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TCR-dependent sensitization of human $\gamma\delta$ T cells to non-myeloid IL-18 in cytomegalovirus and tumor stress surveillance

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Human $\gamma\delta$ T cells contribute to tissue homeostasis under normal conditions and participate in lymphoid stress surveillance against infection and tumors. However, the molecular mechanisms underlying the recognition of complex cell stress signatures by $\gamma\delta$ T cells are still unclear. Tumor cells and human cytomegalovirus (HCMV)-infected cells are known targets of $\gamma\delta$ T cells. We show here that many tumor and CMV-infected cells express caspase-1 inflammasomes and release interleukin (IL)-18. Engagement of the T-cell receptor (TCR) on V δ 2^{neg} $\gamma\delta$ T cells controlled the direct innate immune sensing of IL-18 that enhanced cytotoxicity and interferon gamma (IFN γ) production. This TCR-dependent sensitization to IL-18 was mediated by the upregulation of the innate IL-18 receptor β chain (IL-18R β) expression. These findings shed light on inflammasomes as a unified stress signal of tumor and infected cells to alert $\gamma\delta$ T cells. Moreover, uncovering the TCR-mediated sensitization of $\gamma\delta$ T cells to inflammatory mediators establishes a molecular link between the innate and adaptive immune functions of $\gamma\delta$ T cells that could fine tune the commitment of antigen-experienced $\gamma\delta$ T cells to inflammatory responses.

Introduction

The presence of $\gamma\delta$ T cells, along with $\alpha\beta$ T cells and B cells, in all higher vertebrates suggest that each contributes uniquely to host immune competence and is essential for its maintenance. Human $\gamma\delta$ T cells are a relatively rare immune population in peripheral blood, mostly composed of cells expressing the V γ 9V δ 2 variable regions. Epithelial tissues are substantially enriched in other $\gamma\delta$ T-cell subsets (collectively called V δ 2^{neg} $\gamma\delta$ T cells) that mediate first-line host response to a wide variety of cellular insults triggered by malignancy or bacterial/viral infection, and contribute to tissue homeostasis.^{1,2} The $\gamma\delta$ T cells have been shown to recognize stress-associated proteins, metabolites, and lipids in a classical major histocompatibility complex (MHC)-unrestricted manner.¹ A critical protective role against tumors and infections has been demonstrated *in vivo* using $\gamma\delta$ T-cell-deficient mice^{3,4} as well as in the context of chemotherapy.⁵ Human $\gamma\delta$ T cells can infiltrate tumors and infected tissues, and their expansion in blood correlates with better clinical

outcome in both malignancies and infectious diseases.^{6,7} Notably, they can also regulate $\alpha\beta$ T cells^{8,9} and maintain tissue integrity.¹⁰ *In vitro*, human $\gamma\delta$ T cells can kill transformed cells, infected cells, and microorganisms. To date, phase 1 and 2 clinical trials have been conducted to evaluate the use and efficacy of V γ 9V δ 2 $\gamma\delta$ T-cell-based immunotherapy. Trials against solid tumors revealed mixed results, as they were hampered by fluctuating responses to *ex vivo* stimulation and a strong susceptibility of this population for activation-induced cell death (AICD).¹¹

Interestingly, AICD seems to be reduced for epithelial V δ 2^{neg} $\gamma\delta$ T cells, and increasing evidence supports an important role of this subset for tumor and infection immunosurveillance.¹² Human V δ 2^{neg} $\gamma\delta$ T cells expand in the periphery of individuals during CMV infection in various pathophysiological contexts, including solid-organ and stem cell transplantation,^{13–17} where they develop cytotoxic function and produce proinflammatory cytokines such as tumor necrosis factor α (TNF α) and IFN γ .¹⁸ Importantly, CMV-induced expansion of V δ 2^{neg} $\gamma\delta$ T cells correlates with decreased susceptibility to post-transplant cancers,

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suggesting a role in tumor immunosurveillance *in vivo*.¹⁹ In line with this, such cells display a TCR-dependent cross-reactivity to several solid tumor cell lines and CMV-infected cells, which can be explained by the recognition of common, specific, stress-induced $\gamma\delta$ TCR ligands (e.g., EPCR) expressed by cellular targets in both conditions.¹⁸ However, a complex array of co-stimulatory signals along with TCR engagement seem to be required to fine tune appropriate V δ 2^{neg} $\gamma\delta$ T-cell responses to tissue stress.¹⁸

Inflammasomes are multi-protein complexes that stimulate caspase-1, and initiate inflammatory signaling in response to molecular determinants produced by pathogens and cell stress.^{20,21} Two types of inflammasomes have been described, involving either nucleotide-binding domain leucine-rich repeat-containing receptors (NOD-like receptor [NLRs] family proteins) or the PYHIN family protein (absent in melanoma 2 [AIM2]-like receptors). These serve as anchoring scaffolds onto which protein-complex assembly occurs to process procaspase-1 into active caspase-1. Subsequently, active caspase-1, composed of the hetero-tetramer (p10)₂/(p20)₂, facilitates maturation of pro-IL-1 β and pro-IL-18 into active cytokines to be secreted and prime the inflammatory response. While playing critical roles in host defense against pathogens, NLR functions have also been associated with the progression of metabolic syndrome,^{22,23} thereby contributing to their definition as danger sensors of metabolic perturbations. Interestingly, inflammasome assembly seems to occur in response to a metabolic reprogramming similar to the one triggered by both cellular transformation and viral infection (including CMV).²⁴⁻²⁶ IL-18 is crucially involved in tissue homeostasis.²⁷⁻²⁹ *In vivo* studies have shown IL-18 expression during late stages of tumorigenesis in tumor tissues and the serum of patients with various types of cancer^{30,31} together with an immunoablative role of natural killer (NK) cells.³² Various epithelial cells express NLRs^{33,34}; however, the role of NLRs in the activation of inflammasomes within tissue-derived malignant and infected cells, as well as their direct role in controlling effector functions of intraepithelial lymphocytes (IEL), remains to be defined. We hypothesized that inflammasome activation may represent a unified stress signal triggered by both CMV infection and cellular transformation, which in turn could modulate human V δ 2^{neg} $\gamma\delta$ T-cell response through the secretion of soluble signaling molecules including IL-1 β and IL-18 cytokines. Such a mechanism may represent an additional stress signal recognized by $\gamma\delta$ T cells to sense disturbed tissue integrity.

Results

Tissue-derived cellular targets of human V δ 2^{neg} $\gamma\delta$ T cells secrete mature IL-18

Human V δ 2^{neg} $\gamma\delta$ T cells recognize a wide range of cancer cells as well as CMV-infected endothelial cells through a $\gamma\delta$ -TCR-dependent mechanism.³⁵ We first evaluated whether these cancer cells may secrete inflammasome-dependent inflammatory cytokines including IL-1 β and IL-18, as well as products of antigen-presenting cells (APCs) such as IL-12. We screened

several human cancer cell lines and noticed the secretion of mature IL-18, from glioblastoma U373MG and U343MG, lung adenocarcinoma SKMES-1, and hepatocarcinoma HUH7, as measured by ELISA (ranging from 50 to 200 pg/mL) (Fig. 1A). In contrast to IL-18, mature IL-1 β and IL-12 were not detected from the supernatants of tested cell lines (except minor amounts of IL-12 for HT1080), although both were readily detectable in culture supernatants of the lipopolysaccharide (LPS)/adenosine triphosphate (ATP)-activated monocytic THP-1 cell line used as a positive control (Fig. 1B). We also observed a significant increase of IL-18 secretion from human umbilical vein endothelial cells (HUVECs) following HCMV infection with increased doses of virus (Fig. 1C). Secretion of mature IL-1 β followed that of IL-18 but to a lesser extent, and IL-12 secretion was barely detected from HCMV-infected HUVEC cultures (Fig. 1D). Therefore, both human targets of V δ 2^{neg} $\gamma\delta$ T cells tested here (cancer cells and HCMV-infected cells) secrete caspase-1-dependent cytokines.

