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TCR-independent cytokine stimulation induces non-MHC-restricted T cell activity and is negatively regulated by HLA class I

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Recent evidence suggests that the functional status of T cells activated independently from their TCR differs substantially from classical MHC-restricted T cells. Here, we show that TCR-independent, short-term stimulation *via* the common γ-chain of the IL-2/IL-15 receptor induces non-MHC-restricted cytotoxicity and sustained cytokine secretion in purified $CD4^+$ or $CD8^+$ T cells. NK-like cytotoxicity is directed against MHC class I-negative targets and can be inhibited by classical and non-classical HLA class I molecules. Known inhibitory receptors, such as CD85j (ILT2) and leukocyte-associated Ig-like receptor-1, are not responsible for this HLA-mediated inhibition. NK-like cytotoxicity can be costimulated by NKG2D (CD314) triggering, but 2B4 (CD244) and DNAM-1 (CD226) are not involved. NK-like T cells display an activated phenotype and secrete various cytokines, including IFN-γ, TNF-α, IL-5, IL-13 and MIP-1β. Under normal conditions, HLA class I-mediated inhibition may function as a safety mechanism to prevent unbalanced cytokine production and effector killing mechanisms by T cells that were activated independently from their TCR. Non-MHC-restricted activity represents a functional status rather than a property of distinct T cell subpopulations. Thus, cytokine-induced, non-MHC-restricted T cells may be relevant in immune responses against tumors showing aberrant MHC expression through their capacities of cytokine production and direct tumor cell eradication.

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Introduction

Discoveries in the past decade have confirmed the view that the boundaries between innate and specific immune responses are not static. Rather, both systems influence each other to optimize immune responses and

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Abbreviations: AR: activating receptor · **IR:** inhibitory receptor · **KIR:** killer cell Ig-like receptor · **LAIR:** leukocyte-associated Ig-like receptor

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cells of the antigen-specific arm are able to interact in both systems. The TCR represents the primary mode of T cell stimulation for MHC-restricted, peptide-specific activation. In contrast, various cytokine-based activation protocols have been shown to induce "antigen-independent" T cell responses. Cytokine stimulation of peripheral lymphocytes results in "non-specific" T cell responses detected as non-MHC-restricted cytotoxicity [1–6]. TCR-dependent activation using anti-CD3 mAb, IFN-γ and IL-2 as well as TCR-independent stimulation with IL-2 alone revealed the potential of peripheral T cells to behave like NK cells by switching to a non-MHC-restricted cytotoxicity pattern [6–9]. Several attempts to utilize alternatively activated lymphocytes in clinical settings resulted in occasional regression of tumors in some patients but were accompanied by high toxicity [10, 11].

Recently, we and others demonstrated that the density of MHC class I expression by tumor cells represented one major element in NK-like T cell regulation [6, 12]. Common features of non-MHC-restricted T cells include their polyclonal activation *via* cytokine receptor triggering, the production of various cytokines and their non-MHC-restricted cytotoxicity. Recent evidence suggests the presence of NK-like T cells *in vivo* in the gut of celiac patients. Stimulation by IL-15 increased NKG2D expression on CTL that acquired NKG2D cytolytic pathways but only in effector CTL subsequent to TCR stimulation. These CTL attacked autologous intestinal epithelial cells in a non-MHC-restricted fashion [13, 14].

Recent progress in understanding NK cell regulation through activating receptors (AR) and inhibitory receptors (IR) suggested a relationship between NK cells and non-MHC-restricted T cells displaying NK-like functions. NK cells are regulated by a balance between activation through several receptors, such as NKG2D, and inhibition that is mediated by MHC-specific IR of the killer cell Ig-like receptor (KIR) and C-type lectin families. Some NK receptors are postulated to be responsible for TCR-independent functions of $CD8^+$ T cells [15, 16].

Here, we report the development of non-MHC-restricted (NK-like) activity in highly purified $CD4^+$ or $CD8^+$ T cell populations following TCR-independent stimulation through IL-2/IL-15 receptors that share the γ- (CD132) and the β-chain (CD122). Functional and phenotypic characterization of these T cells revealed that cytotoxicity was inhibited by HLA class I molecules. NK-like T cells did not express KIR, CD94/NKG2A, CD56 or CD161. Moreover, induction of NK-like T cell cytotoxicity was not induced through known AR such as NKG2D, 2B4 (CD244) or DNAM-1 (CD226). These observations confirm recent studies that NKG2D-mediated activity in T cells strictly depends on initial TCR signals [17]. Moreover, $CD4^+$ or $CD8^+$ NK-like T cells secreted a mixed pattern of cytokines such as IFN-γ, TNF-α, IL-5 and IL-13. These observations provide insight into the regulation of cytokine-stimulated T cells characterized by a distinct functional status.

Results

HLA class I molecules inhibit non-MHC-restricted cytotoxicity of purified $CD4^+$ or $CD8^+$ T cells

In previous studies, non-MHC-restricted activity of $CD3^+\alpha\beta TCR^+$ T cells was described for mixed populations following allogeneic or cytokine/PHA stimulation [10]. To demonstrate that non-MHC-restricted activity represents a TCR-independent functional status of $CD4^+$ or $CD8^+$ T cells, analyses were performed using highly purified populations of peripheral $CD4^+$ and $CD8^+$ T cells. PBMC from five healthy donors were separated into $CD4^+$ or $CD8^+$ T cell fractions by negative isolation through depletion of monocytes, B cells, NK cells, γδT cells, and $CD4^+$ or $CD8^+$ T cells. Following activation with 1000 U/mL IL-2/PHA, the cytolytic activity of purified T cells was determined at day 3 and 7 against the HLA class I-negative target cells K562, L721.221 and Daudi (Fig. 1A). Purified $CD4^+$ T cells lysed these hematopoietic target cells but displayed lower cytotoxic activity compared to purified $CD8^+$ T cells. Both populations showed higher cytotoxicity at day 3. In general, T cells lysed hematopoietic tumor cells more efficiently than epithelial RCC26 cells [18]. As expected for polyclonal populations, specificity and levels of cytotoxicity varied between $CD4^+$ and $CD8^+$ T cell populations of one donor and among different donors.

In Fig. 1B, the inhibition of T cell activity by HLA class I molecules is shown using HLA class I transfectants. Cytotoxicity of $CD4^+$ and $CD8^+$ T cells of all donors at day 3 was inhibited by either HLA-C, HLA-E or HLA-G (Supplementary Table 1). Killing of Daudi and L721.221 cells by most T cells was inhibited by their HLA class I-positive counterparts, Daudi-β_2m and L721.112, by more than 40% at both time points. Increased HLA class I levels in RCC26-γ cells resulted in inhibition of lysis, indicating that the density of HLA class I regulates the cytolytic activity of NK-like T cells as described earlier [6].

