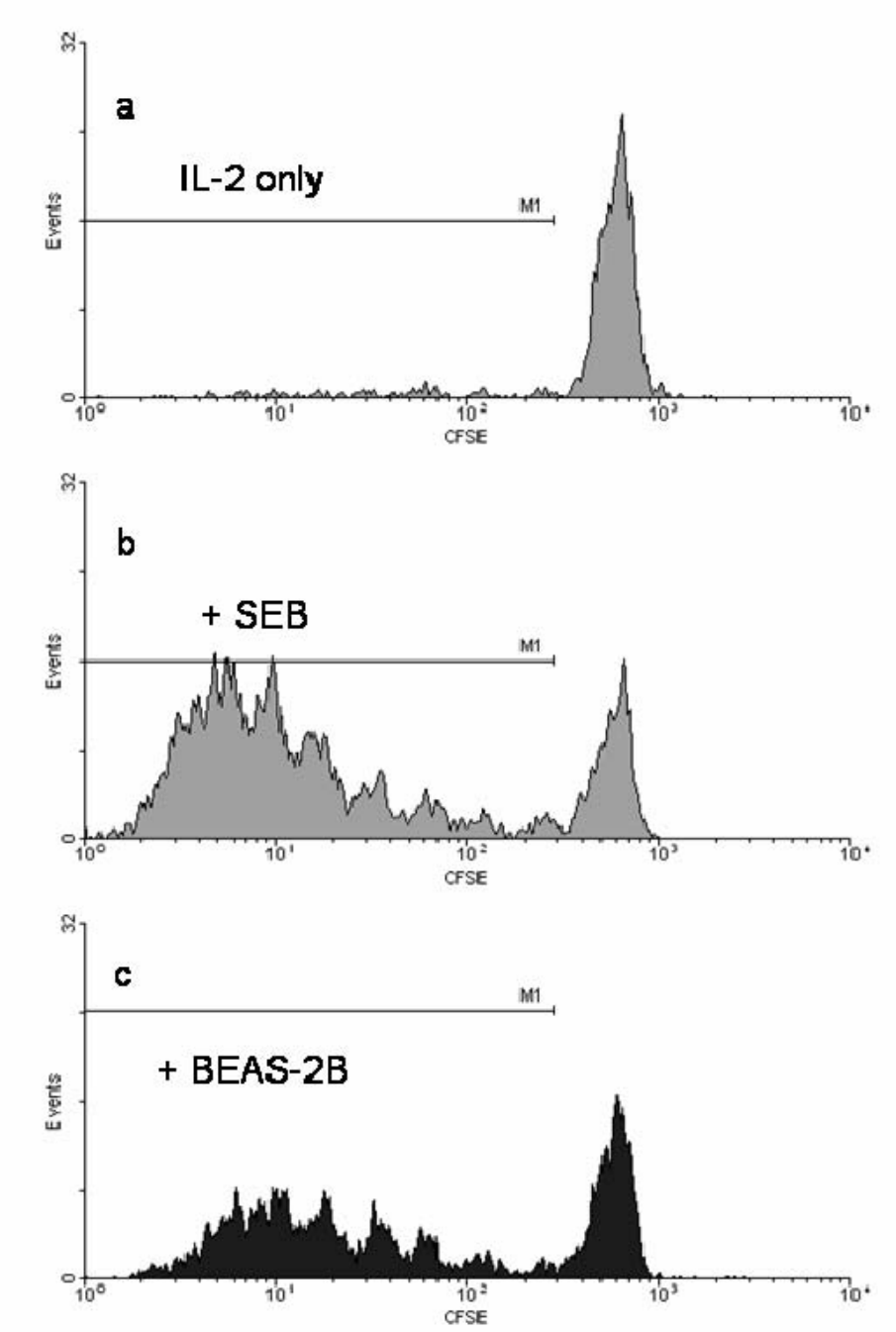

a
IL-2 only
M1
Events
32
0
10^0
10^1
10^2
10^3
10^4
CFSE
b
+ SEB
M1
Events
32
0
10^0
10^1
10^2
10^3
10^4
CFSE
c
M1
+ BEAS-2B
Events
32
0
10^0
10^1
10^2
10^3
10^4
CFSE