$\gamma\delta$ TCR signaling sensitizes V δ 2^{neg} $\gamma\delta$ T cells to soluble IL-18, thereby enhancing IFN γ production

To address whether non-myeloid IL-18 could participate in $\gamma\delta$ T-cell activation by tumor and CMV-infected cells, it was first important to assess the $\gamma\delta$ T-cell response to recombinant IL-18. Therefore, we investigated the direct effect of IL-18 *ex vivo* on fresh peripheral blood mononuclear cells (PBMCs) isolated from CMV-infected kidney transplant recipients that contained at least 15% of V δ 2^{neg} $\gamma\delta$ T cells (mostly expressing V δ 1 and V δ 3) among CD3⁺ T cells (Fig. 1E; Fig. S1).¹³ Among PBMCs, the V δ 2^{neg} $\gamma\delta$ T cells were specifically stimulated through the TCR (by using anti-V δ 1 + anti-V δ 3 Abs), and left untreated or treated with IL-18 or IL-1 β . IL-18, but not IL-1 β , strongly induced IFN γ secretion from bulk PBMCs, but only in the presence of TCR stimulation mediated by both anti-V δ 1 and anti-V δ 3 Abs used either combined (Fig. 1F) or separately (Fig. 1G). Among PBMCs from 7 patients that were tested, none showed an increase of IFN γ secretion after IL-18 treatment alone, conversely to that with co-treatment with IL-18 and $\gamma\delta$ TCR agonists (Fig. 1H). When normalized by the level of IFN γ secreted in the presence of TCR agonists, 4 patients showed a >fold4 increase of IFN γ secretion upon IL-18 stimulation versus no IL-18 (Fig. 1I). Therefore, IL-18 treatment of patient PBMCs acts synergistically with TCR stimulation to enhance IFN γ secretion.

As IL-18-mediated IFN γ secretion from PBMCs might be attributed to indirect effects on other cells among PBMCs ($\alpha\beta$ T and NK cells), we investigated whether purified V δ 2^{neg} $\gamma\delta$ T cell clones and short-term polyclonal cell lines would behave similarly. We used purified V δ 2^{neg} $\gamma\delta$ T-cell clones (expressing V γ 4V δ 5, V γ 8V δ 3, or V γ 2V δ 3 TCRs) and short-term polyclonal cell lines (V δ 1, V δ 1 + V δ 3, and V δ 5) generated from the blood of CMV-infected patients as described in Halary et al.³⁵ First, $\gamma\delta$ T cells were stimulated with increased concentrations of anti-CD3 in the presence or absence of recombinant cytokines IL-18 or IL-1 β (Fig. 2A and B). For all clones tested, the amount of IFN γ secreted was significantly higher in the presence of IL-18, regardless

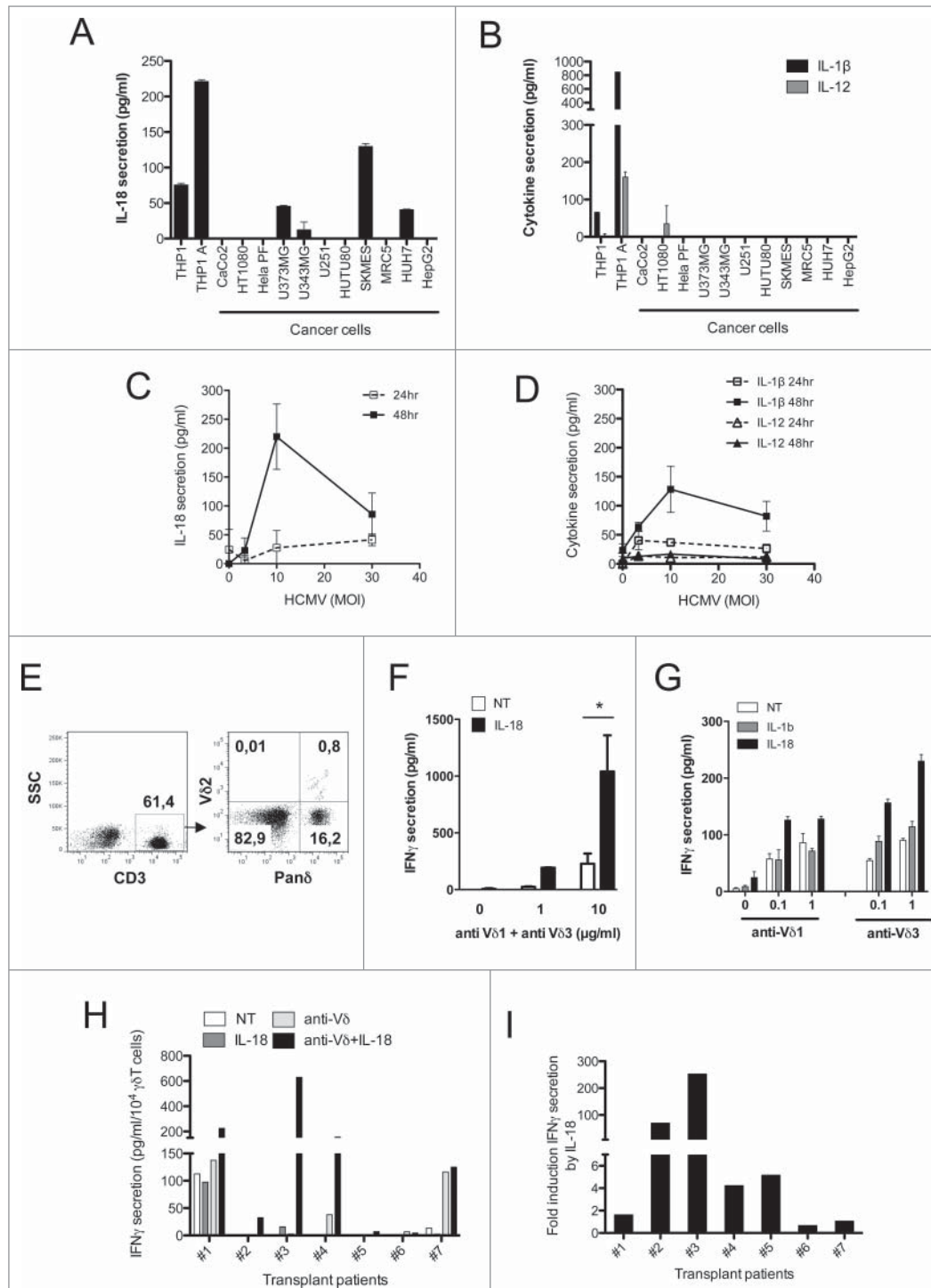


Figure 1. IL-18 is secreted by cancer cells and HCMV-infected cells, and enhances IFN γ production by human V δ ^{neg} $\gamma\delta$ T cells within PBMCs. (A) IL-18 or (B) IL-1 β and IL-12 secretion by cancer cell lines. Cancer cell lines were cultured for 48 h and the secretion of cytokines was measured by ELISA from cell culture supernatants. Results are normalized by the same amount of cells used for each cell line. HUVEC endothelial cells were infected with HCMV at various multiplicities of infection (MOIs), and cell culture supernatant at 24 and 48 h post-infection was used to monitor (C) IL-18 or (D) IL-1 β and IL-12 secretion by ELISA. Data are expressed as concentration of cytokines (pg/mL; mean \pm SD; $n = 3$). (E) Example of Pan δ immunotyping from whole blood. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of a kidney transplant patient and Pan δ populations were quantified using anti-pan δ and anti-V δ 2 antibodies within the CD3⁺ population by flow cytometry. (F) PBMCs isolated from a patient with expanded V δ ^{neg} population ($>12\%$ of CD3⁺) were incubated with various concentrations of anti-V δ 1 and anti-V δ 3 antibodies in the presence or absence of recombinant IL-18 (50 ng/mL) for 24 h at 37°C, then IFN γ secretion was measured by ELISA from cell culture supernatants (mean \pm SD; $n = 3$). (G) Same as in (F) but PBMCs from another patient were treated with either anti-V δ 1 or anti-V δ 3 antibodies at various concentrations in the presence or absence of recombinant IL-18 or IL-1 β (50 ng/mL) for 24 h at 37°C; then, IFN γ secretion was measured by ELISA from cell culture supernatants (mean \pm SD; $n = 3$). (H) IFN γ secretion obtained from several kidney transplant patient's PBMCs was normalized by the same number of V δ ^{neg} $\gamma\delta$ T cells and plotted as raw data. Anti-V δ indicates treatment with combined anti-V δ 1+anti-V δ 3 antibodies (10 μ g/mL each). (I) IL-18 response is represented as fold induction of IFN γ secretion after IL-18 treatment (anti-V δ ^{neg} +IL-18/anti-V δ ^{neg}). *, $P < 0.05$.

of IL-1 β . These results were confirmed on short-term polyclonal cell lines (V δ 1, V δ 1+ V δ 3, and V δ 5) activated with either anti-V δ 1 monoclonal antibody (mAb) or anti-CD3

(Fig. 2C–E). TNF α secretion was also measured, with the same result as for IFN γ secretion (Fig. S2A). The effects of IL-18 were not restricted to V δ 2^{neg} $\gamma\delta$ T cells because they

