Involvement of CD252 (CD134L) and IL-2 in the Expression of Cytotoxic Proteins in Bacterial- or Viral-Activated Human T Cells

Michael Walch, Silvana K. Rampini, Isabelle Stoeckli, Sonja Latinovic-Golic, Claudia Dumrese, Hanna Sundstrom, Alexander Vogetseder, Joseph Marino, Daniel L. Glauser, Maries van den Broek, Peter Sander, Peter Groscurth and Urs Ziegler

*J Immunol* 2009; 182:7569-7579; doi: 10.4049/jimmunol.0800296

http://www.jimmunol.org/content/182/12/7569

Why *The JI*?
- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Speedy Publication! 4 weeks from acceptance to publication

*average

References This article cites 85 articles, 40 of which you can access for free at:
http://www.jimmunol.org/content/182/12/7569.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Involvement of CD252 (CD134L) and IL-2 in the Expression of Cytotoxic Proteins in Bacterial- or Viral-Activated Human T Cells

Michael Walch,‡ Silvana K. Rampini,† Isabelle Stoeckli,* Sonja Latinovic-Golic,* Claudia Dumreise,* Hanna Sundstrom,* Alexander Vogtseder,* Joseph Marino,* Daniel L. Glauser,† Maries van den Broek,§ Peter Sander,†† Peter Groscurth,* and Urs Ziegler3*

Regulation of cytotoxic effector molecule expression in human CTLs after viral or bacterial activation is poorly understood. By using human autologous dendritic cells (DCs) to prime T lymphocytes, we found perforin only highly up-regulated in virus-(HSV-1, vaccinia virus) but not in intracellular bacteria- (Listeria innocua, Listeria monocytogenes, Mycobacterium tuberculosis, Chlamydia pneumoniae) activated CTLs. In contrast, larger quantities of IFN-γ and TNF-α were produced in Listeria-stimulated cultures. Granzyme B and granulysin were similarly up-regulated by all tested viruses and intracellular bacteria. DCs infected with HSV-1 showed enhanced surface expression of the costimulatory molecule CD252 (CD134L) compared with Listeria-infected DC and induced enhanced secretion of IL-2. Adding blocking CD134 or neutralizing IL-2 Abs during T cell activation reduced the HSV-dependent up-regulation of perforin. These data indicate a distinct CTL effector function in response to intracellular pathogens triggered via differing endogenous IL-2 production upon costimulation through CD252. The Journal of Immunology, 2009, 182: 7569–7579.

Cytotoxic T lymphocytes are important effector cells in the immune response to viruses and intracellular bacteria such as Listeria, Mycobacteria, and Chlamydia (1–3). For the induction of a robust T cell effector response toward a certain pathogen, three basic prerequisites have to be met (4). First, Ags have to be taken up, processed, and be presented by professional APCs on MHC molecules for the recognition by a T cell via its Ag-specific TCR. Second, the presence of a costimulatory signal for full T cell activation is necessary. These costimulatory molecules are grouped into two superfamilies: CD28/B7 (5) and TNFR related (6). CD28 is constitutively expressed on naive T cells, and binding to its ligands B7.1 or B7.2 provides a strong activating signal (7). Several new members, both activating and inhibiting, of the CD28/B7 family have been identified (8). The TNFR family is primarily found on activated cells and includes CD30, CD40, 4-IBBL, CD70, and CD252 (CD134L) (9). The interplay of constitutively expressed and inducible costimulatory molecules is thought to be critical in modulating the quantity and quality of a T cell effector response. The third signal is provided by cytokines in which IL-2, IL-12, and the type 1 IFNs are of importance (4). IL-12 is essential to induce IFN-γ and to promote proliferation of activated CD8⁺ cells (10). IL-2 is crucial for maintenance and differentiation of T and NK cells (11–13).

Pathogen elimination is driven by the release of cytotoxic granule contents into the immunological synapse (14) and the secretion of cytokines (15), especially IFN-γ and TNF-α. Effector proteins contained in cytotoxic granula are perforin, several granzymes, and granulysin in a proteoglycan matrix (14). Perforin delivers granules into the target cell cytosol (16). Granzyme B and granzyme A mediate target cell apoptosis (17, 18). Additionally, there is evidence for antiviral activity of these granzymes that is not related to cytosis (19, 20). The third lytic component in cytotoxic CTL granules is granulysin, which exhibits a broad spectrum of antimicrobial activity against bacteria, fungi, and parasites (21). We could recently show that recombiant granulysin exerts lytic activity in vitro against intracellular Listeria without the help of perforin (22). The molecular properties of granulysin enable it to distinguish between eukaryotic and prokaryotic membranes and to target lytic activity against intracellular pathogens without damaging the host cell (23, 24).

