The CD7[−] subset of CD4⁺ memory T cells is prone to accelerated apoptosis that is prevented by interleukin-15 (IL-15)

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Abstract

The CD7[−] subset of CD4⁺ T cells reflects a stable differentiation state of post-thymic helper T cells with CD45R0⁺CD45RA[−] 'memory' phenotype. Here we report that CD4⁺CD7[−] T cells are prone to increased spontaneous apoptosis *in vitro* compared to CD4⁺CD7⁺ T cells. Spontaneous apoptosis is prevented by IL-15, but not by IL-2. Moreover, IL-15 increases Bcl-2 and decreases CD95/Fas expression of CD7[−], but not of CD7⁺ T cells. Because IL-15 is physiologically not secreted but expressed in a membrane-bound form, we cocultured T cells with TNF- α stimulated fibroblasts that expose membrane IL-15. TNF- α stimulated fibroblasts rescue CD4⁺CD7[−] T cells from apoptosis whereas unstimulated fibroblasts do not. Rescue from apoptosis requires cell-cell contact and is abolished by addition of neutralizing antibodies to IL-15. We conclude that membrane IL-15 prevents accelerated apoptosis of CD4⁺CD7[−] T cells. This mechanism may contribute to accumulation of CD7[−] T cells in chronic inflammatory skin lesions. *Cell Death and Differentiation* (2001) 8, 395–402.

Keywords: membrane IL-15; CD4⁺CD7[−] T cells; memory cells; fibroblasts

Abbreviations: FACS, fluorescence-activated cell sorter; MFI, mean fluorescence intensity; TUNEL, terminal deoxynucleotidyl transferase reaction; PI, propidium iodide

Introduction

Absence of CD7 antigen defines a subset of post-thymic CD4⁺ T cells with CD45R0⁺CD45RA[−] memory phenotype. In contrast to the CD4⁺CD7⁺ T-cell population, CD4⁺CD7[−] T cells expand during certain physiological and pathological conditions *in vivo*. For instance, circulating CD4⁺CD7[−] T cells

in the peripheral blood increase in both percentages and absolute numbers during aging^{1–3} and, furthermore, under conditions that are associated with permanent T-cell stimulation such as chronic inflammatory skin diseases,⁴ rheumatoid arthritis,^{5,6} and kidney transplantation recipients.⁷ We moreover found that preferentially CD4⁺CD7[−] T cells infiltrate the skin in diseases associated with chronic immunostimulation.^{4,8}

In vitro evidences indicate that specific downregulation of CD7 antigen expression in T cells reflects a separate and stable differentiation state occurring late in the immune response.¹ It is therefore under discussion whether extended rounds of stimulation will result in expansion of the CD4⁺CD7[−] T-cell population under pathological situations resulting in accumulation of these cells in inflammatory lesions. Alternatively or in addition, delay in cell death of CD7[−] T cells may account for their local accumulation in a variety of chronic inflammatory diseases.

In the present study we report that CD4⁺CD7[−] T cells from the peripheral blood are prone to an increased rate of spontaneous apoptosis *in vitro* compared to CD4⁺CD7⁺ T cells. Addition of recombinant interleukin-15 (IL-15), physiologically expressed in a membrane-bound form on the surface of TNF- α stimulated fibroblasts,⁹ is also capable to prevent spontaneous apoptosis of CD7[−] T cells *in vitro*. CD4⁺CD7[−] T cells compared to CD4⁺CD7⁺ T cells express decreased levels of Bcl-2 and increased levels of CD95/Fas. IL-15 increases Bcl-2 expression and decreases CD95/Fas expression in CD4⁺CD7[−] cells but not in CD4⁺CD7⁺ cells. Rescue of CD4⁺CD7[−] T cells from accelerated apoptosis by membrane-bound IL-15 may be of physiological significance, e.g., in chronic inflammatory lesions where CD7[−] cells preferentially accumulate.