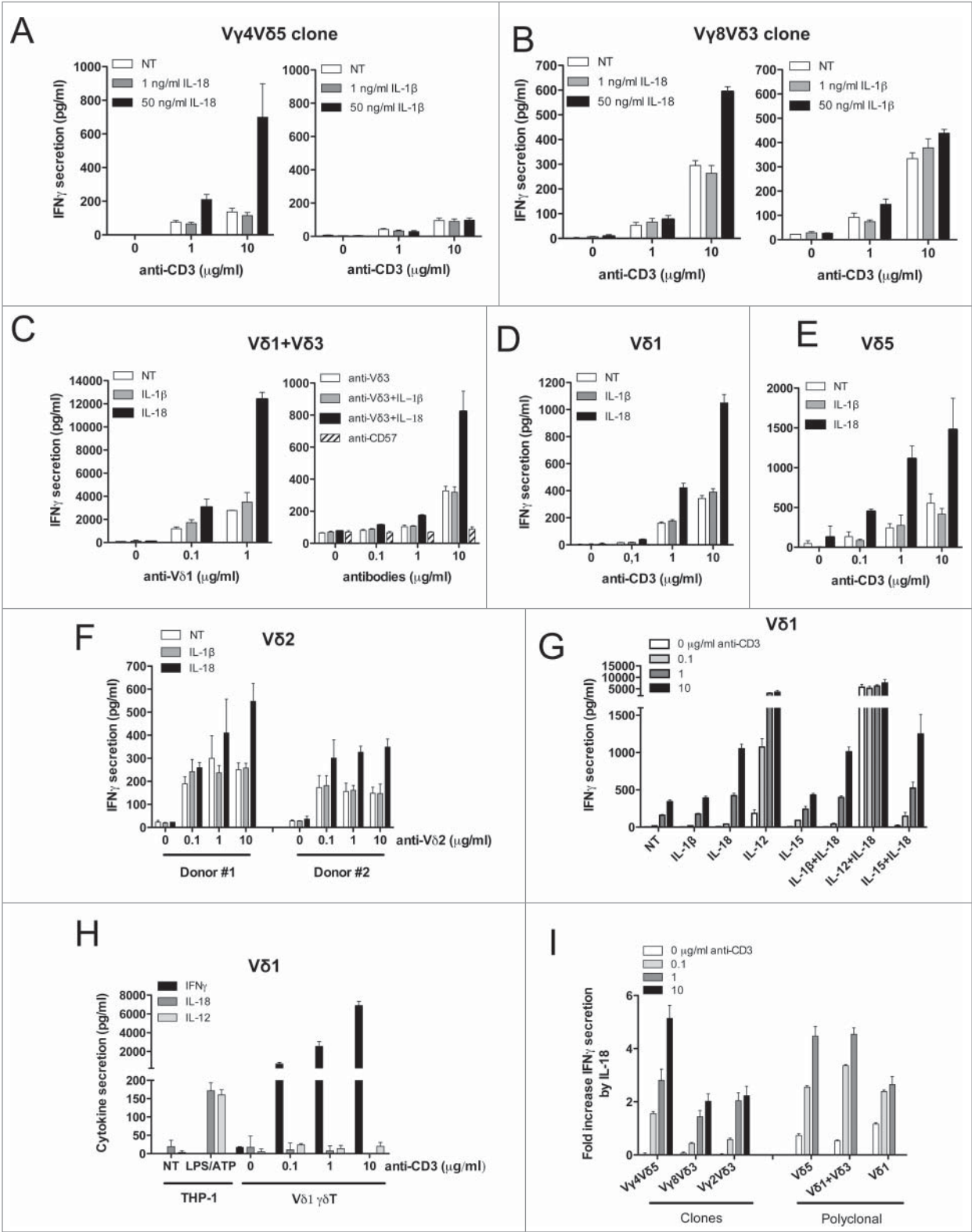


Figure 2. For figure legend, see next page.

were also observed on purified and expanded polyclonal V γ 9V δ 2 T cells isolated from 2 different healthy donors, upon TCR stimulation using anti-V δ 2 antibody (Ab) (Fig. 2F). Therefore, TCR-stimulated $\gamma\delta$ T cells respond directly to IL-18 and likely contribute to the IFN γ production observed in PBMCs.

We next tested whether IL-18 could synergize with others cytokines to increase IFN γ production. IFN γ secretion was measured in the presence of cytokines alone (IL-1 β , IL-18, IL-12, and IL-15) or in combination. When used alone, only IL-12 or IL-18 increased IFN γ secretion following anti-CD3 treatment (using either V δ 1 or V δ 5 polyclonal cell lines; Fig. 2G; Fig. S2B, respectively). The combination of IL-18 and IL-12 had a synergistic effect even in the absence of TCR stimulation (as already observed for $\alpha\beta$ T cells).³⁶ The TCR-mediated effect on the IL-18 response could not be attributed to IL-12 secretion by $\gamma\delta$ T cells themselves because anti-CD3 treatment of V δ 1 polyclonal cell line did not induce any detectable IL-12 in cell culture supernatant (neither IL-18), while inducing IFN γ (Fig. 2H). In conclusion, the TCR-dependent response to IL-18 is a general mechanism among all human V δ 2^{neg} $\gamma\delta$ T cells tested (clones and polyclonal cell lines) that mediates at least a fold2-induction of IFN γ secretion in comparison to TCR signaling alone (Fig. 2I). V δ 2^{neg} $\gamma\delta$ T cells do not require any other co-factor provided by APCs such as IL-12 to respond to IL-18, although this combination can lead to a better response. Strikingly, the $\gamma\delta$ T-cell responsiveness to IL-18 is highly dependent on the strength of $\gamma\delta$ TCR signaling.

$\gamma\delta$ TCR signaling induces the expression of IL-18R β chain

We next tested whether $\gamma\delta$ TCR engagement would increase IL-18 sensing by upregulating expression of the cell membrane receptor IL-18R β (which controls the number of high-affinity IL-18 receptors).³⁷ This was tested on various V δ 2^{neg} $\gamma\delta$ T cells (clones and polyclonal cell lines), upon $\gamma\delta$ TCR stimulation by anti-CD3 agonist Ab. We detected basal expression of IL-18R β in approximately 10% of $\gamma\delta$ T-cell clones (V γ 9V δ 1) and polyclonal cell lines (V δ 1, V δ 1+3, V δ 5) (Fig. 3A). Upon anti-CD3 stimulation, we noticed a dose-dependent increase of the number of IL-18R β -positive cells by at least fold2-(Fig. 3B). These results suggest ¹ a molecular link between $\gamma\delta$ TCR signaling and the expression of innate IL-18R β , and ² that TCR-

mediated responses to IL-18 might be a specific characteristic of $\gamma\delta$ T cells that allows them to enhance IFN γ production and cytotoxicity.

Soluble IL-18 secreted from tumor and infected target cells enhances IFN γ production by V δ 2^{neg} $\gamma\delta$ T cells

Cancer cells and HCMV-infected endothelial cells are both targets of V δ 2^{neg} $\gamma\delta$ T cells, and both can secrete IL-18. Therefore, we tested if conditioned culture supernatant of target cells can promote IFN γ secretion from V δ 2^{neg} $\gamma\delta$ T cells in the presence of TCR signaling. Polyclonal V δ 2^{neg} $\gamma\delta$ T-cell lines were incubated with plate-coated anti-CD3 and the conditioned culture supernatants (CS) of either cancer cells or HCMV-infected HUVEC cells. We first screened various CS with the V δ 1 polyclonal cell line and observed that glioblastoma U373MG, lung adenocarcinoma SKMES-1, and hepatocarcinoma HUH7 were potentially able to enhance IFN γ secretion, which is consistent with the level of IL-18 secreted by these cells (Fig. 1A). At least a fold4-increase was observed at 1 μ g/mL of anti-CD3 stimulation (Fig. 4A). $\gamma\delta$ T-cell responsiveness to CS followed the amounts of U373MG cancer cells used to generate the CS as shown in Figure 4B. Importantly, no effect of CS was observed without TCR stimulation.

Altogether these results suggest that IL-18 secreted by cancer cells could enhance IFN γ production by TCR-activated $\gamma\delta$ T cells. We tested this hypothesis using anti-IL-18 blocking Ab (Fig. 4C). By using various V δ 2^{neg} $\gamma\delta$ T-cell clones and polyclonal cell lines as indicated, we observed that the anti-IL-18 blocking Ab, but not a control anti-IL-10 Ab, significantly decreased the effect of U373MG CS on $\gamma\delta$ T-cell IFN γ production by almost 50% at high anti-CD3 stimulation. In the same manner than for cancer cell-derived CS, CS isolated from HCMV-infected HUVEC cells was able to enhance IFN γ secretion in the presence of anti-CD3 stimulation, in contrast to CS obtained from non-infected endothelial cells. This response was also decreased by almost 50% after using the anti-IL-18 blocking Ab (irrespective to anti-IL10).