IL-2 or IL-15 can induce NK-like activity by T cells

To exclude that NK-like T cell activation by IL-2/PHA was induced primarily by non-physiological PHA-mediated signals, T cells were activated with reduced amounts of IL-2 (500 U/mL) without PHA (Fig. 2A–C). $CD4^+$ as well as $CD8^+$ T cells lysed the three target cells, Daudi,

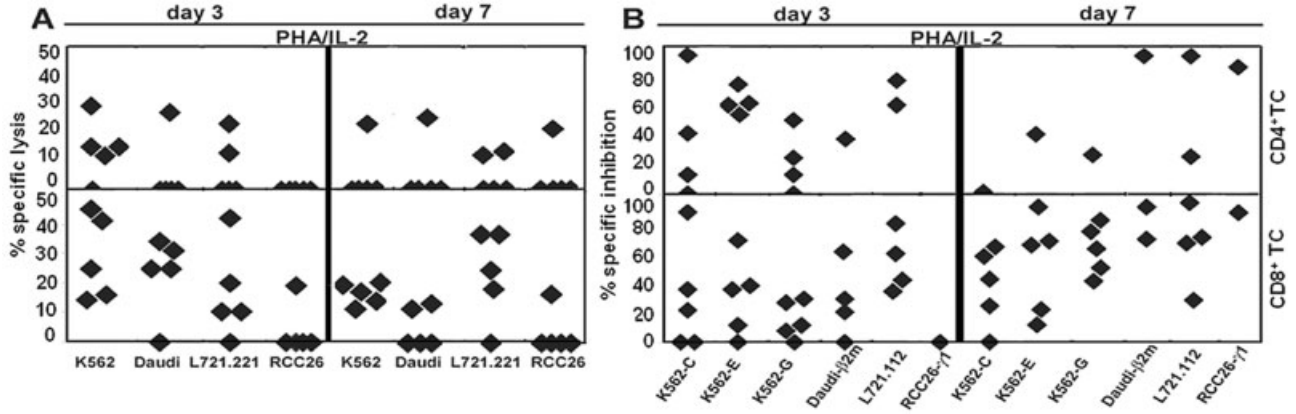

Figure 1. Cytotoxicity and HLA class I-mediated inhibition of $CD4^+$ and $CD8^+$ T cells. (A) $CD4^+$ and $CD8^+$ T cells show cytotoxicity against K562, Daudi, L721.221 cells, weakly against RCC26 cells at day 3 and 7 after stimulation with PHA and IL-2. (B) Cytotoxicity was inhibited by HLA-C, HLA-E and HLA-G expressed on K562 cells and by Daudi-β_2m, L721.112 and RCC26-γ cells. E:T ratio at day 3 and 7 was 40:1 for $CD4^+$ and $CD8^+$ T cells. For the calculation of inhibition, cytotoxicity against HLA class I-negative targets was set to 100% and percent lysis against the HLA-positive counterpart was related to this value. Percent inhibition was calculated by the difference between these two values; percent inhibition was only calculated for lysis values higher than 10%.

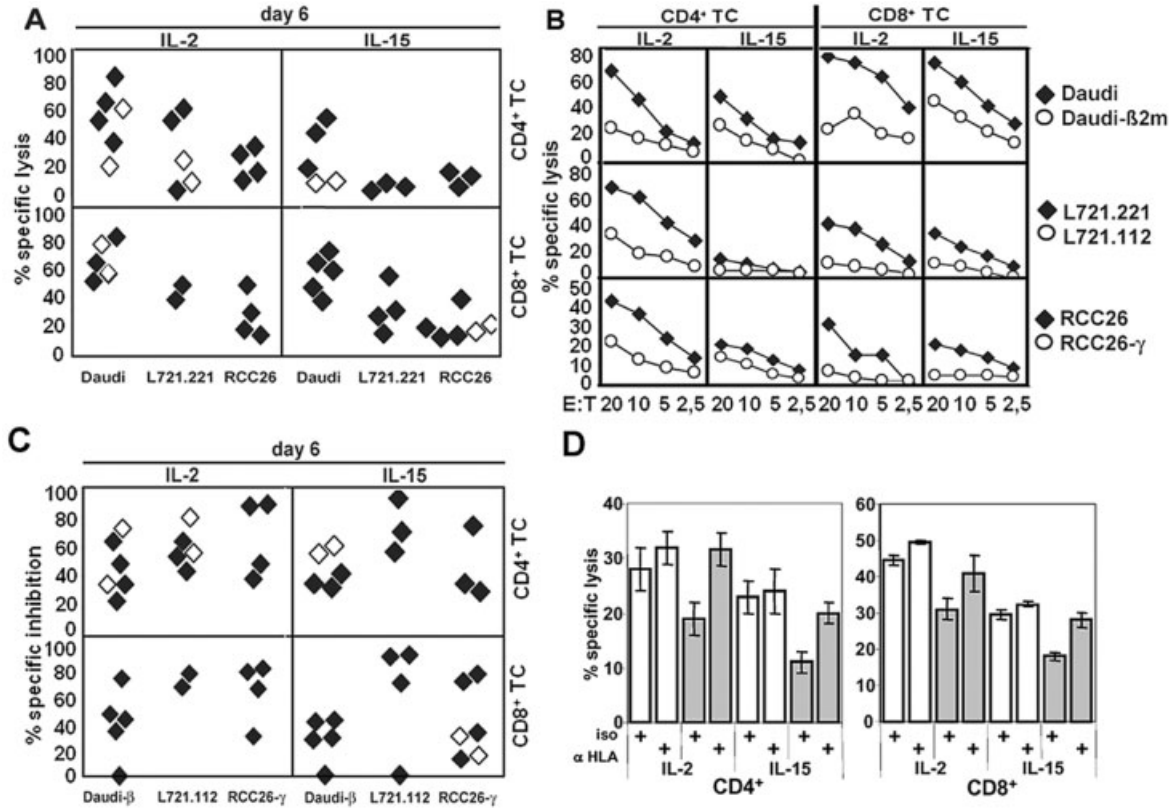

Figure 2. Stimulation with IL-2 or IL-15 alone is sufficient to generate $CD4^+$ or $CD8^+$ NK-like T cells. (A) Cytotoxicity of $CD4^+$ and $CD8^+$ T cells by activated IL-2 (500 U/mL) or IL-15 (5 ng/mL) was measured against Daudi, L721.221 and RCC26 cells. E:T ratio was 20:1 for all donors at day 6. (B) One of the six experiments combined in Fig. 1A is shown in titration curves of lysis by $CD4^+$ and $CD8^+$ T cells of different donors against HLA-negative targets and their HLA-positive counterparts. (C) Inhibition of lysis by HLA class I-positive target cells. E:T ratio was 20:1 for all donors at day 6. Black diamonds represent different donors, white diamonds represent two independent experiments with T cells of the same donor. Percent inhibition was calculated as described for Fig. 1B. (D) Blocking of HLA molecules. $CD4^+$ or $CD8^+$ T cells from two donors were stimulated for 6 days with IL-2 or IL-15. L721.221 (white bars) and L721.112 cells (grey bars) were pre-incubated with isotype MOPC21 or HLA-specific mAb A1.4. Mean values and standard deviations of two donors are shown at E:T ratios of 20:1.