The effector pathway of pathogen elimination differs between viruses. In mice, the elimination of HSV-1 or lymphocytic choriomeningitis virus (LCMV) is dependent on perforin (25, 26) while IFN-γ rather than perforin is essential for the clearance of poxviruses such as vaccinia virus (27, 28). Also, the clearance of

*Division of Cell Biology, Institute of Anatomy, †Institute of Medical Microbiology, ‡Institute of Virology, and 3Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland; ¶National Center for Mycobacteria, Zurich, Switzerland; and 5Division of Cell Biology, Institute of Anatomy, †Institute of Medical Microbiology, ‡Institute of Virology, and 3Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland

Received for publication February 25, 2008. Accepted for publication April 9, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Kurt and Senta Herrmann-Foundation and in part by the Swiss National Science Foundation (3200-068488; 3100AO_120326) to P.S. S.R is supported by grants from Swiss Lung Foundation and Wolfermann-Zangger-Foundation. M.W. is supported by a fellowship from the Stiefel-Zangger-Foundation.

2 Current address: Immune Disease Institute, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115.

3 Address correspondence and reprint requests to Dr. Urs Ziegler, Center for Microscopy and Image Analysis, Winterthurerstrasse 190, 8057 Zurich, Switzerland. E-mail address: ziegler@zmb.uzh.ch

4 Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; aLDC, autologous lymphocyte-dendritic cell; DC, dendritic cell; MOI, multiplicity of infection.
intracellular bacteria is orchestrated by various mechanisms including cytokine release (29), apoptosis of the host cell (2), and direct antimicrobial activity of CTLs (21, 30). In perforin knockout mice the clearance of *Mycobacteria*, *Listeria*, or *Chlamydia* is only slightly impaired (31–34), whereas elimination of these pathogens is drastically hampered in mice deficient in TNF or IFN-γ signaling (35–37). A case report suggests IFN-γ-mediated immunity is of critical importance to control *Mycobacteria* and *Listeria* also in humans (38).

The varying effector requirements to efficiently eradicate different pathogens led to the rationale for the investigation of the pathogen-dependent modulation of the T cell effector function using a well-established in vitro system for human cells. Despite the impact of a specifically adapted effector function, especially in humans, little is known about the concerted regulation of diverse effector proteins in response to different pathogens. The study presented herein is the first comprehensive in vitro investigation of the effector function in human CTLs in response to different intracellular pathogens presented by dendritic cells (DCs) in the context of costimulation and cytokine environment.

**Materials and Methods**

**Isolation and culture of DCs**

Human DCs were generated in vitro from blood-derived adherent PBMCs as previously described (40). Briefly, human PBMCs obtained from venous blood of healthy donors were isolated by Ficoll-Paque (Pharmacia Biotech) density centrifugation. The PBMCs were cultured in RPMI 1640 supplemented with penicillin/streptomycin (all Invitrogen) and 10% heat-inactivated autologous plasma for 2 h. The adherent cells were cultured for 6 days in DC culture medium composed of RPMI 1640 supplemented with penicillin/streptomycin, 10% heat-inactivated autologous plasma, and with rGM-CSF (50 ng/ml; Novartis) and rIL-4 (100 U/ml; R&D Systems).

**Propagation of pathogens and infection of DCs**

*L. monocytogenes*, wild-type strain EGD, and *Listeria innocua*, wild-type strain LL-263, were propagated in tryptic soy broth medium. *Mycobacterium tuberculosis* (strain 1424), a derivative of strain H37Rv carrying the nonrestrictive *rpsL* mutation, were grown in Middlebrook 7H9 (Difco), supplemented with 0.5% Tween, 0.2% glycerol, and OADC (oleic acid, 10% bovine serum fraction V, glucose, and catalase). We used *M. tuberculosis*, strain 1424, carrying a nonrestrictive *rpsL* mutation, since it is parental to several genetically engineered, isogenic mutants deficient in various virulence factors (41, 42). Bacteria were harvested, washed in PBS, and resuspended in RPMI 1640. DCs were infected for 1 h with a multiplicity of infection (MOI) of 10. Subsequently, cultures were washed with PBS and incubated overnight in DC culture medium containing 25 μg/ml gentamicin (Sigma-Aldrich). In some experiments, *Listeria* infection efficiency was tested by lysing DC in ice-cold water and spreading serial dilutions of the lysate on tryptic soy broth agar plates. By doing so, viable...
Listeria could be isolated from DCs up to 48 h postinfection (data not shown).

Chlamydophila pneumoniae, strain VR1310, was cultured and purified as described previously (43). Infection with chlamydia was performed in RPMI 1640 using 10 inclusion-forming units per cell, which were added to DCs and centrifuged onto the cells for 1 h at 1000 g. Subsequently, the supernatant was replaced by antibiotic-free DC culture medium and incubated overnight.

HSV-1, strain F, was grown and titrated in Vero 2-2 cells as previously described (44).