To confirm these results in a more physiological setting of TCR stimulation, which involves the $\gamma\delta$ TCR-dependent recognition of stress antigens expressed by target cells, we used our model of $\gamma\delta$ T-cell activation by cancer cells or HCMV-infected cells.³⁵ We tested whether target cell-release of IL-18 was also involved in $\gamma\delta$ T-cell activation. U373MG and $\gamma\delta$ T-cell

Figure 2 (see previous page). IL-18 alone enhances IFN γ production by purified V δ 2^{neg} $\gamma\delta$ T cells in a TCR-dependent manner. Human V δ 2^{neg} $\gamma\delta$ T-cell clones including (A) V γ 4V δ 5 or (B) V γ 8V δ 3 purified from human PBMCs were cultured through polyclonal activation and incubated with various concentrations of anti-CD3 in the presence or absence of IL-18 or IL-1 β for 24 h at 37°C; then, IFN γ secretion was measured by ELISA from cell culture supernatants (mean \pm SD; n = 3). The same procedure was applied to purified V δ 2^{neg} $\gamma\delta$ T polyclonal cell lines isolated from kidney transplant patients, including (C) V δ 1+V δ 3, or (D) V δ 1, or (E) V δ 5 $\gamma\delta$ T cells. Polyclonal cell lines were incubated with various concentrations of $\gamma\delta$ TCR agonists (anti-V δ 1, or anti-V δ 3, or anti-CD3 antibodies) in the presence or absence of either IL-18 or IL-1 β for 24 h at 37°C. Anti-CD57 was used as negative control for TCR stimulation. IFN γ secretion was then measured by ELISA from cell culture supernatants (mean \pm SD; n = 3). (F) V δ 2 $\gamma\delta$ T polyclonal cell lines were isolated from PBMCs of 2 healthy donors and cultured through polyclonal activation using 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) and IL-2, then incubated with various concentrations of $\gamma\delta$ TCR agonist anti-V δ 2 in the presence or absence of either IL-18 or IL-1 β for 24 h at 37°C. IFN γ secretion was then measured by ELISA from cell culture supernatants (mean \pm SD; n = 3). (G) A V δ 1 $\gamma\delta$ T polyclonal cell line was treated with anti-CD3 in the presence or absence of various cytokines alone or in combination for 24 h at 37°C. IFN γ secretion was then measured by ELISA from cell culture supernatants (mean \pm SD; n = 3). (H) A V δ 1 $\gamma\delta$ T polyclonal cell line treated with anti-CD3 and cell culture supernatant was used to monitor IFN γ , IL-18, or IL-12 secretion by ELISA. LPS/ATP-treated THP-1 cells were used as positive control for cytokine secretion (mean \pm SD; n = 3). (I) The various V δ 2^{neg} $\gamma\delta$ T-cell clone and polyclonal cell line responses to IL-18 are represented as fold induction of IFN γ secretion by IL-18 (IL-18 + TCR agonist/TCR agonist).

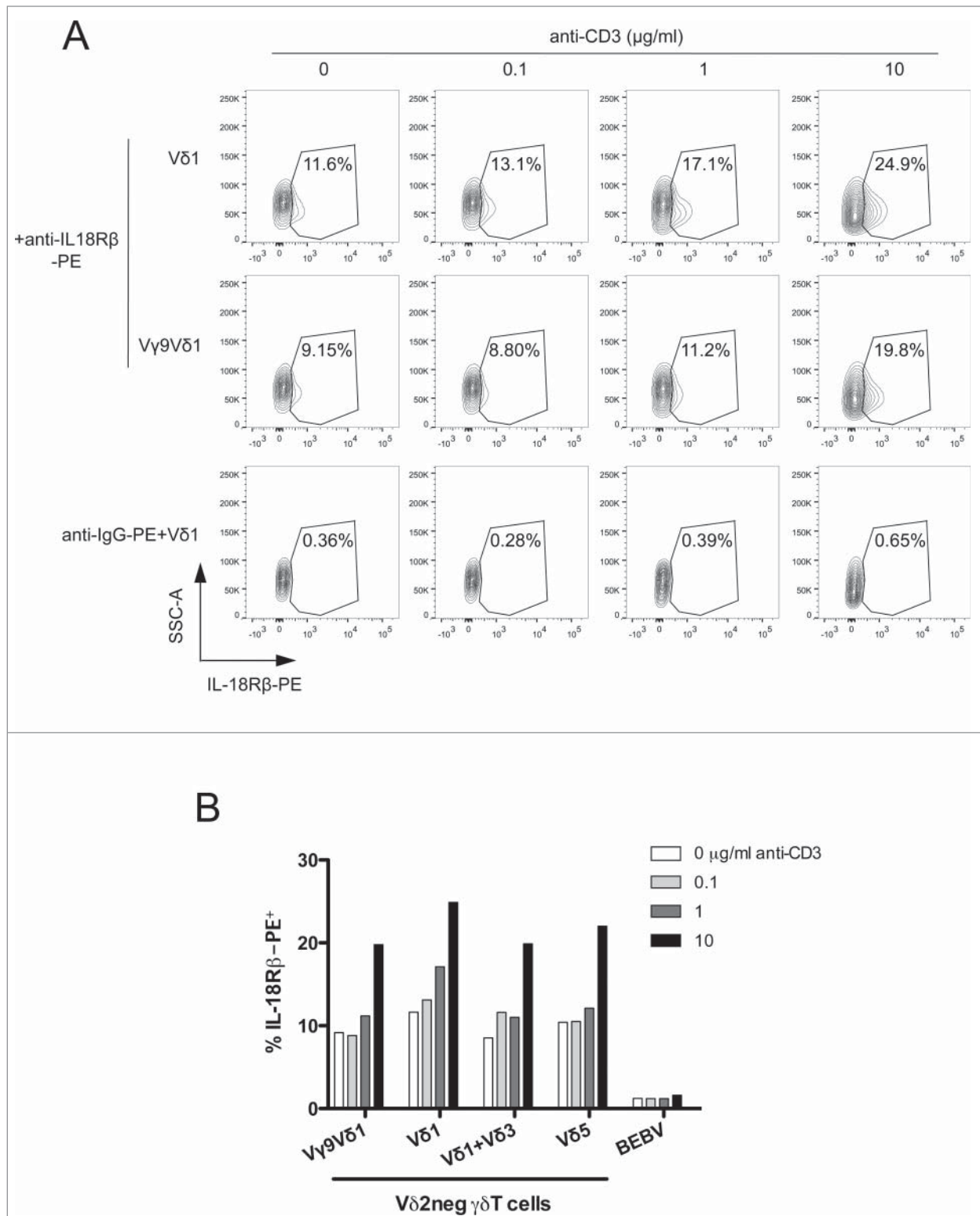


Figure 3. $\gamma\delta$ TCR signaling increases membrane IL-18R β chain expression. (A) A V δ 1 $\gamma\delta$ T polyclonal cell line, V γ 9V δ 1 cell clone, or B-cell lymphoma BEBV cell line were incubated with various concentrations of anti-CD3 for 24 h at 37°C, and immunostained with PE-conjugated anti-IL-18R β or IgG-PE control (for the V δ 1 $\gamma\delta$ T polyclonal cell line). The expression of membrane IL-18R β chain was determined by flow cytometry within the healthy cell population. IL-18R β -PE $^{+}$ cells are shown in the gate with the percentage of positive cells as indicated. (B) Bar graph summarizing IL-18R β -PE $^{+}$ cells (%) from various V δ 2 neg $\gamma\delta$ T-cell clones (V γ 9V δ 1) and polyclonal cell lines (V δ 1, V δ 1+3, V δ 5) after incubation with various concentrations of anti-CD3. Data are representative of at least 3 independent experiments with similar results (mean of at least 5×10^3 cells).