Table 1. Phenotypes of $CD4^+$ or $CD8^+$ NK-like T cells[a)]

	$CD4^+$ T cells		$CD8^+$ T cells	
mAb	Day 3	Day 7	Day 3	Day 7
CD3	100	99 ± 2	100	99 ± 2
CD4	98 ± 3	99 ±3	0	5 ± 3
CD8	2 ± 2	1	100	95 ± 2
CD45RO	92 ± 4	100	95 ± 2	100
CD56	0	0	0	0
CD2	100	100	100	100
KIR-2DL1	1	1	0	0
KIR-2DL2,3	1	1	1	0
KIR-3DL1	0	1	0	0
KIR-2DS4	0	1	1	1
CD94/NKG2A	1	2	2	5
LAIR-1	0	100[b)]	100[b)]	100[b)]
ILT2 (CD85j)	100[c)]	7 ± 1	6 ± 2	10 ± 2
2B4 (CD244)	4 ± 2	4 ± 1	2 ± 2	5 ± 2
NKG2D	6 ± 1	1 ± 1	74 ± 4	68 ± 4
DNAM-1	81 ± 4	75 ± 3	90 ± 4	97 ± 3
CD161	3 ± 1	1	1	1

a) The data represent mean values ± SD of % positive cells of $CD4^+$ or $CD8^+$ NK-like T cells of five donors.
b) $CD4^+$ or $CD8^+$ NK-like T cells expressed low levels of LAIR-1 (MCR = 9 *vs.* MCR = 5 for isotype control at day 7); for $CD8^+$ NK-like T cells, MCR = 19 at day 3, MCR = 9 at day 7 *vs.* MCR = 4 for isotype control.
c) $CD4^+$ NK-like T cells expressed low levels of ILT2, MCR=19 at day 3 *vs.* MCR = 6 for isotype control.

L721.221 and RCC26. In general, IL-15-stimulated T cells showed somewhat reduced cytotoxic potential. In Fig. 2B, the titration curves for inhibition of $CD4^+$ as well as $CD8^+$ T cells are shown comparing HLA class I-negative or low target cells with their HLA-positive or -high counterparts. HLA class I-mediated inhibition is summarized in Fig. 2C for T cells of multiple donors. Cytotoxicity of all T cell populations was inhibited by the HLA class I-positive target cells.

To test whether inhibition of lysis by HLA class I-positive target cells was mediated directly by HLA class I molecules, blocking experiments with an HLA-specific mAb were performed (Fig. 2D). Pre-incubation of HLA class I-positive L721.112 cells with the pan-HLA-specific mAb A1.4 resulted in higher lysis compared to pre-incubation with isotype control mAb. Restoration of lysis did not reach the level seen against the HLA class I-negative cell line L721.221, possibly because of the very high HLA expression on L721.112 cells (data not shown). Moreover, not all HLA molecules may be equally well blocked by this mAb [6].

In contrast to cytotoxicity, the highest proliferation rate was achieved for both T cell subsets with the combination of PHA and IL-2 or IL-15. IL-2 or IL-15 alone induced lower proliferation rates of $CD4^+$ or $CD8^+$ T cells (Fig. 3A). Thus, high levels of cytotoxicity directed against HLA class I-negative target cells were achieved by culturing T cells without PHA using only 500 U/mL IL-2 or 5 ng/mL IL-15, but this increase in cytolytic activity was accompanied by low proliferation.

One prominent mechanism of T and NK cell cytotoxicity is the secretion of perforin following receptor-mediated degranulation. $CD4^+$ T cells did not possess intracellular perforin regardless of the induction modus (Fig. 3B). CD95L-specific mRNA was detected by RT-PCR in $CD4^+$ as well as $CD8^+$ T cells. Both T cell subsets lysed $CD95^-$ variants of Jurkat cells to similar levels, indicating that yet other mechanisms like TRAIL may be involved in this activity (data not shown). $CD8^+$ T cells displayed the highest perforin level following stimulation with IL-2 without PHA, while perforin staining was substantially reduced by PHA. No sig-