Vaccinia virus, strain WR, was propagated on BSC-40 cells (45). Viral infections were performed in RPMI 1640 for 1 h with a MOI of 10. Subsequently, infectious medium was replaced by DC culture medium and incubated overnight. To test the productivity of the HSV-1 infection, DCs, were lysed by three consecutive freeze-and-thaw cycles 0, 24 and 48 h postinfection. Serial dilutions of the lysates were added to Vero 2-2 cells and PFU were determined after 5 days. Forty-eight hours postinfection, up to 3.8 × 10^8 PFU/ml were isolated from human DCs (data not shown).

aLDC culture

DCs matured for 6 days cells were transferred to 6-well plates (10^6 cells well) and infected with the pathogens as above described. Noninfected DCs served as negative control (mock). Freshly isolated autologous, nonadherent PBMCs in RPMI 1640 with 10% autologous plasma were added to the infected DCs at the PBMC/DC ratio of 10:1. For some cytometry experiments PBMCs were MACS depleted of either CD4^+ or CD56^+ cells according to the manufacturer’s recommendations (Miltenyi Biotec) using the following Abs: anti-human CD4 MicroBeads, goat anti-mouse IgG MicroBeads (both Miltenyi Biotec), anti-human CD19 panB Dynabeads (Dynal Biotech/Invitrogen), purified mouse anti-human CD356 (clone 123C3; Dako). The positive MACS selection for some RT-PCR measurements was achieved using anti-human CD4 MicroBeads and anti-human CD8 MicroBeads (both Miltenyi Biotec). Following MACS depletion, cells were routinely assessed by flow cytometry indicating a depletion efficiency of 95–98% (data not shown).

aLDCs were cultured for 6 days before restimulation with freshly infected autologous DCs. To test specificity of the T cell response, cell proliferation was measured in some experiments after restimulation using Click-iT EdU cell proliferation assays (Invitrogen) according to the manufacturer’s instructions or using tritiated thymidine incorporation assay, alternatively, as previously described (46). T cells showed a prominent proliferative response when restimulated with DCs presenting the relevant Ag. The absence of Ag, the presentation of an irrelevant control Ag by DCs, or the presence of an anti-MHC class II Ab (HLA-DR, DP, and DQ; BD Biosciences) reduced the proliferative response significantly (data not shown). At indicated days, cellular samples and supernatant were assayed for phenotype, intracellular cytotoxic proteins, gene expression as determined by real-time PCR, and ELISA detection of cytokines. Some viral aLDCs were grown in presence of a mouse anti-CD134 Ab (1 g/ml, clone ACT35; BD Biosciences), a mouse anti-IL-2 Ab (1 μg/ml, clone F16; Monosan), or a mouse anti-y1 Ab (BD Biosciences) as control. Bacterial-stimulated aLDCs were in some experiments supplemented with hrIL-2 (5 ng/ml; R&D Systems).

Confocal laser scanning microscopy

Cells from virus- and bacteria-stimulated aLDCs were collected at indicated days and directly fixed with 1.5% paraformaldehyde and 1%
Flow cytometry

For intracellular cytotoxic protein staining, cells from HSV-1 and L. monocytogenes activated aLDCs were collected and stained on ice for 15 min with FITC-conjugated mouse anti-human CD4 Ab and allophycocyanin-conjugated mouse anti-human CD8 Ab (both Immunotech/Beckman Coulter) before fixation with solution A (Caltag Laboratories). For intracellular cytokine staining, cell samples were removed and incubated for an additional 6 h at 37°C with brefeldin A (20 μg/ml; Sigma-Aldrich) before CD molecule staining and fixation as described above. After fixation, cells were washed with PBS and incubated for 1 h at room temperature with solution B (Caltag Laboratories) containing one of the following Abs: mouse anti-human perforin (BD Biosciences), mouse anti-human granzyme A (BD Biosciences), PE-conjugated mouse anti-human granzyme B (BD Biosciences), and MHC class II (HLA-DR, DP, and DQ; all BD Biosciences) mAbs directed against human CD40, CD80, CD83, CD86, CD252, and CD134 (BD Biosciences). For detection, the following Abs were used: FITC-conjugated goat anti-mouse Ab (Kirkegaard & Perry Laboratories), PE-conjugated goat anti-mouse Ab (BD Biosciences), and FITC-conjugated polyclonal goat anti-mouse Ab (Jackson ImmunoResearch Laboratories). All Abs were incubated at 4°C for 15 min. Fluorescent-labeled specimens were examined using a confocal laser scanning microscope (CLSM SP1 or SP2; Leica). Percentage of cytotoxic protein-positive cells were determined by counting at least five optical fields (containing >200 cells) in every experiment.