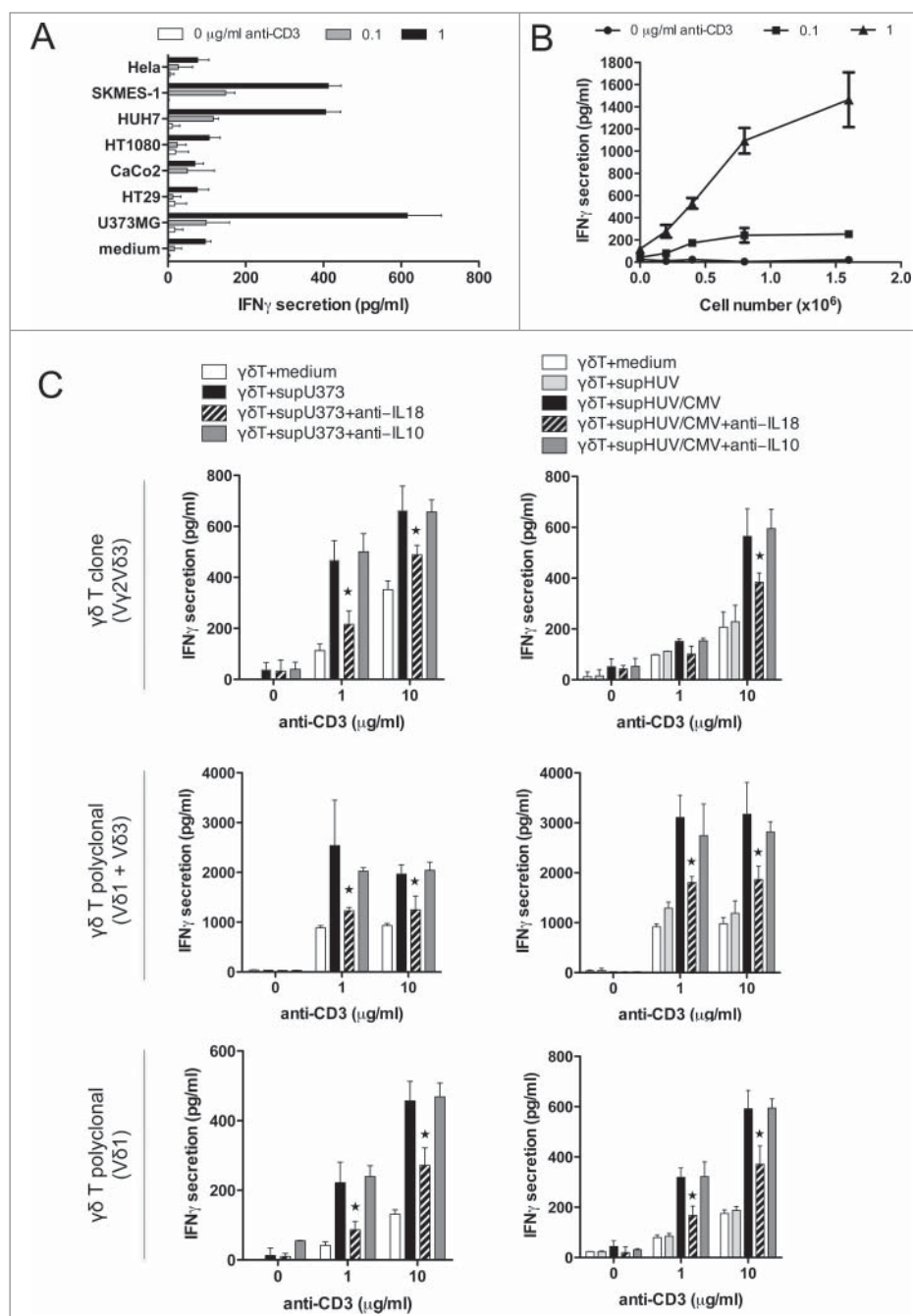


Figure 4. Soluble IL-18 secreted by cancer cells and HCMV-infected cells enhances IFN γ production by human V δ 2^{neg} $\gamma\delta$ T cells, in a TCR-dependent manner. (A) Conditioned culture supernatants of various cancer cell lines, or fresh media, were isolated after 48 h at 37°C, cleared by centrifugation, and incubated with a V δ 1 $\gamma\delta$ T polyclonal cell line in the presence of various concentrations of coated anti-CD3 for 24 h at 37°C. IFN γ secretion was then measured by ELISA from cell culture supernatants (mean \pm SD; n = 3). Results are normalized by the same amount of cells used for each cancer cell line. (B) Conditioned culture supernatants from various amounts of U373MG cancer cells, or fresh media, were isolated after 48 h at 37°C, cleared by centrifugation, and incubated with a V δ 1 $\gamma\delta$ T polyclonal cell line in the presence of anti-CD3 for 24 h at 37°C. IFN γ secretion was then measured by ELISA from cell culture supernatants (mean \pm SD; n = 3). (C) Conditioned culture supernatants of U373MG cancer cells, or HUVECs infected or not with HCMV (MOI 10), or fresh media, were isolated after 48 h at 37°C, cleared by centrifugation, and incubated with V δ 2^{neg} $\gamma\delta$ T cells in the presence of anti-CD3 with or without anti-IL-18 or control anti-IL-10 antibodies (10 μ g/mL). After 24 h at 37°C, IFN γ secretion was measured by ELISA from cell culture supernatants (mean \pm SD; n = 3). As indicated, V γ 2V δ 3 T cell clone, V δ 1, and V δ 1+3 polyclonal cell lines were tested. ★, P < 0.05.

co-culture induced a robust IFN γ secretion that was further inhibited by increasing concentrations of neutralizing anti-IL-18 monoclonal antibody (mAb; in contrast to control anti-IL-10) (Fig. 5A). Moreover, anti-IL-18 mAb also inhibited cytotoxic functions of the $\gamma\delta$ T-cell clone as measured by expression of CD107a using flow cytometry (Fig. S3). In addition, HUVEC and $\gamma\delta$ T-cell co-culture also induced IFN γ secretion but only when endothelial cells were infected by HCMV. Moreover, co-culture in the presence of anti-IL-18 partially but specifically blocked IFN γ secretion, as shown with anti-IL-10 negative control. Therefore, CS and co-culture experiments confirmed that tumor and infected-cell-derived IL-18 enhances $\gamma\delta$ T-cell IFN γ production and cytotoxic function.

IL-18 secretion from tumor and infected target cells depends on caspase-1 activation

The inactive pro-IL-18 precursor must be cleaved through caspase-1-dependent processing to generate an active cytokine that is secreted to prime the adaptive immune response. Prior to this, procaspase-1 itself needs to be activated by proteolytic cleavage upon assembly of NLR inflammasomes. However, in some circumstances including Fas signaling, IL-18 and IL-1 β can be processed by a non-canonical caspase-8-dependent process. To gain insights into the mechanism of IL-18 maturation in cancer cells and HCMV-infected cells, we evaluated caspase-1 cleavage in these cells by Western blotting. THP-1 monocytic cells activated by LPS/ATP (described to induce caspase-1 processing) were used as positive controls to detect protein sizes of procaspase-1 and active cleaved forms of caspase-1 (p45 and p10, respectively). We detected a basal expression of p45 in human cancer cell lines including glioblastoma U373MG, U251, U343MG, and lung SKMES-1 (Fig. 6A). Interestingly, all cancer cell lines tested expressed the ultimate p10 active cleaved fragment, in contrast to