Results

CD4⁺CD7[−] T cells are prone to increased spontaneous apoptosis *in vitro*

We isolated cells of the CD4⁺CD7[−] and CD4⁺CD7⁺ T cell subset, respectively, from peripheral blood mononuclear cells and monitored by Annexin V staining cells that have entered apoptosis during culture *in vitro*. In summary, the number of apoptotic cells is substantially increased within the CD4⁺CD7[−] T cell subset compared to the CD4⁺CD7⁺ subset. After 96 h of culture, less than 20% of CD4⁺CD7[−] cells were found viable compared to 80% viable CD4⁺CD7⁺ T cells. TUNEL assays (Figure 1) and gel electrophoretic analyses (data not shown) revealed increased nuclear DNA

Figure 1 Spontaneous apoptosis of CD4⁺CD7⁺ (A) and CD4⁺CD7⁻ (B) memory T cells *in vitro*. Freshly isolated T-cell subsets were cultured *in vitro* 24 h and fragmented DNA was labeled with FITC-labeled dUTP by terminal deoxynucleotidyl transferase-reaction (TUNEL technique). Cells were analyzed by FACS and percentages of cells undergoing apoptosis was determined

fragmentation indicative for apoptotic cell death within the subset of CD4⁺CD7⁻ T cells compared to CD4⁺CD7⁺ T cells.

IL-15 rescues CD7⁻ T cells from accelerated apoptosis

We asked whether accelerated apoptosis of CD4⁺CD7⁻ T cells *in vitro* can be prevented by certain cytokines. Therefore, isolated CD4⁺CD7⁻ and CD4⁺CD7⁺ T cells were incubated in presence of various cytokines, i.e., IL-2 (1 nM), IL-4 (0.93 nM), IL-6 (1.35 nM), IL-7 (1.13 nM), IL-10 (1.24 nM), IL-15 (0.83 nM), TNF- α (1.16 nM), respectively, (each 15 ng/ml), for 48 h and the number of viable cells was determined. In presence of IL-15, the number of viable CD4⁺CD7⁻ T cells is substantially increased compared to incubation without IL-15 (Figure 2A) indicating that IL-15 rescues CD7⁻ T cells from accelerated apoptosis *in vitro*. The number of viable CD7⁻ T cells in presence of IL-15 is nearly the same as the number of CD4⁺CD7⁺ T cells without supplemented IL-15. The activity of IL-15 towards CD4⁺CD7⁻ T cells is dose-dependent (Figure 2B). In contrast to CD4⁺CD7⁻ cells, the viability of CD4⁺CD7⁺ T cells was not significantly altered by IL-15 in this range of concentration. IL-2 (1 nM) did not significantly alter the number of viable CD7⁻ T cells (Figure 2A), even not in unphysiologically high concentrations of 10 nM (data not shown). The other cytokines tested did not show any effect on survival of CD4⁺CD7⁻ T cells during 48 h of culture (data not shown).

To confirm the specificity of IL-15 signaling, experiments were performed to block the IL-2 receptor β -chain utilizing the antagonistic anti-human IL-2R β mAb (clone Mik- β 2).¹⁰ Added prior to cytokine addition, the IL-2R β specific mAb significantly ($P < 0.01$) neutralised the anti-apoptotic effect of IL-15 on CD4⁺CD7⁻ T cells in a dose-dependent manner (Figure 2C). Blocking of IL-15 specific signaling utilizing the antagonistic Mik- β 2 antibody was shown by Tinhofer and coworkers.¹¹ This indicates that protection of CD4⁺CD7⁻ cells from spontaneous apoptosis by recombinant IL-15 can be abolished by blocking of receptor signaling pathway.

IL-15 and IL-2 receptor subunits are differentially expressed on cell surface of CD4⁺CD7⁻ T cells and CD4⁺CD7⁺ T cells

IL-15 and IL-2 receptor share the β and γ (CD122/CD132) subunit, high-affinity binding of IL-15 and IL-2, however, depends on the cytokine specific α -chains.¹² To test whether the different sensitivities of CD4⁺CD7⁻ T cells and CD4⁺CD7⁺ T cells towards IL-15 depend on different expression levels of the IL-15 receptor subunits, the profiles of the IL-15R α , β , and γ -chains on the cell surface were analyzed by flow cytometry. IL-15R α expression was found to be expressed in a significantly ($P < 0.001$) higher percentage of CD4⁺CD7⁻ T cells ($60 \pm 3\%$) compared to CD4⁺CD7⁺ cells ($6 \pm 5\%$). In addition, we observed a higher expression of the IL-2R α chain in CD4⁺CD7⁻ T cells ($30 \pm 4\%$) compared to CD4⁺CD7⁺ T cells ($9 \pm 3\%$) (Figure 2D). On the other hand, IL-2R β (CD122) and IL-2R γ (CD132) subunits were expressed in a similar level on both, CD4⁺CD7⁻ and CD4⁺CD7⁺ T cells, respectively.