Figure 4

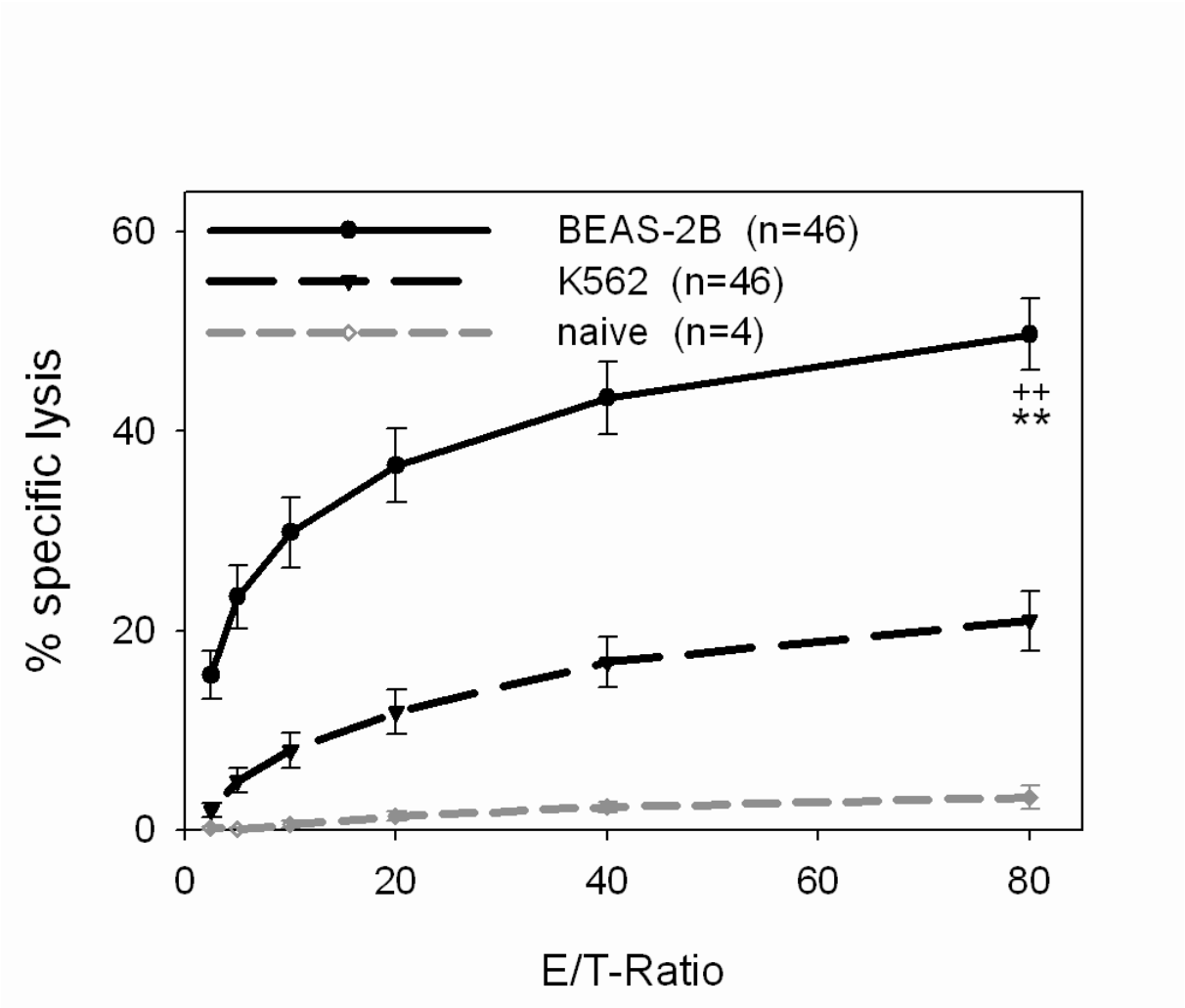

BEAS-2B (n=46)
K562 (n=46)
naive (n=4)
++
**
% specific lysis
E/T-Ratio
0
20
40
60
80