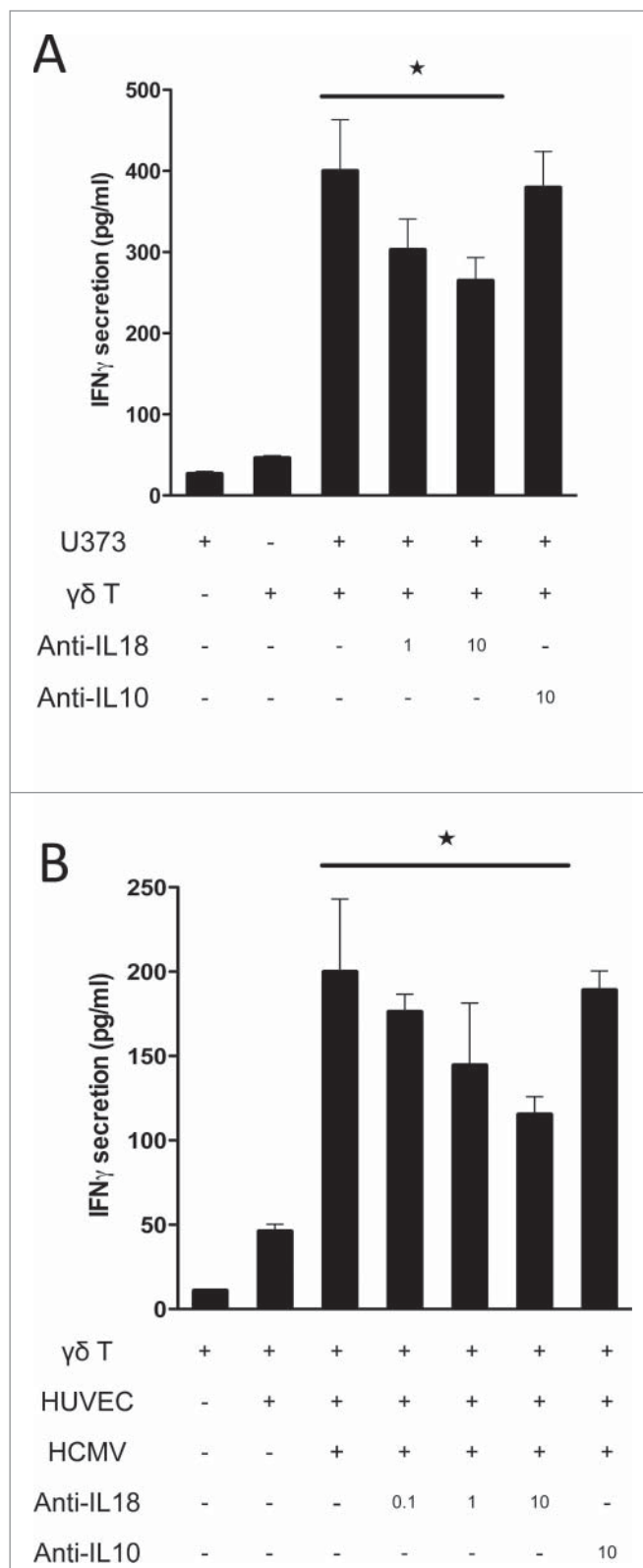


Figure 5. IL-18 produced by cancer cells and HCMV-infected cells contributes to IFN γ production by human V δ 2^{neg} γ δ T cells in co-culture. **(A)** U373MG cancer cells or **(B)** HUVECs uninfected or infected with HCMV (MOI 10) were cultured for 48 h at 37°C, and then co-cultured with a human V γ 4V δ 5 T-cell clone in the presence or absence of increased concentrations of anti-IL-18 or control anti-IL-10 antibodies. After 24 h at 37 °C, IFN γ secretion was measured by ELISA from cell culture supernatants (mean \pm SD; $n = 3$). \star , $P < 0.05$.

further cleaved in HCMV-infected cells into the detectable p35 cleaved form. Cell lysates were incubated with a Biotinyl-conjugated peptide that binds specifically to the active catalytic site of caspase-1 (Biotin-YVAD-fmk), and the active p10 fragment could only be precipitated from HCMV-infected HUVECs lysates. Therefore, caspase-1 is expressed and cleaved in both types of tissue-derived target cells of V δ 2^{neg} γ δ T cells (cancer cells and HCMV-infected cells).

Because ¹ secreted IL-18 from target cells induced IFN γ production by V δ 2^{neg} γ δ T cells and ² caspase-1 was expressed and cleaved in target cells, we tested whether inhibition of active caspase-1 might impair IL-18 secretion and, therefore, V δ 2^{neg} γ δ T-cell responsiveness. In Fig. 6C (left), we prepared conditioned cell CS of U373MG cancer cells cultured in the absence or presence of a specific caspase-1 pharmacological inhibitor (Ac-YVAD-fmk), and subsequently tested the IFN γ response of a polyclonal V δ 1 γ δ T-cell line to YVAD-treated CS. The addition of Ac-YVAD-fmk to U373MG cancer cells strongly decreased the ability of YVAD-treated CS to induce IFN γ production (>50 %), even at the lowest concentration tested (11 μ mol/L). Both 11 and 33 μ mol/L Ac-YVAD-fmk concentrations did not induce cytotoxicity of U373MG cancer cells (data not shown). Moreover, treatment of V δ 1 cells with Ac-YVAD-fmk in fresh media did not impair IFN γ secretion (Fig. 6C, right). Similar results were obtained when using HCMV-infected cells instead of cancer cells (Fig. 6D). We then tested the effect of caspase-1 inhibition by Ac-YVAD-fmk on U373MG cancer cells directly co-cultured with V δ 5 γ δ T cells (Fig. 6E). Consistent with results obtained using CS, the use of Ac-YVAD-fmk inhibited IFN γ secretion at least as efficiently as neutralizing anti-IL-18 mAb. As a control, Ac-YVAD-fmk did not inhibit the anti-CD3-induced IFN γ production by V δ 5 γ δ T cells and, thus, had no direct effect on γ δ T-cell activation. Therefore, caspase-1 activity in both target cells (cancer and CMV-infected cells) regulates the secretion of soluble molecules that trigger IFN γ production by γ δ T cells, following TCR signaling. Caspase-1 inhibition as well as IL-18 blocking experiments resulted in a reproducible ~50% reduction of IFN γ production, which suggest that other caspase-1-independent soluble molecules besides IL-18 may be active on γ δ T cells in these conditions.

Discussion

Human V δ 2^{neg} γ δ T cells combine innate and adaptive features, and although we and others have shown that they display a

the non-transformed MRC5 lung cell line used as a negative control. Therefore, processed and active caspase-1 is expressed to various extents in all cancer cell lines. In Figure 6B, we observed that human endothelial cells also express the inactive form p45 that is

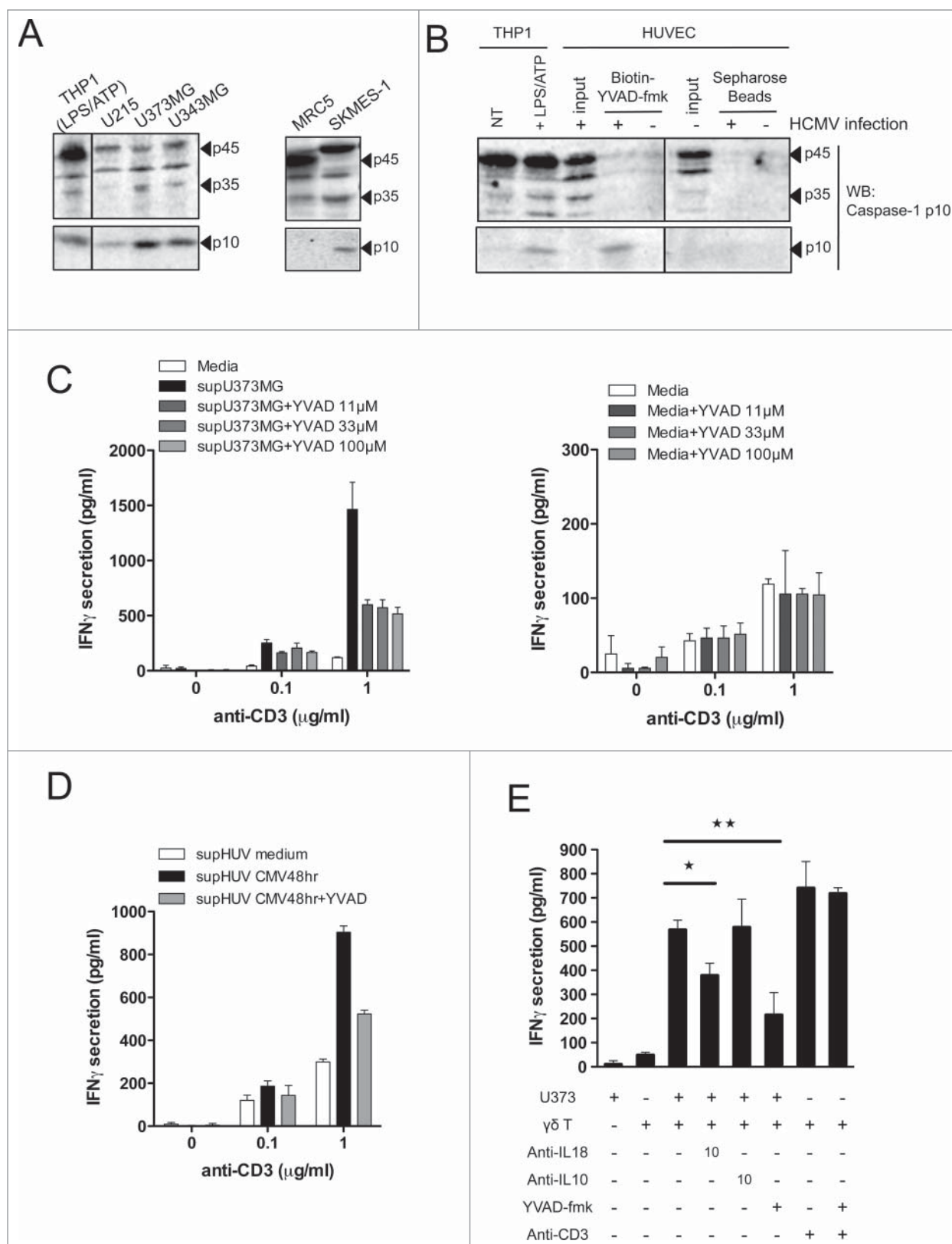


Figure 6. For figure legend, see next page.