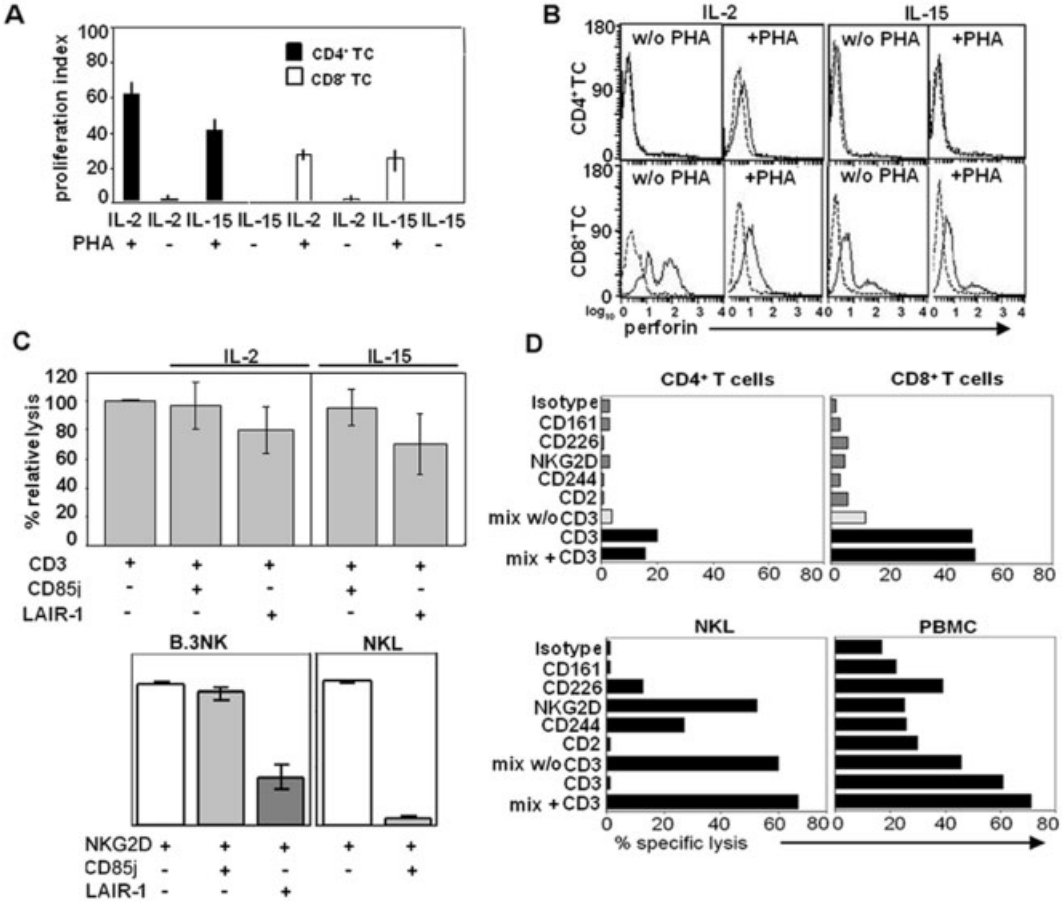

Figure 3. PHA increases proliferation but reduces the amount of perforin in $CD8^+$ T cells; LAIR-1 and ILT2 are unable to inhibit NK-like cytotoxicity. (A) Proliferation indices of $CD4^+$ or $CD8^+$ T cells from four donors were determined by [^{3}H]thymidine incorporation at day 6; cpm values of the lowest proliferating population (IL-15 stimulation) of each donor were fixed as factor 1; ^{3}H incorporation of all other populations was calculated according to this baseline value and expressed as multiplication index. (B) Perforin production was measured by intracellular staining using anti-perforin mAb. $CD4^+$ and $CD8^+$ T cells from one donor were cultured for 6 days with IL-2 or IL-15 and with or without PHA. Isotype control curves are given by dashed lines, perforin by solid lines. (C) $CD4^+$ and $CD8^+$ T cells from four donors were cultured with IL-2 or IL-15 for 6 days. Lysis of anti-CD3-coated P815 cells was defined as 100% T cell lysis; lysis of anti-CD3/anti-CD85j- or anti-CD3/αLAIR-1-coated P815 cells is expressed as percent lysis according to this value. Two NK lines, B.3NK and NKL, served as functional controls. Lysis of anti-NKG2D-coated P815 cells was defined as 100% and lysis of anti-NKG2D/anti-CD85j- or anti-NKG2D/anti-LAIR-1-coated P815 cells is expressed as percent lysis according to this value. (D) $CD4^+$ or $CD8^+$ T cells were generated with PHA/IL-2 and stimulated at day 15 with mAb against AR. Cytotoxicity against mAb-coated P815 cells was measured at E:T ratios of 40:1 ($CD4^+$) and 30:1 ($CD8^+$), respectively. The NK leukemia line NKL was used as positive control for activation by CD226, NKG2D and CD244 (E:T ratios of 20:1). Unseparated PBMC are shown as positive control for NK-containing populations (28% NK cells, 72% T cells, E:T ratio 30:1).

nificant differences were detected following stimulation with IL-15 alone or with PHA.

HLA class Ia- and -Ib-mediated inhibition of T cells is not accompanied by expression of known IR

Remarkably, none of the KIR2D/3D (CD158) receptors were detected on NK-like T cells (Table 1), although inhibition by HLA-Cw*0304, a KIR2DL2/3 (CD158b) ligand, was observed for both $CD4^+$ and $CD8^+$ T cells (Fig. 1B). Leukocyte-associated Ig-like receptor (LAIR)-1 and CD85j (ILT2), which can be expressed by both T cells and NK cells, were weakly expressed at day 7 and 14 (LAIR-1) or by a small subpopulation of $CD4^+$ or $CD8^+$ T cells (ILT2). To test whether LAIR-1 and CD85j were functional, IL-2- or IL-15-activated $CD4^+$ and $CD8^+$ T cells were stimulated with anti-CD3 mAb combined with anti-LAIR-1 or anti-CD85j mAb (Fig. 3C). No inhibition of TCR/CD3-mediated cytotoxicity could be triggered *via* these receptors. In contrast, cross-linking of CD85j or LAIR-1 efficiently inhibited NKG2D-mediated activation of two different NK lines, indicating that the functionality of IR can be evaluated with redirected lysis experiments.

NKG2D costimulates proliferation and cytotoxicity in some NK-like T cells

This non-MHC-restricted cytolytic pattern is strikingly similar to NK cell activity. Therefore, the expression of receptors known to regulate NK activity was analyzed. The quality of the $CD4^+$ and $CD8^+$ T cell separations was controlled by flow cytometry at days 3 and 7. $CD4^+$ and $CD8^+$ T cells displayed stable purity of 98–99% throughout the culture period (Table 1). Thus, NK-like cytotoxicity was exerted by *bona fide* $CD4^+$ or $CD8^+$ T cells that were further characterized with respect to their expression of known AR and IR.

CD56 was not found on $CD4^+$ or $CD8^+$ T cells, although it can be expressed by subsets of activated T cells [19]. As expected, the costimulatory receptor 2B4 (CD244) was induced on $CD8^+$ but not on $CD4^+$ T cells. Another costimulatory receptor, DNAM-1 (CD226), was found on $CD4^+$ as well as $CD8^+$ NK-like T cells [20]. NKG2D was expressed by 71–76% of $CD8^+$ NK-like T cells, consistent with the constitutive expression of NKG2D on virtually all human $CD8^+$ T and NK cells [21]. $CD4^+$ as well as $CD8^+$ T cells displayed a $CD45RO^+$ memory phenotype. Diversity of the TCR repertoire of all T cell populations was maintained throughout the *in vitro* culture period, which underlines their polyclonal character (data not shown).

The detection of AR, such as NKG2D, 2B4 (CD244) and DNAM-1 (CD226), on $CD8^+$ NK-like T cells suggested that these receptors may be involved in this T cell activity analogous to their known function in NK cells. However, none of these receptors alone or in combination was able to induce cytolytic activity in $CD4^+$ or $CD8^+$ T cells (Fig. 3D). In contrast, these receptors were able to trigger cytotoxicity by the control NKL line and NK-containing PBMC. Non-specific activation was excluded by using non-activating control mAb against CD2 expressed by T and NK cells and CD161 expressed by NKL and peripheral NK cells. TCR/CD3 triggering was sufficient to induce cytotoxicity but combinations of CD3 with all other AR did not enhance lytic activity.

To elucidate the role of NKG2D as potential costimulatory receptor, the response of NK-like T cells was analyzed to the skin-specific NKG2D ligand, ULBP4, artificially expressed by C1R cells. Increased lysis of ULBP4-expressing cells compared to control vector or wild-type C1R cells was observed for IL-15-activated $CD4^+$ T cells and IL-2- or IL-15-activated $CD8^+$ T cells in one of three donors (Fig. 4A) and the NKG2D-dependent NK line, NKL (Fig. 4B). ULBP4 also enhanced the lytic activity of $CD4^+$ as well as $CD8^+$ T cells that were expanded by stimulation with autologous PHA blasts. Proliferation was slightly enhanced by ULBP4 in IL-15-activated $CD4^+$ T cells of one donor (Fig. 4C), which