Figure 5

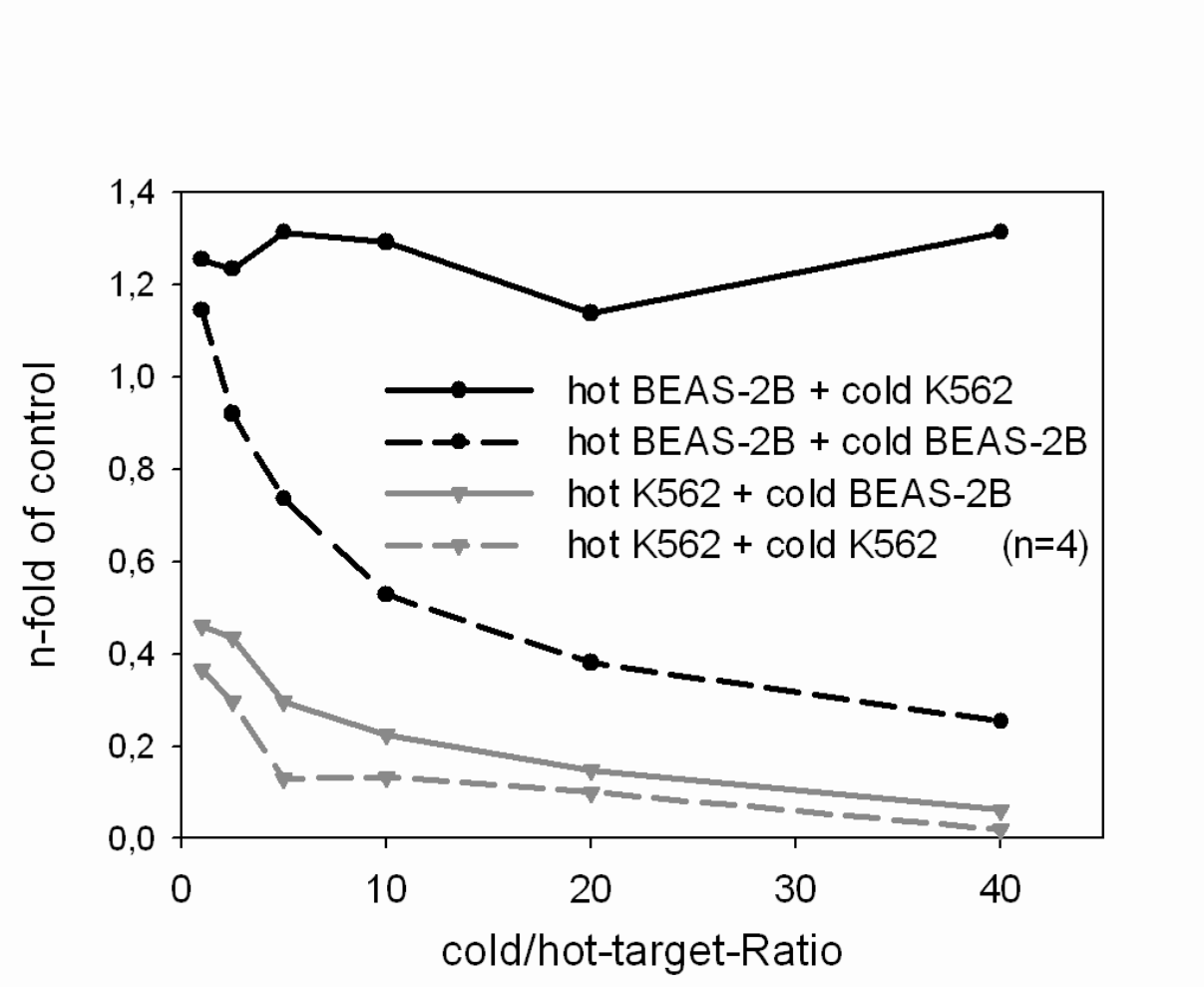

1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
n-fold of control
0
10
20
30
40
cold/hot-target-Ratio
hot BEAS-2B + cold K562
hot BEAS-2B + cold BEAS-2B
hot K562 + cold BEAS-2B
hot K562 + cold K562
(n=4)