dual reactivity against tumor and CMV-infected cells,^{18,38} the mechanisms by which they participate in lymphoid stress surveillance are poorly defined. Further knowledge of the mechanisms underpinning their stress surveillance function has clear relevance for the control of malignancy and virus infection in humans. Here, we report a molecular link that orchestrates the adaptive and the innate arm of $\gamma\delta$ T cells through the $\gamma\delta$ TCR-dependent sensing of soluble inflammatory molecules. Specifically, $\gamma\delta$ TCR stimulation modulates the sensitivity to the inflammatory cytokine IL-18, whose production is controlled by activation of caspase-1 inflammasomes in stressed non-myeloid human cells (malignant cells and HCMV-infected cells). $\gamma\delta$ T-cell sensitivity to IL-18 is controlled by the expression level of the innate membrane receptor IL-18R β . The combination of $\gamma\delta$ TCR and IL-18R signaling ultimately leads to enhanced IFN γ production and cytotoxicity. Importantly, tissue-derived and soluble IL-18 seems sufficient to enhance $\gamma\delta$ T-cell effector functions as no other co-factor expressed by APCs, such as IL-12, are required.

Altogether, our data suggest that, in addition to the $\gamma\delta$ TCR recognition of specific stress antigens expressed by tumor and infected cells,¹⁸ epithelial V δ 2^{neg} $\gamma\delta$ T cells fine tune their effector functions by directly sensing cues in the form of soluble molecules including innate cytokines. This observation illustrates an intricate array of molecular events involved in the lymphoid stress surveillance mediated by the immune system, which may explain the complex stress signature recognized by some V δ 2^{neg} $\gamma\delta$ T-cell clones.¹⁸ This array of events comprises both innate-like components (innate cytokine receptor-mediated and perhaps others) and adaptive components (TCR-specific).

The low diversity of $\gamma\delta$ TCRs is hypothesized to allow responses to a wide range of stress antigens. The TCR-dependent innate sensing mechanism we report herein would add an extra level of safety control whether to engage in potent cytotoxicity and, therefore, potentially irreversible destruction of epithelial structures, only when disturbed epithelial cells display a complex membrane stress signature. This could include stress antigens, inflammatory signals, and co-stimulating/inhibitory receptors and adhesion molecules (NKG2D, KIRs, CD100, JAML, etc.) that are all necessary to fine tune the $\gamma\delta$ T-cell-mediated immune outcome.^{2,39} In the context of tissue homeostasis, such mechanisms may restrain autoinflammation, as disturbed

epithelial cells releasing all signals including inflammatory cytokines would be rapidly eliminated, thereby avoiding harmful activation of myeloid cells.

We show here that secreted inflammatory soluble molecules can originate directly from stressed epithelia or endothelium, and not only from APCs. Engagement of the $\gamma\delta$ TCR orchestrates the expression level of innate immune sensors deployed to fine tune a stress-level appropriate response. Therefore, V δ 2^{neg} $\gamma\delta$ T cells may act as autonomous sentinels of epithelial structures by answering back promptly to tissue threats without needing other immune cells including APCs. Activation of caspase-1 inflammasomes in target cells becomes pivotal for the secretion of innate soluble molecules. Active caspase-1 holds several functions beyond its seminal cytokine regulation. Therefore, we can also consider a putative role in regulating the expression of $\gamma\delta$ TCR stress ligands (for e.g., through post-translational modifications).

Peripheral V γ 9V δ 2 $\gamma\delta$ T cells sense inflammatory cytokines (IL-1 β) produced by caspase-1 inflammasomes in APCs, and trigger a Th17 response in the presence of IL-23.⁴⁰ However, sensing of innate cytokines has not yet been demonstrated for epithelial V δ 2^{neg} $\gamma\delta$ T cells, nor has the relationship between cytokine sensing and $\gamma\delta$ TCR signaling in the context of epithelial immunosurveillance. We found that IL-18 is secreted by various cancer cell lines (unlike IL-1 β) and by HCMV-infected endothelial HUVEC cells. It has been shown previously that murine CMV infection triggers the AIM2 inflammasome in mouse macrophages followed by caspase-1 activation and the subsequent maturation and secretion of IL-18. In turn, IL-18 mediates IFN γ production by NK cells *in vivo* that confers protection to infected mice.⁴¹ Similarly, we show here that HCMV triggers caspase-1 activation in non-immune human endothelial cells to secrete mature IL-18, which in turn enhances IFN γ production by V δ 2^{neg} $\gamma\delta$ T cells. Such observations provide new perspectives related to the intrinsic response of human cells to HCMV infection, and raise the question of which inflammasome(s) is responsible for IL-18 production by infected cells.

Interleukin-18 has a contrasting role in cancer *in vivo*, being either protective at early stage or pro-tumoral at later stages of disease progression. Interestingly, various epithelial cells express NLRs that assemble into inflammasome complexes to activate caspase-1.^{33,42} In turn, active caspase-1 in colon epithelial cells

Figure 6 (see previous page). Active caspase-1 in cancer cells and HCMV-infected cells regulates the release of soluble molecules that enhance IFN γ production by human V δ 2^{neg} $\gamma\delta$ T cells. **(A)** Various cancer cell lines or non-transformed lung-derived MRC5 were cultured for 48 h, and lysed in denaturing buffer. Proteins were quantified to load the same amount of proteins per lane and then analyzed by SDS-PAGE immunoblotting (Western blotting) using anti-human caspase-1 p10 Ab. The p45 pro-caspase-1, p35, and p10 small catalytic subunit are indicated by dark arrowheads. LPS/ATP-treated THP-1 cells were used as the positive control for the presence of procaspase-1 p45 and p10. **(B)** HUVECs uninfected or infected with HCMV (MOI 10 for 48 h) were lysed in immunoprecipitation buffer (IP), and proteins were quantified to load the same amount of proteins per lane. Input indicates loading of cell lysates. THP-1 cells that were untreated or treated with LPS/ATP were used as positive control for the presence of procaspase-1 p45 and p10 using the anti-human caspase-1 p10 Ab. In parallel, solubilized caspase-1 p10 fragments contained in cleared supernatants after HUVEC cell lysis were precipitated by the addition of biotinyl-VAD-fmk; thereafter, biotinylated complexes were recovered by adding streptavidine-Sepharose beads, and the Sepharose-bound complexes were analyzed by Western blotting (WB) using anti-human caspase-1 p10 Ab. **(C)** U373MG cancer cells or **(D)** HUVECs that were uninfected or infected with HCMV (MOI 10) were cultured for 48 h at 37°C with or without various concentrations of Ac-YVAD-fmk (40 μ mol/L for HUVECs). Conditioned culture supernatants (to the left) or fresh media (to the right) were then cleared by centrifugation and incubated with a V δ 1 $\gamma\delta$ T polyclonal cell line for 24 h at 37°C, in the presence of anti-CD3. IFN γ secretion then was measured by ELISA from cell culture supernatants (mean \pm SD; $n = 3$). **(E)** U373MG cancer cells were cultured for 48 h at 37°C with or without various concentrations of Ac-YVAD-fmk, and then co-cultured with a human V γ 4V δ 5 T-cell clone in the presence or absence of anti-IL-18, control anti-IL-10, or anti-CD3 antibodies. After 24 h at 37°C, IFN γ secretion was measured by ELISA from cell culture supernatants (mean \pm SD; $n = 3$). ★, $P < 0.05$ and ★★, $P < 0.01$.

protects mice against colitis-associated colorectal cancer (CAC) *in vivo* through IL-18 secretion, which participates in intestinal tissue homeostasis.^{27,28} Moreover, these cytokines (IL-1 β /IL-18) enhance the efficacy of chemotherapy-induced anticancer immunity in mice.^{5,43} At late stages of cancer progression in humans, IL-18 is detected in the serum of patients along with others inflammatory cytokines (TNF α , IL-6).³¹ In mice, an immunosuppressive role by ablating NK cells has been reported for IL-18.³² However, clinical administration of IL-18 appears to be relatively safe and beneficial against various solid tumors.³¹ This correlates with our *in vitro* observations that IL-18 improves human $\gamma\delta$ T-cell antitumor function or may wipe out inflammation due to inflammatory cytokines. Surprisingly, cancer cell lines tested here constitutively express large amounts of caspase-1 that is cleaved into active fragments. Consistent with this, these cells also secrete mature IL-18 that triggers IFN γ secretion by $\gamma\delta$ T cells.