IL-15 alters Bcl-2 and CD95/Fas expression in CD4⁺CD7⁻ T cells but not in CD4⁺CD7⁺ T cells

We assessed the levels of Bcl-2, Bcl-xL, and Bax protein expression in freshly isolated cells of the CD4⁺CD7⁻ and CD4⁺CD7⁺ T-cell subset, respectively, by flow cytometric determination of mean fluorescence intensities (MFI). As shown in Figure 3A, Bcl-2 protein levels are significantly ($P < 0.05$) lower in CD4⁺CD7⁻ T cells (MFI, mean $\pm$ SD: 3.25 ± 0.53) compared with CD4⁺CD7⁺ T cells (MFI, mean $\pm$ SD: 5.03 ± 0.61). No differences were found in the levels of Bcl-xL and Bax protein expression (data not shown). Moreover, CD95/Fas is expressed at a significantly ($P < 0.001$) higher level (MFI, mean $\pm$ SD: CD4⁺CD7⁻: 7.83 ± 0.33 ; CD4⁺CD7⁺: 2.54 ± 0.13) and in a significantly ($P < 0.001$) higher percentage of cells of the CD4⁺CD7⁻ subset ($79.3 \pm 4\%$) compared to cells of the CD4⁺CD7⁺ subset ($34.7 \pm 5\%$) (Figure 3B). CD95L is not expressed in freshly isolated T cells neither of the CD4⁺CD7⁻ nor of the CD4⁺CD7⁺ subset (data not shown).

CD4⁺CD7⁻ T cells and CD4⁺CD7⁺ T cells were cultured in presence of recombinant IL-15 and IL-2 (15 ng/ml each), respectively, for 72 h and assayed for Bcl-2 and CD95/Fas expression by FACS analysis. Upon incubation with IL-15, CD4⁺CD7⁻ T cells express increased amounts of Bcl-2 compared to incubation without IL-15 (Figure 4). On the

Figure 2 IL-15 rescues CD4⁺CD7⁻ cells from spontaneous apoptosis *in vitro*. (A) Cells of the CD4⁺CD7⁻ and of the CD4⁺CD7⁺ subset were cultured for 48 h in medium without supplements, supplemented with IL-2 (1 nM) or with IL-15 (0.8 nM), (each 15 ng/ml). Apoptotic cells were assessed by FACS analysis after annexin V-FITC and propidium iodide (PI) staining. This procedure enables the detection of viable (annexinV-FITC-/PI-), early apoptotic (annexinV-FITC+/PI-), late apoptotic (annexinV-FITC+/PI+) and necrotic (annexinV-FITC-/PI+) cells. (B) Dose-dependence of IL-15 to inhibit CD4⁺CD7⁻ cells from spontaneous apoptosis *in vitro*. CD4⁺CD7⁻ and CD4⁺CD7⁺ T cells were cultured for 48 h in medium with increasing concentrations of IL-15 (0–100 ng/ml). The percentages of apoptotic cells were determined by FACS analysis after staining with the combination of annexin V-FITC and propidium iodide (PI). The results reflect the mean $\pm$ SD of triplicate determinations. (C) Blocking of IL-15R signaling via IL-2R β chain neutralizes antiapoptotic effect of IL-15 on CD4⁺CD7⁻ T cells. CD4⁺CD7⁻ and CD4⁺CD7⁺ T cells were pretreated with increasing concentrations (0–20 μ g/ml) of anti-IL-2R β mAb (clone Mik- β 2) or an isotype-matched control mAb 30 min prior to stimulation with IL-15. After washing, CD4⁺CD7⁻ and CD4⁺CD7⁺ T cells were cultured for 48 h in medium with IL-15 (15 ng/ml) or in medium without supplements, respectively. The percentages of apoptotic cells were determined by FACS analysis after staining with annexin V-FITC and propidium iodide (PI). The results reflect the mean $\pm$ SD of triplicate determinations. (D) Flow cytometric analysis of the IL-15R and IL-2R subunits in CD4⁺CD7⁻ and CD4⁺CD7⁺ T cells. Cells were stained with antibodies specific for the receptor subunit or with an isotype-matched control

other hand, the expression level of CD95/Fas protein is decreased in these cells in presence of IL-15. Noteworthy, the expression levels of Bcl-2 and of CD95, as monitored by MFI, in CD7⁻ cells upon stimulation with IL-15 are nearly similar to those in CD7⁺ cells without IL-15 stimulation. IL-2 did not alter significantly the protein levels of Bcl-2 and CD95/Fas in CD7⁻ T cells. The amounts of Bcl-xL and Bax protein were similar in CD4⁺CD7⁻ cells stimulated with IL-15 and IL-2, respectively, compared to incubation in medium without added cytokine (data not shown). In contrast to CD4⁺CD7⁻ T cells, the levels of Bcl-2 and CD95/Fas in CD4⁺CD7⁺ T cells are not altered after incubation with IL-15 or IL-2 (Figure 4).