Figure 6

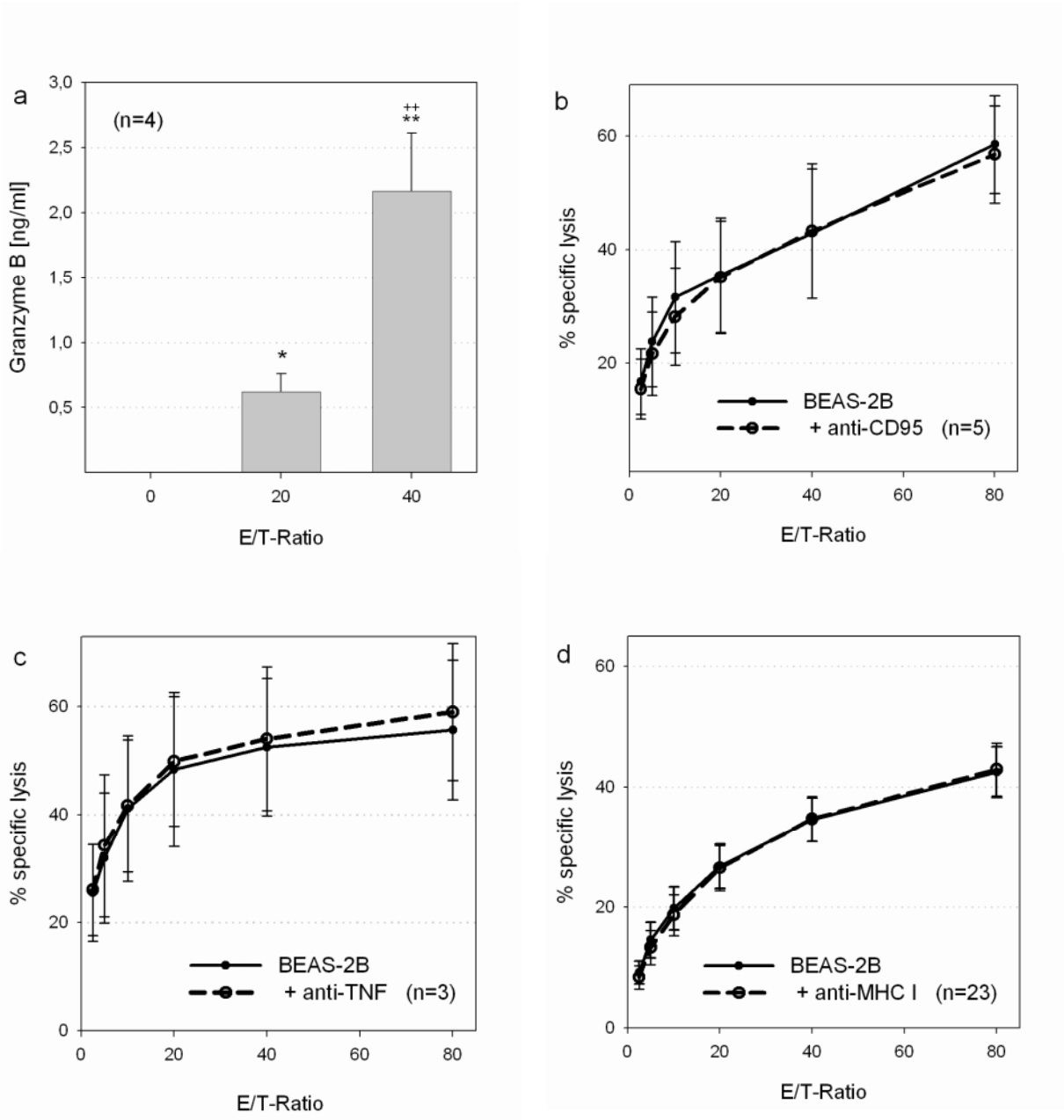

a
(n=4)
Granzyme B [ng/ml]
3,0
2,5
2,0
1,5
1,0
0,5
*
++
**
0
20
40
E/T-Ratio
b
% specific lysis
60
40
20
BEAS-2B
+ anti-CD95 (n=5)
0
20
40
60
80
E/T-Ratio
c
% specific lysis
60
40
20
0
BEAS-2B
+ anti-TNF (n=3)
0
20
40
60
80
E/T-Ratio
d
% specific lysis
60
40
20
0
BEAS-2B
+ anti-MHC I (n=23)
0
20
40
60
80
E/T-Ratio