Quantitative RT-PCR

Total RNA was isolated from cells using the Absolutely RNA MicroPrep kit from Stratagene according to the manufacturer’s instructions. First strand cDNA synthesis was performed using SuperScript II reverse transcriptase and oligo(dT) primers (Invitrogen). Real-time PCR was performed using the Power SYBR Green Master Mix (Applied Biosystems) in an iCycler (Bio-Rad Laboratories). Reactions were performed in triplicate by using standard reaction conditions, and the relative amount of mRNA was normalized to GAPDH. For detection the following forward/reverse primer pairs were used: AGAAAGAACACACAGAGCGCCCT/ACAGCAGGTCATATTGAGGAGTT for perforin; TCTTCCTGGACCACCCTGCACTCCGGAGTT for granzyme A; mouse anti-human perforin, mouse anti-human granzyme A, mouse anti-human IFN-γ, mouse anti-human IL-2 (all PE-conjugated; BD Biosciences), PE-conjugated mouse anti-human granzyme B (Caltag Laboratories), and guinea pig anti-human granzyme antisera (22). Granulysin was detected using a PE-conjugated goat anti-guinea pig Ab (Jackson ImmunoResearch Laboratories). All Abs were diluted in 0.1% BSA in PBS. DNA was labeled with 1 μg/ml DAPI (4,6-diamidino-2-phenylindol-dihydrochloride; Roche) in PBS for 15 min at room temperature. Fluorescent-labeled specimens were examined using a confocal laser scanning microscope (CLSM SP1 or SP2; Leica). Percentage of cytotoxic protein-positive cells were determined by counting at least five optical fields (containing >200 cells) in every experiment.

Results

Differential effector function in PBMCs after viral compared with bacterial activation

To study the influence of different Ags presented by DCs on the expression of effector molecules in PBMCs, we generated aLDC cultures that were activated with a variety of intracellular pathogens. DCs were infected with intracellular bacteria (L. monocytogenes, L. innocua, M. tuberculosis, C. pneumoniae) or with viruses (HSV-1, vaccinia virus) and used as stimulator cells in aLDCs. At
day 6 CTLs were restimulated using freshly infected DCs. Intracellular cytotoxic molecule expression and localization were evaluated using confocal laser scanning microscopy. Representative images of HSV-1-stimulated effector cells at day 11 of stimulation are shown in Fig. 1A; corresponding images of L. monocytogenes-activated cells are shown in Fig. 1B. Viral-activated PBMCs showed high expression of perforin, granzyme A and B, as well as granulysin. In L. monocytogenes-stimulated cells, granulysin and granzyne B expression was similar, whereas the expression of perforin and granzyme A was lower than in viral-activated cells.

Quantification (percentage positive cells) of the cytotoxic expression in PBMCs activated by different intracellular pathogens is shown in Fig. 1C. Granulysin and granzyme B expression was induced similarly by all bacteria and viruses. This was in contrast to perforin and to granzyme A where higher expression levels upon viral activation were found.

Additionally, for L. monocytogenes and HSV-1 the amount of IFN-γ in the supernatant of aLDCs was assessed by ELISA. The time course of IFN-γ secretion is illustrated in Fig. 1D. Data of one donor are presented, as the individual IFN-γ responses differed by a factor of >10 (range, 10–130 ng/ml) and therefore the experimental data of all donors were not pooled. In all donors, the supernatants of either L. monocytogenes- or HSV-1-activated cultures contained significant amounts of IFN-γ compared with mock-infected controls. However, in three of four donors bacterial activation led to a significantly higher IFN-γ secretion compared with HSV-1-activated cells, as shown in Fig. 1D. One donor showed a converse response. Overall, these data indicate a pathogen-dependent modulation of the effector function.

Cell type-specific characterization of CTLs after HSV-1 or L. monocytogenes activation

On the basis of the promising confocal data presented in the above section, we decided to perform three-color cytometry, allowing a more accurate quantification as well as phenotype characterization of the effector cells after L. monocytogenes or HSV-1 activation. To evaluate the impact of CD4+ T cell help in the modulation of the effector function, CD4+ cells were depleted by positive MACS selection in some control cultures (Fig. 2B). Furthermore, to minimize Ag presentation and possible costimulation by contaminating B cells as well as to avoid bystander activation of NK and NKT cells, some cultures were depleted of CD19+ or CD56+ cells, respectively, for comparison to bulk cultures where all nonadherent PBMCs were present (data not shown).

At indicated time points cells from aLDCs were stained for CD4 and CD8 as well as for intracellular effector proteins. Percentage of effector protein positive cells within the gated CD4 and CD8 population was determined. Representative cytometry measurements of perforin, granzyme A, granulysin, and granzyme B expression at day 11 and of intracellular IFN-γ at day 9 of a bulk culture are presented in Fig. 2A. The presented histograms demonstrate perforin up-regulated higher in HSV-1 activated CD8+ cells (solid line) compared with L. monocytogenes (dotted line) or mock-stimulated cells (filled graph). Granzyme A appears also to be expressed higher in virus-activated cells. On the other hand, granulysin and IFN-γ are found to greater extents in bacterial-stimulated CD8+ cells.