Accelerated apoptosis of CD4⁺CD7⁻ T cells is inhibited by membrane-bound IL-15 exposed on the surface of TNF- α stimulated dermal fibroblasts

IL-15 is physiologically expressed in a membrane-bound form on the surface of keratinocytes,¹³ monocytes,¹⁴ and on TNF- α stimulated fibroblasts.⁹ Beside some established tumor cells lines,¹⁵ IL-15 is not secreted into the culture supernatant. We therefore asked whether membrane IL-15 on the surface of normal cells prevents spontaneous apoptosis of CD4⁺CD7⁻ T cells. Because CD4⁺CD7⁻ T cells preferentially infiltrate the dermis,⁸ we cocultured CD4⁺CD7⁻ T cells with dermal fibroblasts that expose membrane IL-15 after TNF- α stimulation (20 ng TNF- α /ml, 72 h). Unstimulated fibroblasts do not express membrane IL-15.⁹ Coculture with TNF- α stimulated fibroblasts substantially reduced the number of apoptotic cells within the CD4⁺CD7⁻ subset but not within the CD4⁺CD7⁺ subset whereas coculture of unstimulated dermal fibroblasts only marginally altered the number of viable CD4⁺CD7⁻ T cells (Figure 5A). TNF- α alone did not rescue CD7⁻ T cells from apoptosis making it unlikely that this effect is due to a TNF- α activity directly towards CD4⁺CD7⁻ T cells.

Moreover, we incubated TNF- α stimulated fibroblasts with a neutralizing anti-IL-15 antibody or, as control, with an isotype matched control antibody prior to coculture with CD4⁺CD7⁻ T cells. As shown in Figure 5A, the number of apoptotic CD4⁺CD7⁻ T cells in presence of the anti-IL-15 mAb was dramatically increased ($P < 0.01$) and nearly the same as incubated without TNF- α treated fibroblasts indicating that protection of CD4⁺CD7⁻ cells from spontaneous apoptosis by TNF- α stimulated fibroblasts is nearly completely abolished by neutralization of IL-15. Incubation with an isotype matched control antibody did not alter the number of apoptotic CD4⁺CD7⁻ T cells.

In a second set of experiments, CD4⁺CD7⁻ T cells were physically separated from the monolayer of TNF- α stimulated fibroblasts by a TranswellTM membrane insert. In this situation, the same numbers of apoptotic CD4⁺CD7⁻ T cells were found as in cultures of CD4⁺CD7⁻ T cells without TNF- α stimulated fibroblasts (Figure 5B). This indicates that

antibody. Results are given as per cent stained cells within the CD4⁺CD7⁻ and CD4⁺CD7⁺ T-cell subsets, respectively. Mean values $\pm$ SD of three experiments are presented

Figure 3 (A) Analysis of Bcl-2 expression. Freshly isolated CD4⁺CD7⁺ and CD4⁺CD7⁻ T cells (closed histograms) were analyzed for Bcl-2 expression utilizing anti-Bcl-2 mAb 6C8. The open histograms indicate background staining with isotype-matched control antibodies. (B) Analysis of CD95/Fas expression. Isolated CD4⁺CD7⁺ and CD4⁺CD7⁻ T cells, respectively (closed histograms), were stained with anti-CD95/Fas specific antibody (clone UB2) and analyzed by FACS. The open histograms indicate background staining with an isotype-matched control antibody

Figure 4 Altered expression of Bcl-2 and CD95/Fas after incubation of CD4⁺CD7⁻ T cells with IL-15. Freshly isolated CD4⁺CD7⁻ and CD4⁺CD7⁺ T cells were seeded in microwell plates (10⁵ cells/well) and incubated for 72 h in medium without supplements or supplemented with recombinant IL-2 (15 ng/ml) or IL-15 (15 ng/ml). Cells were stained (A) with an anti-human CD95/Fas antibody (clone UB2) and (B) with an anti-human Bcl-2 antibody (clone 6C8) and subjected to FACS analysis. An isotype matched control antibody served as control. The results represent the mean $\pm$ SD from three experiments

protection of CD4⁺CD7⁻ cells from apoptosis is due to lymphocyte–fibroblast contact and is not mediated by

soluble factors. Taken together, we conclude that membrane IL-15 on the surface of TNF- α treated fibroblasts rescues CD7⁻ T cells from accelerated apoptosis.