Major differences exist between epithelial immune cells in their regulation of IFN γ production by the innate cytokines IL-18 and IL-12/IL-15. NK cells respond to IL-18 and IL-12, both independently and together, to produce IFN γ and develop cytotoxicity to cancer cells.^{44,45} The combination of both cytokines slightly increases IFN γ production and cytotoxicity due to the fact that NK cells constitutively express high amounts of cytokine membrane receptors (IL-18R and IL-12 β 2).⁴⁵ In contrast, $\alpha\beta$ T cells have been reported to require a combination of both cytokines IL-18 and IL-12 to synergistically induce IFN γ production in the presence or absence of TCR engagement. This synergism is mediated by the induction of IL-18R on naive T cells following IL-12 signaling, and by subsequent regulation of IL-12R β 2 by IL-18.^{46,47} Importantly, naive T cells from IL-12^{-/-} mice fail to produce IFN γ when stimulated with anti-CD3 and IL-18,⁴⁷ although we cannot exclude a IL-18 response in the absence of IL-12 in other settings with differently activated $\alpha\beta$ T cells. Therefore, our results suggest that $\gamma\delta$ T cells may display an alternative scenario regarding the regulation of IFN γ production by innate cytokines: like NK cells, $\gamma\delta$ T cells do not need APC products (IL-12, IL-15) in the presence of IL-18, although they do require an additional signal for being sensitized that is provided by a $\gamma\delta$ TCR-dependent upregulation of the IL-18 receptor. Together, $\gamma\delta$ T cells join NK cells for their capacity to sense tissue-derived molecules alone, although their sensitivity would be more finely tuned by $\gamma\delta$ TCR engagement. Because IL-12R β 2 signals through JAK2/TYK2-dependent activation of STAT4 to upregulate gene expression of IL-18R β in $\alpha\beta$ T cells,⁴⁸ it is tempting to speculate that the $\gamma\delta$ TCR has evolved to connect to the same JAK2/TYK2 signaling pathway, thereby sensitizing $\gamma\delta$ T cells to tissue-derived soluble triggers. This putative difference between $\gamma\delta$ and $\alpha\beta$ T cells regarding the IL-18 response mechanism is intriguing and deserves further investigation.

Of note, the $\gamma\delta$ TCR sensitizes not only to soluble IL-18 but also to other unknown soluble molecules that originate from disturbed tissue-derived cells. Additional soluble triggers of $\gamma\delta$ T cells might be metabolites that originate from dysregulated metabolic pathways, as disturbed target cells undergo major and

similar metabolic changes during carcinogenesis and CMV infection that are consistent with inflammasome activation.²⁴ Such small molecules may constitute new DAMPS, and their identification as well as their innate sensor machineries (presumably pattern recognition receptors) would constitute attractive tools to promote first-line $\gamma\delta$ T-cell-mediated antitumor or anti-infectious immune responses. Such knowledge may, therefore, offer new clinical strategies of anticancer-or anti-infectious immunotherapy.

Materials and Methods

Human $\gamma\delta$ T cell clone and line generation and functional assays

Peripheral blood mononuclear cells were generated from blood samples collected for medical care with the approval of the local medical ethics committee. Primary $\gamma\delta$ T-cell lines (V δ 1, V δ 1+3, V δ 5) and V δ 2^{neg} $\gamma\delta$ T-cell clones (V γ 8V δ 3, V γ 2V δ 3) were generated and cultured following the same methods as described previously.^{14,35} For co-culture functional assays and IFN γ secretion, primary T-cell lines and clones (5×10^4 cells per well) were incubated with monolayers of tumor cell lines or HUVECs that were uninfected or infected with the human CMV clinical strain TB40-E as described.³⁵ After 24 h at 37°C, supernatants were isolated by centrifugation, and cytokine secretion (IFN γ and TNF α) was measured by ELISA. Alternatively, primary $\gamma\delta$ T-cell lines and clones (5×10^4 cells per well) were left untreated or treated with recombinant cytokines IL-18/IL-1 β /IL-15 (50 ng/mL) or IL-12 (20 ng/mL). From co-culture experiments, the degranulation potential of $\gamma\delta$ T cells was analyzed by measuring the expression of CD107a. Cells were co-cultured for 6 h in the presence of PE-conjugated mAb to CD107a (H4A3; BD Biosciences) and brefeldin A (10 μ g/mL) and then were collected and stained with phycoerythrin-indodicarbocyanine-conjugated mAb to $\gamma\delta$ TCR (IMMU510; Beckman Coulter); then, the expression of CD107a on $\gamma\delta$ TCR⁺ cells was analyzed by flow cytometry. In some experiments, anti-IL-18 or anti-IL-10 Abs were added to culture media at various concentrations. Alternatively, conditioned culture supernatants from tumor cell lines and HUVECs uninfected or infected with HCMV obtained after 48 h were isolated by centrifugation and kept frozen at -80°C. Then, primary $\gamma\delta$ T-cell lines and clones (5×10^4 cells per well) were incubated with immobilized anti-CD3 (OKT3 clone) in a 96-well plate. The plate was then centrifuged (2,500 rpm, 1 min) and media was replaced by conditioned culture supernatants for 24 h at 37°C. $\gamma\delta$ T cells in the 96-well plate were then pelleted by centrifugation (2,500 rpm, 1 min) and the supernatant was used to measure cytokine secretion by ELISA.

Immunoblotting and recovery of active caspase-1

Immunoblotting was carried out as described previously.⁴⁹ For recovery of active caspase-1, pellets of HUVECs uninfected or infected with HCMV were suspended in IP buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 50 mmol/L NaF,

0.3% NP-40, 0.1 mmol/L Na₃VO₄, 20 µg/mL leupeptin, 20 µg/mL aprotinin, 1 mmol/L phenylmethyl sulfonyl fluoride [PMSF]). After centrifugation at 16,500 rpm/20 min at 4°C, cleared lysates were incubated with biotinyl-VAD-fmk (30 µmol/L) for 30 min at 37°C to precipitate the active cleaved fragment p10 of caspase-1. The biotinyl-VAD-fmk/caspase-1 p10 complex was recovered by using streptavidin-Sepharose beads (SIGMA), adding 30 µL of the 1:1 streptavidin-Sepharose suspension per 250 mL of IP buffer for 3 h at 4°C.⁵⁰ Beads were pelleted by centrifugation (3,000 rpm/10 min at 4°C) and washed 3 times in cold IP buffer before adding an SDS loading buffer on top of the beads. Beads were then heated for 5 min, pelleted, and the supernatant was analyzed by SDS-PAGE/immunoblotting using an Ab that detects the p10 small subunit of processed human caspase-1 (sc515; Santa Cruz Biotechnology). For positive control of caspase-1 cleavage, THP-1 cell lines in suspension were treated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) for 12 h at 37°C. Adherent cells were cultured with fresh media (RPMI) and then treated with 1 µg/mL LPS for 6 h, followed by 5 mmol/L ATP (for 30 min).

Reagents and antibodies

Anti-human CD3 Ab was purchased from Biolegends (clone OKT3, 317302); anti-human Caspase-1 p10 Ab (C-20) from Santa Cruz Biotechnology (sc515); anti-human IL-18 blocking Ab from MBL (D044-3, clone 125-2H); anti-human IL-10 blocking from Mabtech (9D7); anti-human IL-18Rβ-PE conjugated from R&D Systems (FAB118P). Fluorescent dye-conjugated secondary Abs were purchased from LI-COR Biosciences. ELISA kits to measure IFNγ and TNFα secretion were purchased from Mabtech (3420-1H-20 and 3510-1H-20;

respectively). Mature human recombinant IL-1β and IL-15 were obtained from Peprotech (200-01B and 200-15; respectively); human recombinant IL-12 and mature human IL-18 from MBL (JM-4161-10 and B003-5; respectively). Caspase-1 inhibitor Ac-YVAD-fmk was purchased from Calbiochem (caspase-1 inhibitor VI; 218746), and biotinyl-VAD-fmk from Enzo Life Sciences (ALX-260-098). LPS from *Escherichia coli* serotype 055:B5 (L6529) and ATP were purchased from SIGMA.

Statistical analysis

Most data were presented as the mean ± SD from at least 3 independent experiments. Statistical comparisons between different treatments were done by unpaired *t*-test, where *P* < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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