All assessed effector molecules were found at higher levels in CD8+ cells following either antigenic stimuli. The quantification (percentage of positive cells in M1) of the measurements (Fig. 2B) indicated furthermore that CD4 depletion significantly reduced the HSV-dependent up-regulation of perforin at day 11. Perforin expression in CD8+ cells from CD4-depleted cultures was also reduced at day 6, as was the granzyme A expression at day 11; in any case, these differences just missed the confidence level of 95%. Depletion of CD19+ or CD56+ cells did not significantly impact the outcome of the effector function (data not shown).

These data confirm a pathogen-dependent regulation of perforin and to some extent also for granzyme A. Furthermore, there is evidence that CD4+ T cell help is involved in the modulation of a distinct effector function. 

FIGURE 4. Enhanced surface expression of CD252 is found in HSV-1-infected DCs. A, DCs were infected with HSV-1 or L. monocytogenes and after 16 h stained for costimulatory molecules as well as MHC classes I/III and analyzed by cytometry. B, Activated cells at day 6 and day 11 were stained for CD4, CD8, and CD134 and analyzed by three-color cytometry. Representative dot plots and histograms at day 11 of stimulation to demonstrate gating strategy are presented. Filled histograms represent mock-stimulated cells; solid lines correspond to HSV-1 activation and dotted lines to L. monocytogenes activation. The quantified data are illustrated as mean fluorescent intensity ratio (infected/mock). Means (±SD) of the mean fluorescent intensity ratios of four donors are shown.
Transcriptional regulation of effector molecules in activated T cells

To evaluate mRNA expression of cytotoxic molecules in activated T cells, quantitative RT-PCR at day 11 was performed. Most noticeable were the mRNA levels of perforin, as unlike with protein levels, there was no significant difference in cells from HSV-1-activated cultures compared with *L. monocytogenes* activation (Fig. 3A). CD4+ depletion did also not significantly impact the perforin mRNA expression. Overall, the induction of perforin mRNA was rather weak following both antigenic stimuli (only up to 3.4-fold compared with mock induction) in respect to other cytotoxic proteins (up to ~40-fold for granzyme A and granulysin and even up to 60-fold for granzyme B). The induction of granzyme A, granulysin, granzyme B, as well as of IFN-γ (Fig. 3A) transcription was comparable to the protein levels found at day 11 (Fig. 2B), indicating mainly transcriptional regulation of these genes following pathogen activation. CD4+ depletion reduced the amount granzyme A mRNA as already observed for the protein expression. Depletion of CD19+ or CD56+ cells did not influence the mRNA expression of any of the effector molecules (data not shown).

To clarify the difference in the transcriptional and translational regulation of perforin, we performed a time course analysis of perforin and granzyme A mRNAs in positively MACS selected CD4+ and CD8+ cells. As shown in Fig. 3B, a significant amount of perforin mRNA was already detected in CD8+ cells at day 0 (in relation to the housekeeping gene GAPDH). On the other hand, no perforin protein was detected in naive T cells (data not shown). Perforin mRNA increased rather slightly upon activation in both CD4+ and CD8+ cells, which is in sharp contrast to the perforin protein expression (Fig. 2B). Granzyme A mRNA (Fig. 3B) as well as protein (data not shown) were very low in naive T cells. The antigenic stimulus triggered a steep increase in the mRNA levels in CD8+ cells, consistent with the increase in granzyme A protein. These data provide evidence for a posttranscriptional regulation step in the pathogen-dependent expression of perforin.

**CD252 is enhanced in HSV-1 compared with *L. monocytogenes*-infected DCs**

To unravel the mechanistic background of the different expression pattern after viral and bacterial stimulation, the expression level of costimulatory and presenting molecules in DCs following viral or bacterial infection was examined. DCs were infected with *L. monocytogenes* or HSV-1 and were assessed for surface expression of a variety of costimulatory molecules as well as MHC classes I and II. As indicated in Fig. 4A, there is a similar surface expression of most tested costimulatory molecules (CD40, CD80, CD83, CD86) with the exception of CD252 (CD134L), which was significantly more highly expressed on HSV-1-infected DCs compared with *L. monocytogenes*-infected DCs. Additionally, there is a tendency for higher expression of MHC class I following viral infection and MHC class II following infection with *Listeria*. This difference in Ag presentation might also contribute to some extent to the divergent effector function in viral- or bacterial-activated T cells.

**Viral stimulation enhances IL-2 secretion**

As the cytokine environment in aLDCs during viral or bacterial activation could also influence the modulation of the effector function of CTLs, the supernatants of the cultures were screened for various cytokines. Fig. 6, A and B, shows the time course of cytokine secretion of a representative donor in *Listeria* and HSV-1 activated aLDCs. HSV-1 activation of aLDCs significantly enhanced IL-2 secretion compared with *L. monocytogenes* stimulation (Fig. 6A). The increased IL-2 production was effectively blocked with the anti-CD134 Ab. The Th2 cytokine IL-4 was