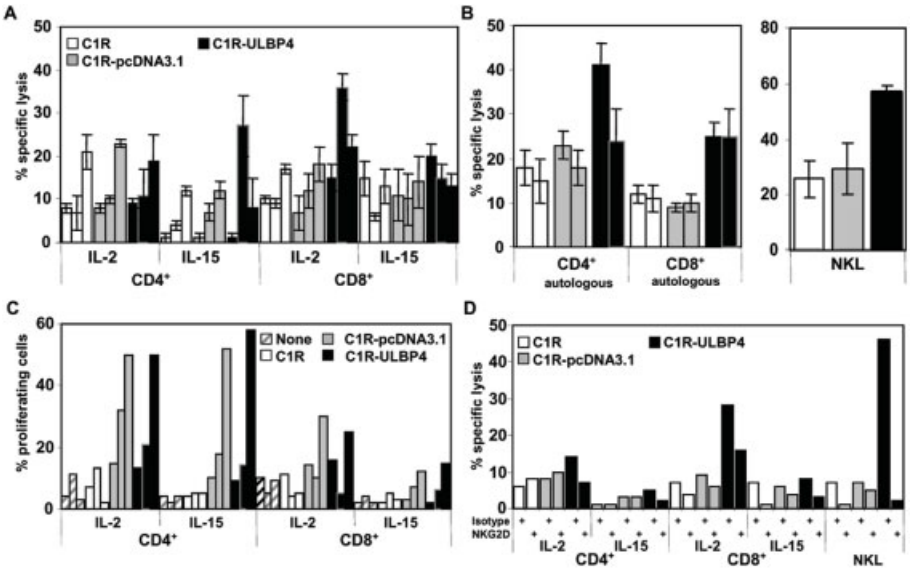

Figure 4. NKG2D is involved in target recognition and costimulation of $CD4^+$ and $CD8^+$ NK-like T cells. (A) Cytotoxicity of $CD4^+$ and $CD8^+$ T cells of three donors at day 6 after IL-2 or IL-15 stimulation was tested against wild-type, pcDNA3.1 or C1R-ULBP4 cells; E:T ratio of all effector cells was 20:1. (B) Cytotoxicity of $CD4^+$ and $CD8^+$ T cell lines at day 6 after stimulation with autologous PHA-blasts and NKG2D-responsive control NKL cells was analyzed against the same target cell set at E:T ratios of 20:1. (C) Proliferation of freshly isolated and CFSE-labeled $CD4^+$ and $CD8^+$ T cells of three donors was measured by flow cytometry 96 h after culture alone, with irradiated wild-type C1R, vector control C1R-pcDNA3.1 or C1R-ULBP4 cells. Percentage of proliferating $CD3^+$ T cells was calculated as percent T cells with decreased CFSE staining compared to all $CD3^+$ cells. (D) Reduction of cytotoxicity against three target cells by anti-NKG2D blocking mAb compared to isotype mAb was determined for $CD4^+$, $CD8^+$ T cells and NKL control cells at E:T ratios of 20:1; one of three representative experiments is shown.

correlated with the presence of NKG2D$^+$ T cells in these different T cell populations (Supplementary Fig. 1B).

Interestingly, vector control C1R cells induced substantial proliferation in all populations compared to wild-type C1R cells. Thus, other molecules may impinge on NK-like T cell proliferation since C1R sublines differ in their stimulatory capacity even in the absence of additional NKG2D ligands. Nevertheless, lysis of C1R-ULBP4 cells by all four T cell populations and NKL cells was partially blocked by NKG2D-specific mAb (Fig. 4D). Blocking of 2B4 (CD244) had no effect on cytotoxicity of these effector cells (data not shown).

CD4$^+$ NK-like T cell lines and clones secrete mixtures of Th1 and Th2 cytokines

The cytokine secretion patterns of T cell populations derived from three donors were analyzed in parallel to cytotoxicity and proliferation. C1R-ULBP4 cells served as indicator for NKG2D-mediated enhancement of cytokine secretion. IFN-γ, IL-2 and low levels of IL-10 were exclusively secreted by CD4$^+$ T cells following stimulation with vector control C1R and C1R-ULBP4 cells (Fig. 5A). In addition, substantial amounts of IL-5 and IL-13 and low amounts of IL-4, GM-CSF, IL-8 and TNF-α were secreted only by the IL-2-activated CD4$^+$ population (Fig. 5B). In contrast, MIP-1β was detected in all four T cell populations. Vector control C1R cells strongly stimulated secretion of cytokines analogous to induction of proliferation (Fig. 4C).

In general, these cytokine secretion patterns were confirmed by T cell clones and polyclonal T cell lines following 24 h stimulation with vector control or C1R-ULBP4 cells (Fig. 6A, B). However, after 72 h of stimulation, three CD8$^+$ clones displayed secretion of IFN-γ, IL-8 and IL-13 while only one CD8$^+$ clone acquired secretion of MIP-1β, GM-CSF, IL-5, IL-4, IL-10, indicating different kinetics for cytokine and chemokine secretion in CD4$^+$ *vs.* CD8$^+$ NK-like T cells (Supplementary Fig. 3). NKG2D on CD4$^+$ T cells may be associated with immediate IFN-γ, MIP-1β and IL-8 secretion since clone D8 but not D6 was NKG2D$^+$ and 2B4$^+$ (Table 2). Clones C4 and D4 were NKG2D- and 2B4$^+$ while both co-receptors were missing on clone G9 (Table 3). For these CD8$^+$ T cell clones, the secretion patterns did not correlate with NKG2D expression. None of the clones displayed cytotoxicity against Daudi, K562, L721.221 or C1R cells (data not shown).

In contrast to cytokine stimulation only, CD4$^+$ T cells of one donor stimulated with autologous PHA blasts showed substantial ULBP4-mediated enhancement of all tested cytokines and chemokines. In this autologous, antigen-free system, NKG2D-expressing CD4$^+$ T cells (Supplementary Fig. 1B) may have acquired the ability to respond to NKG2D ligands most likely due to additional TCR signals.

Discussion

In previous studies, antigen-independent T cell stimulation by cytokines focused primarily on proliferation and differentiation of T cell subpopulations [22]. Cytokines such as IL-2 and IL-15 which bind to receptors sharing the IL-2/IL-15R β- and γ-chain have been shown to be crucial for the process of memory T cell generation,

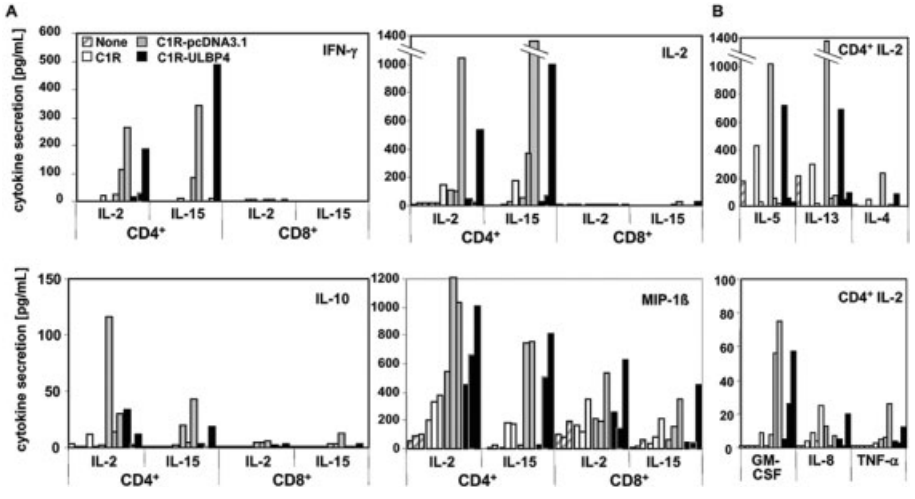

Figure 5. Cytokine and chemokine secretion by CD4$^+$ and CD8$^+$ NK-like T cells is induced by C1R cells and partially enhanced by NKG2D costimulation. (A) Secretion of IFN-γ, IL-2, IL-10 and MIP-1β is shown for IL-2- or IL-15-activated CD4$^+$ and CD8$^+$ T cells of three donors after 72 h stimulation without or with irradiated wild-type, pcDNA3.1 or C1R-ULBP4 cells in parallel to proliferation experiments shown in (Fig. 4.) (B) Secretion of IL-5, IL-13, IL-4, GM-CSF, IL-8 and TNF-α is only shown for IL-2-activated CD4$^+$ T cells in response to the same set of target cells since none of the other T cell populations produced these cytokines (data not shown).