Figure 7

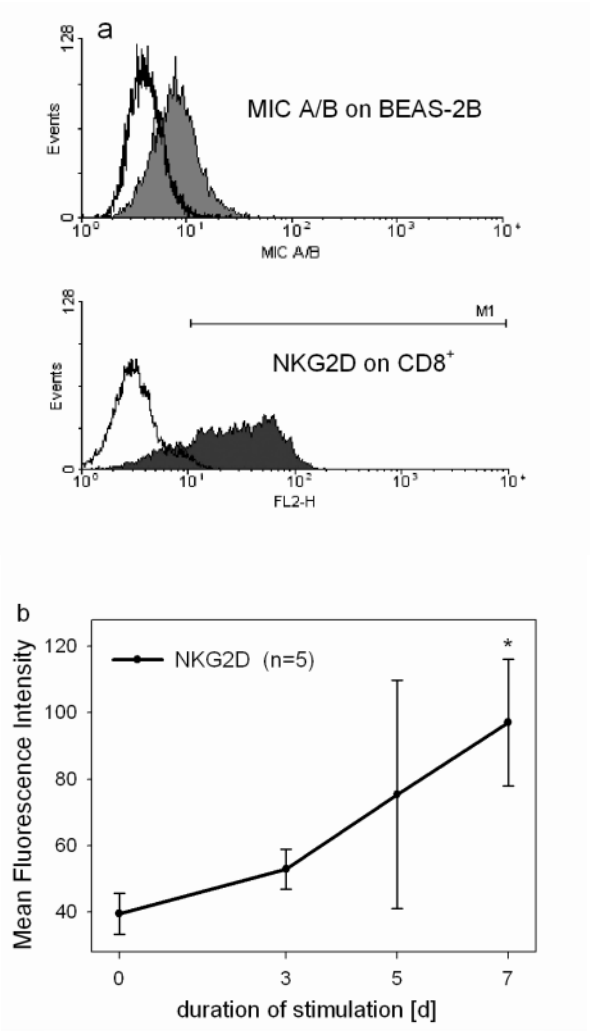
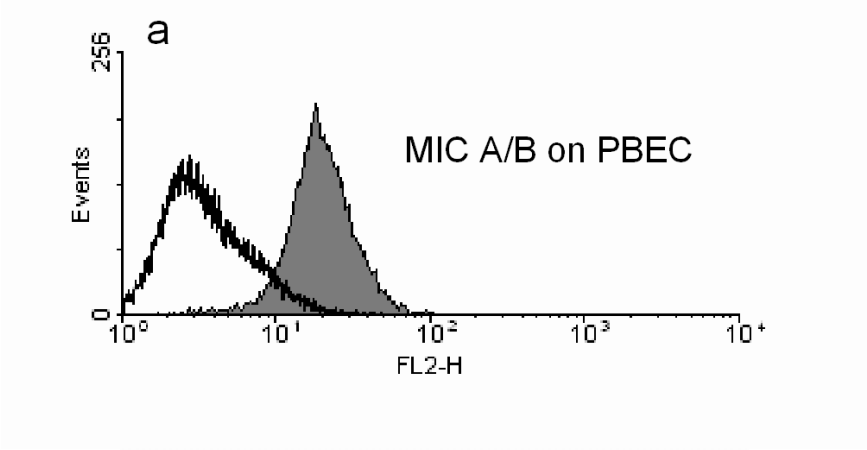
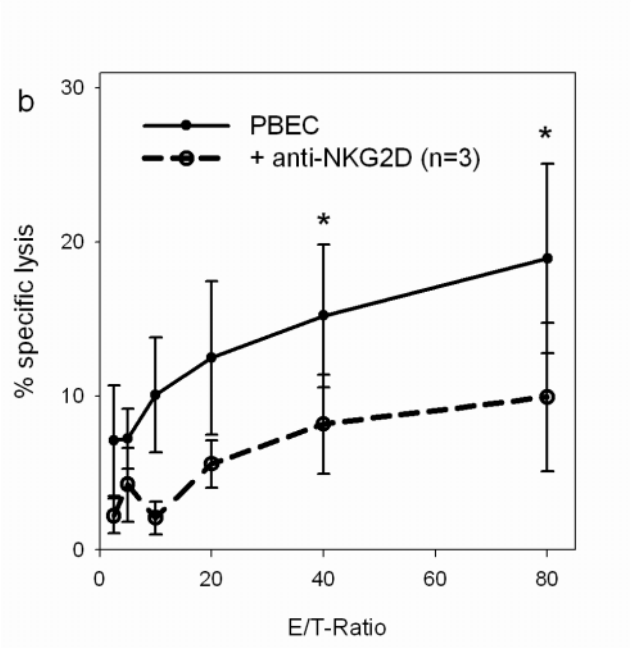
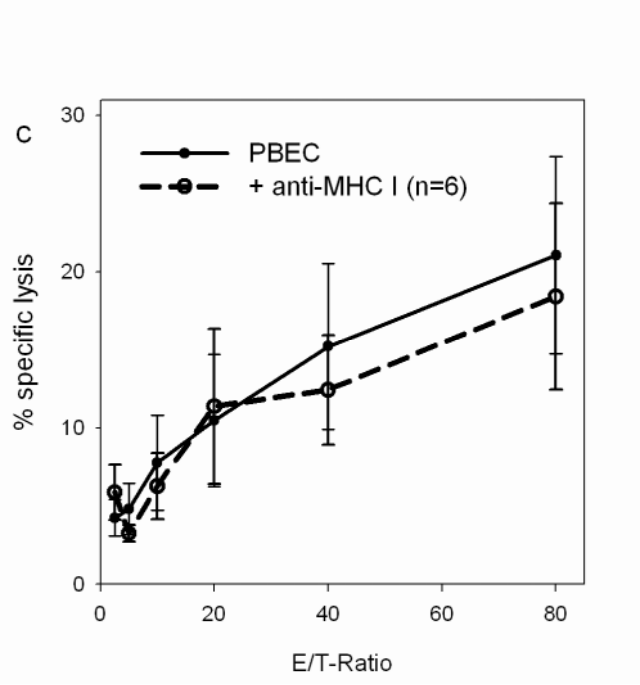

a
MIC A/B on BEAS-2B
Events
MIC A/B
M1
NKG2D on CD8+
FL2-H
b
NKG2D (n=5)
Mean Fluorescence Intensity
duration of stimulation [d]
*
C
BEAS-2B
specific lysis (n-fold of control)
**
+ IgG
+ NKG2D
+ MHC I
(n=8)
(n=8)
(n=23)

Figure 8


a
256
Events
0
MIC A/B on PBEC
10^0
10^1
10^2
10^3
10^4
FL2-H


b
30
20
10
0
% specific lysis
PBEC
+ anti-NKG2D (n=3)
*
*
0
20
40
60
80
E/T-Ratio


c
30
20
10
0
% specific lysis
PBEC
+ anti-MHC I (n=6)
0
20
40
60
80
E/T-Ratio