Discussion

We report here that the majority of CD4⁺CD7⁻ T cells more rapidly enters apoptosis *in vitro* compared to cells of the CD4⁺CD7⁺ subset. This observation is based on FACS analyses of cells stained for Annexin V, on electrophoretic analyses of nuclear DNA fragmentation and on TUNEL tests. In accordance with the phenotype of apoptosis prone cells, CD4⁺CD7⁻ T cells express increased levels of CD95/Fas protein and decreased levels of Bcl-2 compared to CD4⁺CD7⁺ T cells. No differences in the levels of Bcl-xL and Bax expression in these T cell subsets were observed. Decreased Bcl-2 and increased CD95/Fas protein levels reflect on the one hand the phenotype of chronically activated CD4⁺CD7⁻ T cells, on the other hand may contribute to accelerated apoptosis of these cells *in vitro*. Low Bcl-2 levels associated with increased numbers of apoptotic cells *in vitro* are found under various physiological and pathological conditions, e.g., in T cells from aging blood donors¹⁶ who constitutively have elevated numbers of CD4⁺CD7⁻ cells in the peripheral blood compartment,^{1–3} in T cells from patients with autoimmune diseases,¹⁷ and with acute and chronic viral infections^{18,19} including HIV,^{20,21} in T cells from patients with allogeneic bone

Figure 5 (A) TNF- α -stimulated dermal fibroblasts rescue CD4⁺CD7⁺ T cells from spontaneous apoptosis *in vitro*. Freshly isolated CD4⁺CD7⁺ T cells were cocultured for 48 h with dermal fibroblasts, or with fibroblasts prestimulated for 72 h with TNF- α (20 ng/ml). Cocultures in the presence of irrelevant mouse IgG mAb and cells cultured in medium without supplements were used as controls. Apoptotic cells were monitored by annexin V and propidium iodide (PI) staining and FACS analysis. Results are expressed as the mean $\pm$ SD of triplicate determinations. (B) Cell-cell contact with TNF- α -stimulated fibroblasts rescues CD4⁺CD7⁺ T cells from apoptosis. Isolated CD4⁺CD7⁺ T cells were cocultured for 48 h with TNF- α -stimulated fibroblasts as described for (A). Alternatively, cocultured T cells were cultured physically separated from fibroblasts utilizing a TranswellTM membrane plate. Cell death was monitored by FACS analysis after annexin V and propidium iodide (PI) staining. The results represent the mean $\pm$ SD from three experiments

marrow transplantation,²² and in synovial T cells from joints affected with rheumatoid arthritis.^{23,24} Supplementation of T-cell cultures with IL-2R γ -chain cytokines upregulates Bcl-2 and Bcl-xL expression and decreases the number of apoptotic cells *in vitro* implying that cytokine deprivation may be responsible for accelerated apoptosis of these cells.^{25,26} Whereas IL-15 is active, IL-2 in concentrations of 1–10 nM, in contrast, fails to rescue CD4⁺CD7⁺ T cells from apoptosis *in vitro*. We speculate that the ineffectiveness of IL-2 towards CD4⁺CD7⁺ T cells may be due to the quiescent phenotype of these cells that is characterized by a low, if any, spontaneous

proliferative capacity *in vitro* and a substantial hyporesponsiveness to both IL-2 and TCR/CD3 mediated stimuli compared to CD4⁺CD7⁺ T cells.²⁷ IL-2 in high concentrations can compensate for diminished IL-2 receptor signaling in anergic mouse T cells²⁸ and exerts its anti-apoptotic effect preferentially in cycling cells.^{19,24} Cells of the CD4⁺CD7⁺ T cell subset, however, express a quiescent, nonproliferating, anergic phenotype *in vivo*. Chronic stimulation by IL-15 may contribute to anergy of CD4⁺CD7⁺ T cells as IL-15 skews T-cell clones from apoptosis to anergy.²⁹ Recent reports suggest that IL-2 and IL-15 may exert their anti-apoptotic properties on T cells by different receptor pathways.^{30–34} Here, we demonstrate that the majority of CD4⁺CD7⁺ T cells express IL-15R α -chain in addition to IL-2 R α , β , γ chains. Our data reconcile with previous observations³⁵ that CD4⁺CD45RO⁺ T cells constitutively express all IL-2 receptor subunits in addition to high expression of IL-15 R α chain.³⁶ In accordance to previous data,³⁷ we speculate that although both IL-15 and IL-2 R α -chains are expressed on the cell surface CD7⁺ cells preferentially respond to IL-15 due to the significant higher affinity of IL-15 than IL-2 to the corresponding α -chain of the receptor. Accordingly, some immune cell subsets appear to respond preferentially to one cytokine, IL-2 or IL-15.³⁸ Zhang *et al.*³⁹ reported similar effects of IL-15 on mouse CD8⁺ T cells. On the other hand, continuous IL-15 stimulation is known to downregulate IL-15R α chain and to upregulate IL-2R α chain expression in T cells.⁴⁰