Table 2. Phenotypes of $CD3^+CD4^+$ T cell clones of two donors

	$CD3^+CD4^+$ T cells		$CD3^+4^+$ and $CD3^+8^+$ T cells[a]	
			>70%/30%[a]	<70%/30%
	$NKG2D^+$	$NKG2D^-$	$NKG2D^{-/+}$	$NKG2D^-$
	$2B4^+$	$2B4^-$	$2B4^{-/+}$	$2B4^+$
Donor 1 (*n*=42)	1[b]	3[b]	35	3
Donor 2 (*n*=20)	3	2	13	2

[a] These cultures contained mixtures of $CD4^+$ and $CD8^+$ T cells with respect to NKG2D and 2B4 expression.
[b] Some of these clones (D6, D8) were functionally analyzed.

Table 3. Phenotypes of $CD3^+CD8^+$ T cell clones of two donors

	$CD3^+CD8^+$ T cells				$CD3^-CD8^-$ NK cells
	$NKG2D^+$	$NKG2D^-$	$NKG2D^+$	$NKG2D^-$	$NKG2D^+$
	$2B4^+$	$2B4^-$	$2B4^-$	$2B4^+$	$2B4^+$
Donor 1 (*n*=47)	16[a]	30[a]	1	2	0
Donor 2 (*n*=25)	3	9	3	8	1

[a] Some of these clones (D4, C4 and G9) were functionally analyzed.

proliferation, homeostasis and immune reconstitution [23–27]. Both cytokines can stimulate differential proliferation of naive T cells and induce a memory T cell phenotype independent of antigen exposure [28, 29]. Initial studies using combinations of common CD132 cytokines demonstrated that antigen-independent stimulation generated $CD69^+$ activated T cells in the absence of TCR triggering [30]. Moreover, IL-15 can restore antigen responsiveness of tolerant $CD8^+$ T cells although the molecular mechanism remains elusive in this murine model [31]. In our studies, TCR-independent activation through cytokine receptors induced a novel functional status in T cells, leading to non-MHC-restricted NK-like activity. Here, we define the phenotypic and functional properties of highly purified $CD4^+$ and $CD8^+$ T cells following stimulation by PHA and IL-2, IL-2 or IL-15 alone, whereby isolated signals *via* CD122/CD132 induced the strongest NK-like activity.

Among HLA class I-negative target cells, Daudi cells were killed more efficiently than K562 and L721.221 cells. These cells vary in expression of ligands for AR, such as CD48, ULBP1–3 and MICA/B, and adhesion molecules like LFA-3 and ICAM-1 (unpublished observations). Therefore, they may also differ in the expression of other surface molecules relevant for the induction of NK-like T cell activity. Primary leukemia cells (CLL, AML and ALL) were also lysed by NK-like cells, suggesting that such T cells may possess anti-leukemic activity in an allogeneic setting (Supplementary Fig. 2). Epithelial tumor cells (RCC26) were lysed by NK-like T cells of all donors but sometimes only to a low extent. In concordance with previous studies, the non-specific cytotoxicity of NK-like T cells affects tumor cells of hematological as well as non-hematopoietic origins and the individual HLA class I levels represent important parameters for the regulation of NK and NK-like T cells [32, 33].

Although expression of NKG2D, CD244 and CD226 was observed on $CD8^+$ NK-like T cells, cytotoxicity could not be induced by isolated signals through these receptors. However, cytotoxicity was enhanced by ULBP4-expressing C1R cells, suggesting that NKG2D may have costimulatory effects in the context of adequate target cells for NK-like T cells such as allogeneic LCL. Consistent with previous studies, the IL-15-mediated conversion to NKG2D-dependent function required TCR pre-activation and, therefore, seems to be restricted to the effector CTL pool [13–15, 34]. NKG2D expression on $CD4^+$ T cells is postulated to be associated with autoimmunity and senescence [35]. Combined TCR and cytokine receptor signaling pathways may account for the observed differences suggesting that cytokine stimulation alone is insufficient to couple NKG2D and CD244 receptors to their downstream signaling pathways. In a similar fashion, triggering of the IR CD85j and LAIR-1 on NK-like T cells did not inhibit CD3-mediated cytotoxicity. Despite their *bona fide* T cell phenotype, NK-like T cells are different from NK-CTL because they are not HLA-E-restricted and blocking of their TCR/CD3 complex does not influence NK-like cytotoxicity [36, 37] (Supplementary Fig. 1A).

The inhibitory effect of HLA class I molecules on NK-like cytotoxicity could be assigned to HLA-C, HLA-E and