**Blocking CD134-CD252 costimulation reduces the HSV-1-dependent up-regulation of perforin**

As CD252 displays higher expression in HSV-1 compared with *Listeria*-infected DCs, the costimulatory interaction of CD252-CD134 in aLDCs was assessed using an anti-CD134 Ab that was previously demonstrated to reduce the perforin-dependent cytolytic activity of human PBMCs (47). The introduction of the blocking Ab during priming and restimulation in aLDCs significantly reduced the HSV-1-dependent up-regulation of perforin as shown in confocal microscopy (Fig. 5), thus indicating involvement of this costimulatory pathway in the pathogen-driven induction of perforin expression. There was a tendency toward a CD134 blocking effect regarding the granzyme A; however, these changes in expression were not statistically significant. The expression of granulysin, granzyme B, and the secretion of IFN-γ (not shown) were also not significantly influenced by the Ab.
equally produced in cultures activated by HSV-1 and *L. monocytogenes*-aLDCs and was also efficiently reduced by the anti-CD134 Ab.

*L. monocytogenes* as Ag in aLDCs resulted in a prominent increase in the secretion of IL-1α, IL-12, TNF-α (Fig. 6B), and IL-4 but not IL-2 (Fig. 6A). Mock activation did not result in significant cytokine secretion over background level in culture medium (Fig. 6B).

To investigate which T cells contribute predominantly to the IL-2 secretion in aLDCs, three-color cytometry was performed. As shown in Fig. 6C, CD4⁺ cells appear to be the main T cell source of the IL-2 secretion.

**Blocking endogenous or supplementing exogenous IL-2 compensates for the pathogen-dependent differences in the effector function**

To test the influence of IL-2 during the initial priming, HSV-1-activated aLDCs were cultured in the presence of a neutralizing anti-CD134 Ab, whereas mock- or bacterial-stimulated cultures were supplemented with hrIL-2 for 6 days. We focused on the initial

---

**FIGURE 6.** Distinct cytokine environment is found in HSV-1- and *L. monocytogenes*-activated aLDCs. The time course as well as anti-CD134 Ab blocking effect on IL-2 and IL-4 secretion in viral and bacterial activated aLDCs measured using ELISA are depicted in A. The time course of proinflammatory cytokine secretion in HSV-1- or *L. monocytogenes*-activated aLDCs is shown in B. One representative donor with mean (±SD) of measurements in triplicate is presented. C. Activated PBMCs at day 3 and day 9 were stained for CD4, CD8, and intracellular IL-2 and analyzed by three-color cytometry. Mean (±SD) of three donors are shown.

**FIGURE 7.** Blocking endogenous or supplementing exogenous IL-2 compensates for the pathogen-dependent differences in the effector function. Activation of aLDCs with HSV-1 was performed in presence of a blocking anti-CD134 Ab or control Ab, and mock or *L. monocytogenes* stimulated cultures were supplemented with hrIL-2 or left untreated. Effector cells were stained for intracellular cytotoxic protein expression and quantified by counting positive cells. Mean (±SD) of four donors per group are presented; *p* values were calculated using *t* test for unpaired samples.
priming, as IL-2 levels were especially high during the first days of viral activation. As seen in Fig. 7, rhIL-2 resulted in significant up-regulation of perforin ($p = 0.002$) and granzyme A ($p = 0.052$) in Listeria-activated effector cells to a similar expression level as observed in mock-stimulated, rhIL-2-treated cells. We did not detect an additional impact on the production of granulysin or granzyme B in Listeria-activated PBMCs, indicating that pathogen activation drives the granulysin and granzyme B expression to a maximum level and therefore no additional IL-2 effect can be observed.

The inverse effect was observed in HSV-1-activated cultures, where the neutralization of IL-2 reduced significantly the up-regulation of perforin and granzyme A and had no effect on the granulysin or granzyme B production. These findings clearly indicate a relevant role of the endogenous IL-2 production in the modulation of the pathogen-dependent effector function.

Discussion

The efficient control of different intracellular pathogens requires distinct effector pathways comprising the production of cytotoxic molecules released into the immunological synapse and the secretion of several cytokines. Mice require intact signaling of TNF-α and IFN-γ to control and eliminate intracellular bacteria such as Listeria, Mycobacteria, or Chlamydia, whereas the perforin/granzyme B pathway was less important (31, 35–37, 48). Also in humans, impaired IFN-γ or IL-12 signaling was associated with fatal mycobacterial infections (49, 50) as well as with high susceptibility to listerial and mycobacterial infection (38). The role of perforin in immunity toward intracellular bacteria in humans is unclear. In vitro perforin was necessary to efficiently kill intracellular Mycobacteria together with recombinant granulysin (21). On the other hand, it was shown that CD4$^+$ and CD8$^+$ T cells kill intracellular Mycobacteria independent of perforin (51). In vivo, the role of perforin for the elimination of Mycobacteria is even more nebulous. Cells in dermal granuloma of leprosy lesions were CD4$^+$ and CD3$^+$ and contained granulysin but were negative for perforin (52). Furthermore, FACS sorted CCR7, Mycobacteria-(Ag85A) specific CD8$^+$ cells of tuberculosis patients showed very low perforin expression at the time of diagnosis. However, after 4 wk of treatment, the percentage of Mycobacteria-specific CD8$^+$ cells expressing perforin increased to $\sim 40\%$ (53). In humans, several perforin mutations have been associated with familial hemophagocytic lymphohistiocytosis (FHL) (54, 55). However, there is no evidence for an overall higher susceptibility to intracellular bacteria and some severe cases of mycobacterial infection reported in patients with FHL could be accounted for the impairment of granule exocytosis as well as the deregulated activation and proliferation of macrophages rather than for the impaired function of the A91V mutated perforin (55, 56).