Our observations complement a recent report by Agrawal *et al.*⁴¹ that demonstrates profound *in vitro* apoptosis of T cells isolated from inflammatory, non-malignant tissues and of tumor-infiltrating lymphocytes derived from a B cell non-Hodgkin's lymphoma. These T cells exhibit properties with striking similarities to those of the CD4⁺CD7⁺ T cell subset, including: (i) accumulation in a variety of chronic inflammatory lesions;⁴ (ii) decreased activation capacity via the TCR/CD3 pathway but strong activation via the CD2 pathway or by CD28 costimulation;^{27,42} (iii) marked frequency of spontaneous apoptosis *in vitro*; and (iv) failure of IL-2 to inhibit spontaneous apoptosis *in vitro*.

The anti-apoptotic effect of IL-15 on T cells through upregulation of Bcl-2 expression is well described (reviewed by Waldmann *et al.*¹⁵ and Akbar *et al.*²⁵), e.g., on T cells from HIV-infected persons^{20,43} and on TCR $\gamma\delta$ intraepithelial T cells.⁴⁴ In recent years, more details of how the IL-15 signaling pathway is coupled to the Bcl-2 survival pathway have been elucidated. IL-15 signals are transmitted through the cytoplasmic domains of the β and γ receptor chains to janus kinases, especially Jak3,⁴⁵ that are linked to the STAT pathway, including STAT5a, STAT5b, STAT1 α , STAT3 and other so far unidentified DNA-binding proteins.⁴⁶ STAT3 binds to the Bcl-2 promoter thereby enhancing the transcription rate and finally the amount of Bcl-2 protein.⁴⁷

Less is known about the linkage between the IL-15 signaling pathway and the CD95/Fas pathway. Bulfone-Paus *et al.*³⁰ reported that IL-15 efficiently inhibits apoptosis induced by anti-Fas antibodies. In the absence of T-cell receptor triggering, however, IL-15 induces a quiescent phenotype characterized by G0/G1 arrest and downregulation of CD95/Fas expression.³¹ This is in accordance to our

observations in so far that IL-15 downregulates CD95/Fas protein expression in CD4⁺CD7⁺ T cells. Other studies, however, imply that IL-2R γ -chain cytokines do not inhibit CD95-CD95L mediated cell death in CD4⁺ and CD8⁺ T cells (reviewed in Akbar *et al.*²⁵).

It is an obvious discrepancy that CD4⁺CD7⁺ T cells *in vitro* undergo extensive apoptosis but *in vivo* do not show evidence for preferred susceptibility to apoptosis. This observation supports the speculation that loss of the *in vivo* microenvironment may result in increased entry into apoptosis *in vitro*. Stroma cells are known to be basically capable in maintaining T-cell survival by providing environmental factors.^{25,48} We here show that spontaneous apoptosis of CD4⁺CD7⁺ T cells *in vitro* is counteracted by skin-derived fibroblasts that are prestimulated with TNF- α . This effect is due to membrane-bound IL-15 exposed on the surface of TNF- α treated fibroblasts. This conclusion is based on the observation that: (i) TNF- α stimulated, but not unstimulated fibroblasts, are effective towards CD4⁺CD7⁺ T cells; (ii) TNF- α stimulated, but not unstimulated fibroblasts, express membrane IL-15;⁹ (iii) rescue from apoptosis requires membrane contact of CD4⁺CD7⁺ T cells to TNF- α stimulated fibroblasts; and (iv) the apoptosis protective activity of TNF- α stimulated fibroblasts is abolished by addition of neutralizing anti-IL-15 antibody. It is unlikely that TNF- α exhibits anti-apoptotic activities directly towards CD4⁺CD7⁺ T cells because TNF- α without cocultured dermal fibroblasts did not rescue CD7⁺ T cells from apoptosis. Whereas IL-15 is physiologically membrane-associated and not secreted in detectable amounts into the culture medium,^{9,13,14} addition of soluble, recombinant IL-15 substitutes the anti-apoptotic activity of TNF- α stimulated fibroblasts.