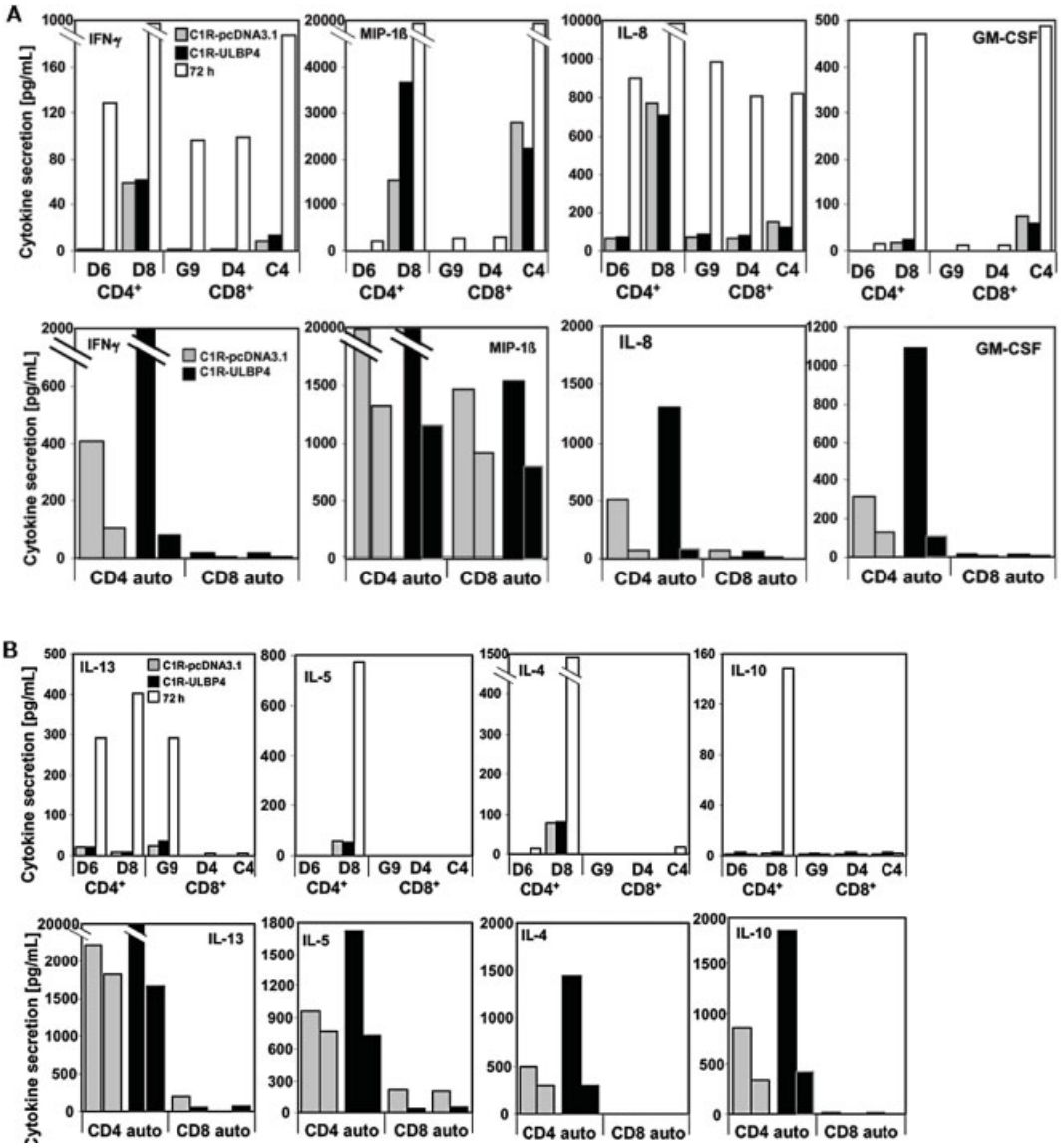

Figure 6. $CD4^+$ rather than $CD8^+$ NK-like T cell clones secrete cytokines and chemokines. (A) Secretion of IFN-γ, MIP-1β, IL-8, GM-CSF of two $CD4^+$ and three $CD8^+$ T cell clones and corresponding T cell lines of two donors is shown 24 h after stimulation with vector control or ULBP4-transfected C1R cells and 72 h after restimulation with autologous PHA-blasts. (B) Secretion of IL-13, IL-5, IL-4 and IL-10 for two $CD4^+$ and three $CD8^+$ T cell clones and corresponding T cell lines of two donors is shown 24 h after stimulation with vector control or C1R-ULBP4 cells and 72 h after restimulation with autologous PHA-blasts.

HLA-G for most effector populations. Variations between different donors may result from the fact that various T cell clones were expanded during culture. Remarkably, none of the known IR specific for HLA-C or HLA-E were expressed by $CD4^+$ or $CD8^+$ NK-like T cells. Thus, their negative regulation seems to be mediated by, as yet, undefined receptors that bind to HLA class Ia and Ib molecules. In previous studies, HLA-Cw6 was identified as a common inhibitory molecule for unseparated NK-like T cell populations [6] and, here, we demonstrate that HLA-Cw3 also serves as a potent inhibitor of cytotoxicity of purified $CD4^+$ or $CD8^+$ NK-like T cells.

Stimulation with IL-2 or IL-15, in the absence of TCR-mediated signals, was sufficient to increase intracellular perforin expression only in $CD8^+$ but not $CD4^+$ NK-like T cells. Perforin levels detected following different stimulations (IL-2 *vs.* IL-15) of $CD8^+$ non-MHC-restricted T cells correlated with the degree of cytotoxicity, suggesting that signaling *via* CD132/CD122 alone was able to induce perforin-mediated NK-like activities in peripheral T cells. In addition to cytotoxicity, NK-like T cells secreted a broad spectrum of cytokines and $CD4^+$ T cell clones differed from $CD8^+$ T cell clones. Cytokine secretion was not directly induced *via* AR, but NKG2D-mediated costimulation increased

secretion of IFN-γ, MIP-1β, IL-8 and GM-CSF after contact with human cells, suggesting that cell-cell contact by different receptors is necessary for this activity.

Various approaches for lymphocyte activation have been designed with the goal to induce potent effector cells against tumors for therapeutical application. For instance, cytokine-induced killer cells were generated using exogenous IL-2, IL-7 or IL-12 [8] or a combination of anti-CD3 specific mAb, IFN-γ and IL-2 [1, 38]. Following TCR-dependent, but antigen-independent stimuli, cytotoxic activity directed against tumors *in vitro* and in a SCID mouse/human lymphoma model [3] could be observed. Moreover, antigen responsiveness of $CD8^+$ CTL was restored by IL-15 stimulation, indicating that cytokine stimulation may provide a rescue mechanism for anergized tumor-specific CTL [31].

Clinical studies using IL-2-activated LAK cells with predominant NK phenotypes revealed occasional high but variable and unpredictable clinical responses [39–42]. Clinical benefits from the adoptive transfer of LAK cells to cancer patients were limited by the failure of many tumors to regress and the high toxicity of a systemic IL-2-application. This toxicity and the inability to understand the mechanisms by which LAK cells could inhibit the growth of some tumors, led to a switch towards the application of antigen-specific CTL in adoptive immunotherapy. One consequence of these therapies was the selective outgrowth of tumor cells with low or aberrant HLA class I expression which were no longer susceptible to specific MHC-restricted T cells [43–45]. Our results demonstrate that the NK-like T cell activity could be useful in therapy settings to combat tumor cells that escape surveillance by MHC-restricted CTL through down-regulation of HLA class I expression. A complementary immunotherapy using both CTL and NK-like T cells might prevent the selection of escape variants through alternate susceptibility to one or the other type of effector cell.

Recently it has been proposed that NK-like T cells could play a role in autoimmune disease where IL-15 is released in high amounts by affected tissues [46]. For celiac disease patients, a conversion of intestinal CTL to an NKG2D-mediated NK-like cytotoxic activity has been postulated upon exposure to inadequate amounts of IL-15 after gluten exposure [13, 14]. These experiments highlight the *in vivo* relevance of the transformation of normal MHC-restricted T cells into NK-like T cells. Thus, our novel findings of MHC-mediated inhibition of cytokine-induced and antigen-independent NK-like T cell activity contribute to define the receptor-ligand interactions involved in positive and negative regulation of this supplementary T cell function.

Materials and methods

Effector cells and target cells

PBMC were separated into $CD4^+$ and $CD8^+$ T cells by depleting all other subpopulations using magnetic bead separation (Dynal Biotech, Oslo, Norway), according to the manufacturer's instructions, and culture in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/mL penicillin, 100 μg/mL streptomycin (complete medium), 15% heat-inactivated pooled human serum, 1000 or 500 U/mL recombinant IL-2 (Proleukin; Cetus Corp., Emeryville, CA) or 5 ng/mL IL-15 (PromoCell, Heidelberg, Germany) with or without 1% PHA (Difco Laboratories, Detroit, MI). T cell cloning was performed by limiting dilution of $CD4^+$ or $CD8^+$ T cells of two donors and antigen-free stimulation with autologous irradiated PHA-blasts and IL-2 (500 U/mL). T cell clones were restimulated once at day 14 and characterized at day 9 after restimulation.