In CTL activated by intracellular bacteria we found an effector function involving high expression of granulysin and granzyme B as well as intermediate expression levels of granzyme A and very low expression of perforin. Three-color cytometry revealed that the effector molecule was predominantly expressed by CD8$^+$ cells. The modulation of the effector function after bacterial activation was independent of CD4$^+$ T cell help. Contaminating B cells and NK/NKT cells also did not influence the characteristic of the effector function (data not shown). In the supernatant of Listeria activated aLDCs, high levels of both IFN-γ and TNF-α were detected. This effector profile is in agreement with the reported requirement to effectively eliminate intracellular bacteria in mice and humans with a major contribution of granulysin, IFN-γ, and TNF-α and an assumed minor role of perforin. There are some studies reporting perforin protein up-regulation following in vitro activation of CTLs with Mycobacteria (51, 57, 58). These seemingly conflicting data are clarified by the protocol chosen by these groups, in which exogenous IL-2 was used after priming to maintain the Mycobacteria-specific CTLs. It was an interesting finding that both granzymes (granzyme B high, granzyme A intermediate) were induced by llisterial Ags, which could not be satisfactorily explained by the existing literature and therefore is a topic of ongoing investigations.

The effective clearance of viruses also requires a specific effector function in CTLs. Perforin-deficient mice are strikingly impaired in the control of HSV-1 infection (25), LCMV (59), and ecmetromelia (mousepox) virus (60). Immunity against mousepox is additionally impaired in mice deficient in granzyme A (61). Perforin-deficient mice, however, are not hampered in the clearance of other viruses, including vaccinia virus, vesicular stomatitis virus, murine cytomegalovirus, Semliki Forest virus (59), cowpox virus (60), rotavirus (62), and murine herpesvirus-68 (63).

Consistent with these results from mouse knockout studies, we found high up-regulation of perforin, granzyme A, and granzyme B expression in human CTLs after HSV-1 activation. Importantly, the modulation of the distinct effector function required CD4$^+$ T cell help, especially for the HSV-1-dependent up-regulation of perforin but also to some extent for the induction of granzyme A. However, vaccinia virus-activated human CTLs also displayed a significantly elevated perforin expression compared with bacterial stimulated CTLs, whereas in mice perforin seemed not to play a major role in vaccinia virus clearance. Our finding of low IFN-γ levels in HSV-1-activated aLDCs is also not fully consistent with the existing literature, as IFN-γ was shown to be important to prevent reactivation of HSV-1 from latency in sensory neurons (64). In any case, the IFN-γ levels (3.8–45.4 ng/ml) in the supernatant of HSV-1-activated cultures were significantly higher than in mock cultures (0.1–1.3 ng/ml), and cytometry revealed $>16\%$ of the CD8$^+$ cells positive for the cytokine after 11 days of stimulation.

The transcriptional regulation of perforin and effective perforin protein expression seemed to be uncoupled. There was no significant difference in RNA levels between HSV-1- or L. monocytogenes-activated PBMCs both in the presence or absence of CD4, unlike the perforin protein expression, which differed significantly between those groups. Furthermore, perforin mRNA but no protein was already present in significant amounts in naive CD8$^+$ T cells in contrast to granulysin A mRNA, which was undetectable in naive T cells. The presence of significant amounts of perforin mRNA in naive T cells as well as in Listeria-activated CTLs does not correspond with the low protein expression in those cells. This difference in mRNA and protein levels indicates a posttranscriptional regulation step, as this was demonstrated for murine NK cells that translate perforin from a preexisting mRNA pool upon activation (65).

The mRNA amount in PBMCs correlated on the other hand with the protein expression for granulysin A, granulysin, granzyme B, and for IFN-γ following stimulation with either pathogen, indicating mainly transcriptional regulation of these genes. CD4 depletion led to a significant reduction of the granulysin A mRNA level in HSV-1-activated PBMCs, demonstrating that CD4$^+$ T cell help is important for the transcriptional induction of granulysin A. Consistent with our results on the transcriptional regulation after Listeria activation, a recent study showed that Mycobacteria triggered the mRNA expression of IFN-γ, granzyme B, and, to a lesser extent, granulysin, while the expression of perforin was not induced (66). The varying mRNA expression profile of the different cytotoxic proteins following an antigenic stimulus is in agreement with results demonstrating the genes for perforin, granzymes A, B,
and C, as well as IFN-γ were differentially expressed during primary activation of naive CD8+ T cells (67).