Under certain physiological and pathological conditions, particularly during chronic inflammation, CD4⁺CD7⁺ memory T cells accumulate in chronic inflammatory lesions. The molecular basis therefore and the role of fibroblasts in sustaining proliferation of attracted leukocytes at the site of chronic inflammation is poorly understood. Leukocyte-fibroblast interactions are known to be mediated by a panel of specific adhesion molecules induced by proinflammatory cytokines on both leukocytes and fibroblasts. TNF- α , a potent mediator of inflammatory functions, induces fibroblasts to express matrix metalloproteinases, chemokines and adhesion molecules (for review: Baker⁴⁹). We recently found that upon prolonged incubation with TNF- α dermal fibroblasts expose membrane-bound IL-15 on the cell surface whereas unstimulated fibroblasts do not.⁹ Here we show that membrane-bound IL-15 on the cell surface of TNF- α stimulated fibroblasts rescues CD4⁺CD7⁺ memory T cells from apoptosis. This T-cell subset, moreover, contributes by secretion of IL-5, in addition to IL-4 and IFN- γ , to attract an eosinophilic infiltrate^{50,51} that on their part is believed to play a substantial role in epithelial cell damage by eosinophil-derived products. This mechanism fits well with the observation that skin infiltrating CD4⁺CD7⁺ T cells accumulate within the chronic inflammatory lesion, a site with locally high concentrations of TNF- α and with eosinophilic infiltrates. Moreover, this mechanism implies a crucial role of TNF- α stimulated

fibroblasts not only in sustaining proliferation of attracted, activated CD7⁺ T cells but also in prevention of apoptosis of cells of the CD7⁺ subset. This process is potentially important because the cytokine cross-talk of mononuclear cells and dermal fibroblasts likely contributes to the chronicity of inflammatory skin diseases. Our results may additionally have impact on the understanding of consequences of increased and prolonged secretion of proinflammatory cytokines like TNF- α in dermal lesions.

Material and Methods

Cells and cytokines

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by Ficoll-Hypaque density centrifugation. T-cell-enriched populations were obtained from PBMC by passage through a nylon wool column (Wako Chemicals, Neuss, Germany). The proportion of contaminating CD14⁺ monocytes and CD19⁺ B cells in the T-cell preparation was <1%. Memory helper T cells were obtained using the high-gradient magnetic cell separation system MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, nylon-non-adherent lymphocytes were simultaneously incubated with an anti-CD8 monoclonal antibody (mAb) (Becton-Dickinson, Mountain View, CA, USA), anti-CD16 mAb (Immunotech, Hamburg, Germany), and anti-CD45RA mAb (Becton Dickinson) for 20 min at 4°C and washed in phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA). The cells were incubated with a goat-anti-mouse IgG coupled to magnetic beads (Miltenyi Biotec), washed, and passed through a MACS column to isolate CD8⁺CD16⁺CD45RA⁺ lymphocytes by negative selection. T cells obtained were homogeneously stained for CD4 (>98%) and CD45RO (>98%), and were found free of CD8, CD45RA, and CD16 expression (<1%) as monitored by fluorescent-activated cell sorter (FACS) analysis. Isolated CD4⁺CD45RA⁺CD45RO⁺ memory T cells were separated into CD7⁺ and CD7⁺ subpopulations by an additional MACS separation procedure. Cells were labeled with anti-CD7 mAb (Becton Dickinson) followed by incubation with an anti-anti-mouse antibody conjugated with magnetic beads and sorted by MACS procedure. The CD7⁺ cell fraction routinely contained <1% CD7⁺ T cells and the CD7⁺ cell fraction >98% CD7⁺ T cells.

Human dermal fibroblasts were obtained from normal skin of patients undergoing plastic surgery. Fibroblasts were initially grown in DME medium, 10% (v/v) fetal calf serum (FCS). For use in coculture experiments, fibroblasts of passages 4 to 6 were subcultured in RPMI 1640 medium supplemented with L-glutamine (2 mM) and 10% (v/v) FCS (Gibco, Karlsruhe, Germany). Fibroblasts were grown to near confluence in 24-well plates (1.5 × 10⁵ cells/well) and incubated with 20 ng/ml TNF- α (R&D Systems, Wiesbaden, Germany) for 3 days. To avoid cellular contact of T cells with fibroblasts, culture wells containing a TranswellTM-COL filter membrane (0.4 μ m pore diameter, Corning, Wiesbaden, Germany) were used.