Cytotoxicity was assessed using the HLA class I-negative lymphoblastoid cell line L721.221 and its hemizygous variant, L721.112, expressing only haplotype A1/B8/Cw7. An HLA-E-expressing transfectant of the HLA class I-negative erythroleukemia line K562 was generated by cotransfecting of K562-HLA-E [47] with a fusion construct of the β_2m/HLA-B8 leader sequence. K562-C expresses Cw*0304; K562-G contains a mutated leader peptide (VTAPRTLLL) to exclude HLA-E stabilization by HLA-G-derived peptides. HLA class I-negative Burkitt lymphoma cell line Daudi is β_2m-deficient; Daudi-β_2m was generated by transfection with β_2m-encoding cDNA that leads to HLA-A14, -A66, -B58, -Cw6, -Cw3 expression (kindly provided by P. Parham, Stanford University, Palo Alto, CA). RCC26 renal cell carcinoma line was established from a primary tumor (HLA-A*0201/*3303, -B*4101/*5101, -Cw*1502/*1701); RCC26 was retrovirally transduced with the human IFN-γ cDNA [18]. All cells were cultured in complete medium; selection media contained 0.5 mg/mL G418 or hygromycin B (Invitrogen, Carlsbad, CA).

Immunophenotyping of effector and target cells

T cells were characterized with these mAb: FITC-conjugated anti-human CD3 (UCHT1), CD25 (B1.49.9), CD122 (CF1), PE-labeled CD4 (13B8.2), CD8 (B9.11), CD158a (2DL1, EB6), CD158b1/b2 (2DL2,3, GL183), CD158e1 (3DL1, Z27.3.7), CD161 (NKRP-1A, 191B8), CD158i (2DS4, FES172), NKG2A (CD159, Z177), CD69 (TP1.55.3) and CD45RO (UCHL1). PC5-labeled TCRαβ (BMA031), CD244 (2B4, C1.7; Beckman-Coulter, Westbrook, ME) and CD25 (B1.49.9) and allophycocyanin-conjugated CD4 (13B8.2) and CD56 (NKH-1) were purchased from Beckman-Coulter. CD94-FITC (HP-3D9) and LAIR-1-PE (DX26) were obtained from BD Pharmingen (San Diego, CA); CD85j-FITC (ILT2, VMP55.1) was obtained from DAKO (Glostrup, Denmark).

Lymphocytes were incubated for 45 min on ice with mixtures of mAb, washed with PBS/5% FBS, fixed with PBS/1% paraformaldehyde and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA). Indirect fluorescence analysis was performed for NKG2D (149810; R&D

Systems, Minneapolis, MN) and CD226 (DNAM-1, DX11; BD Pharmingen); MOPC21 was used as IgG1, UPC10 as IgG2a isotype control (Sigma, Deisenhofen, Germany). Cells were incubated with 5 μg/mL mAb for 45 min on ice, washed with PBS/5% FBS, incubated with PE-conjugated goat-anti-mouse $F(ab')_2$-IgG (Dianova, Hamburg, Germany) (1:100 dilution) for 30 min and analyzed by flow cytometry.

Cell-mediated cytotoxicity and blocking with monoclonal antibodies

Cell-mediated cytotoxicity was quantified in standard 4-h ^{51}Cr-release assays [48]. Target cells were labeled 90 min with $Na_2{}^{51}CrO_4$, washed twice and 2×10^3 cells were exposed to effector cells for 4 h at the E:T ratios indicated. Spontaneous release was determined by incubating target cells alone, maximal release was determined by directly counting labeled target cells. Duplicate or triplicate measurements of four-step titrations of effector cells were used in all experiments.

Redirected lysis experiments using FcR^+ P815 (ATCC, Manassas, VA) were performed with unconjugated mAb (100 ng/mL, 2×10^3 P815 cells): CD161 (DX12; BD Pharmingen), CD226 (DX11; BD Pharmingen), NKG2D (149810; R&D Systems), CD244 (2B4, C1.7; Beckman-Coulter), CD2 (P. Rieber, University Dresden, Germany) and CD3 (rat IgG2b, E. Kremmer, GSF). mAb against CD85j (ILT2; DAKO) and LAIR-1 (Pharmingen) were used at 200 ng/mL. MOPC21, UPC10 and 4G1 (rat IgG2b) are isotype controls. P815 cells were pre-incubated for 30 min at room temperature. For HLA blocking, L721.221 and L721.112 cells were incubated 30 min at 37°C with HLA-specific mAb A1.4 (Olympus, Hamburg, Germany) or isotype mAb (MOPC21; 10 μg/mL).

NKG2D-dependent stimulation assay

The skin-specific NKG2D ligand ULBP4 [49] was cloned into pcDNA3.1(+) (Invitrogen) and transfected into C1R cells (expressing HLA-Cw*0403, ULBP2 and ULBP3; data not shown). After CFSE labeling, 0.125×10^6–0.6×10^6 T cells/mL were incubated for 96 h with irradiated wild-type C1R, vector control C1R-pcDNA3.1 or C1R-ULBP4 cells (E:T ratio 2:1). Proliferation was analyzed and calculated as percent decrease in CFSE; counterstaining was performed with CD3, CD56, CD4 or CD8. For cytokine analyses, supernatants were harvested at 24 h in parallel to proliferation and cytotoxicity assays.

Cytokine and chemokine secretion

Cytokines were quantified by multiplex protein arrays according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Microspheres coated with cytokine-specific mAb were incubated for 30 min at room temperature, with 50 μL supernatant. After three washing steps, biotinylated detection mAb were added, incubated for 30 min at room temperature, followed by streptavidin-PE incubation. A two-laser array reader (Luminex χ-Map) simultaneously quantifies 11 cytokines; standard curves (3.91–3.2×10^4 pg/mL) and concentrations were calculated with Bio-Plex Manager3.1. Cytokines and chemokines tested were IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, GM-CSF, IFN-γ, TNF-α, MIP-1β.

Intracellular perforin staining

T cells were incubated with directly labeled CD4 (PE) and CD3 (Cy5). Cells were fixed with PBS/1% PFA, washed and permeabilized with 0.35% saponin. After addition of perforin-FITC (δG9) or IgG2b-FITC (27-35, isotype control; Becton Dickinson), T cells were washed twice with 0.1% saponin and analyzed by flow cytometry.

Thymidine proliferation assay

NK-like T cells were cultured with PHA/IL-2, IL-2 alone, PHA/IL-15 or IL-15 alone. At day 6, 0.1×10^6 T cells were incubated in 200 μL medium with 1 μCi [^{3}H]thymidine (NEN-Dupont). After 18 h, proliferation was quantified by measuring incorporation of thymidine in a β-counter (Topcount; Packard).

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