An interesting finding was the significant higher surface expression of CD252 (CD134L, OX40L) on HSV-1-infected DCs compared with Listeria-infected DCs. Additionally, costimulation via the CD252-CD134 axis proved to influence the HSV-1-dependent induction of the perforin expression. CD252 is expressed on activated DCs as well as B cells (68). Mice lacking CD252 have impaired CD4+ T cell responses involving both Th1 and Th2 cytokine production (69). Furthermore, mice deficient in CD134 signaling have a reduced CD4+ T cell response to influenza and LCMV, as indicated in a decreased proliferation and cytokine production (70). As confirmed in our study, resting T cells do not express CD134. After induction via TCR/CD3 signaling, CD134 initially appears 12–24 h after stimulation. Furthermore, APCs co-expressing CD252 with B7-1 markedly induced expression of IL-2 in CD4+ cells (71). Additionally, CD134+ cells were found in HSV-1-infected mouse corneas, indicating that CD134+ cells contribute to immunological control of HSV-1 in infected tissues (72).

Another factor contributing to the modulation of a specific effector function in CTL is the cytokine environment (4). A key cytokine in the development of a CTL and NK cell response is IL-12 (73). IL-12 is produced by activated phagocytes and DCs. In early host defense IL-12 is essential to induce IFN-γ and to promote proliferation and differentiation of activated CD8+ cells (10). IL-1α is also produced by activated APCs and acts as a proinflammatory cytokine (74). Finally, TNF-α is another classical proinflammatory cytokine that is functionally important in macrophage activation and phagocyte recruitment to the site of infection (75). We found these proinflammatory cytokines significantly increased in the supernatant of L. monocytogenes-activated aLDCs, which is in agreement with recent results showing L. monocytogenes triggers a robust IL-12, IFN-γ, and TNF-α response (76).

The low IL-1 and IL-12 titers in viral-activated cultures were unexpected (77, 78). Human herpes virus 6 infection induced dramatic functional abnormalities in APCs, including a selective suppression of IL-12 (79). Furthermore, human CMV inhibited the IL-1 and TNF-α signaling pathways (80). To our knowledge, however, for HSV-1 such viral escape mechanisms have not yet been described.

IL-4 levels were similar in bacterial- and viral-activated cultures. HSV-1-specific CD4+ T cell clones were previously shown to simultaneously produce IL-2, IL-4, and IFN-γ after HSV-1 activation in aLDCs (81). Listeria-specific CD4+ cells were also demonstrated to produce large quantities of IL-4 after antigenic stimulation (82), indicating the simultaneous presence of both Th1 and Th2 cytokines.

However, the culture supernatants of HSV-1-activated cultures contained significantly higher amounts of IL-2 than after L. monocytogenes stimulation, which was significantly reduced by an anti-CD134 Ab. Cytometry demonstrated 36% of HSV-1-activated CD4+ cells positive for the cytokine. Finally, CD4+ cell depletion decreased the HSV-1-dependent perforin induction. Taken together, the evidence clearly suggests CD4+ cells as the main source of this cytokine in our in vitro system. IL-2 is critical in the regulation of perforin expression (83) by its capability to induce perforin expression in resting T cells (84), which is even more significant in synergy with TCR/CD3 complex ligation (85). In the context of Ag presentation by DCs, the significantly higher IL-2 levels in HSV-1-activated aLDCs provides a conclusive explanation for the markedly higher perforin expression in HSV-1-activated CTLs.

As a model, we propose that HSV-1 is processed and presented by human DCs via MHC class I as well as class II, and Ag presentation is accompanied by enhanced surface expression of CD252 (CD134L). Ag presentation to, and costimulation via CD134 of, CD4+ Th cells triggers increased expression and secretion of IL-2. Elevated levels of IL-2 in conjugation with MHC class I Ag presentation to CD8+ CTLs is finally the driving force of robust perforin and granzyme A expression. On the other hand, infection of DCs with L. monocytogenes induces a proinflammatory cytokine response with elevated IL-1, IL-12 and TNF-α. The proinflammatory cytokines and, especially, low levels of IL-2 together with Ag presentation modulate the distinct effector response with high expression of granulysin, granzyme B, and IFN-γ.

Acknowledgments

We are very grateful to Mirjam Christen and Gery Barmettler for excellent technical assistance. We thank Andreas Schaffner, Markus Schneemann, and Gabriele Schoedon (Medical Clinic B Research Unit, Department of Medicine, University Hospital of Zurich) for providing us with L. monocytogenes and L. innocua. We are very grateful to Judy Lieberman for helpful discussions and advice. Furthermore, we thank Judy Lieberman that some of the revision experiments could generously be performed in her laboratory.

Disclosures

The authors have no financial conflicts of interest.

References