IL-2 was purchased from Chiron (Ratingen, Germany), IL-4, IL-6, IL-7, IL-10 and IL-15 from R&D Systems, and neutralizing anti-IL15 antibody from Biosource, Ratingen, Germany. Murine anti-human IL-2R β antibody (clone Mik- β 2) (PharmMingen, San Diego, CA, USA) was used to block IL-15 signaling.¹⁰

Immunophenotyping

For detection of surface antigens, T cells were incubated with a primary antibody, subsequently with a species-matched fluorescence-

labeled secondary antibody, each 20 min at 4°C. For detection of intracellular antigens, cells were fixed in 2% (v/v) paraformaldehyde in PBS and subsequently permeabilized with 0.5% (v/v) saponin, 0.5% (w/v) BSA in PBS prior to antibody incubation. Cells were analyzed by flow cytometry (Coulter EPICS XL) using the system II software (Coulter Electronics, Miami, FL, USA). Mean fluorescence intensities (MFI) represents the ratio of mean fluorescence intensities of specific antigen staining and irrelevant control staining.

To detect IL-15R α and IL-2R $\alpha\beta\gamma$ proteins, the following antibodies were used: mouse anti-human IL-2R α (clone M-A251), IL-2R β (clone Mik- β 2), and IL-2R γ (clone AG184) antibody (PharMingen) and affinity-purified polyclonal goat antibodies N-20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) recognizing the amino-terminal region of IL-15R α chain. 1×10^6 cells were incubated with the antibody (1 μ g/ml) and with a nonreactive (isotype-matched) negative control antibody (Becton Dickinson), respectively. Phycoerythrin (PE)-labeled rabbit anti-mouse (1 μ g/ml), and FITC-labeled rabbit anti-goat (1 μ g/ml) were used as secondary antibodies. For detection of apoptosis-associated proteins the following antibodies were used: anti-CD95 mAb UB2 (10 μ g/ml; Immunotech, Marseille, France), hamster anti-CD95L mAb 4H9 (10 μ g/ml; Immunotech), affinity-purified polyclonal rabbit antibodies N20 (2 μ g/ml; Santa Cruz Biotechnology) recognizing the amino-terminal region of Bax, mouse monoclonal IgG₁ antibody H-5 (2 μ g/ml, Santa Cruz Biotechnology) directed against the amino-terminus of Bcl-xL, and hamster mAb 6C8 (2 μ g/ml; PharMingen) recognizing Bcl-2. Fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit, goat anti-mouse, and goat anti-hamster IgG (1 μ g/ml) were used as secondary antibodies. Species- and isotype-matched irrelevant antibodies were purchased from Becton Dickinson.

Detection of apoptosis

Apoptotic cell death was assessed by double-staining with annexin V (AV) and propidium iodide (PI) using TACS Annexin V Apoptosis Detection Kit (Genzyme) and subsequent flow cytometric analysis. Nucleosomal DNA fragments were detected by using the Apoptotic DNA Ladder Kit (Roche Diagnostics, Mannheim, Germany). To monitor cells that harbor fragmented DNA, DNA was labeled *in situ* by terminal deoxynucleotidyl transferase-reaction in presence of fluorescein-dUTP (TUNEL) using the *In Situ* Cell Death Detection Kit (Roche Diagnostics). Cells were subsequently analyzed by flow cytometry.

Statistical analysis

Data represent the mean $\pm$ SD of at least three independent experiments. Statistical analysis was performed using two-tailed Student's test.

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References

1. Reinhold U and Abken H (1997) CD4+CD7⁺ T cells: a separate subpopulation of memory T cells? *J. Clin. Immunol.* 17: 265–271
2. Ginaldi L, De Martinis M, Modesti M, Loreto F, Corsi MP and Quaglini D (2000) Immunophenotypical changes of T lymphocytes in the elderly. *Gerontology* 46: 242–248

3. Kukel S, Reinhold U, Oltermann I and Kreysel HW (1994) Progressive increase of CD7⁺ T cells in human lymphocytes with ageing. *Clin. Exp. Immunol.* 98: 163–168
4. Moll M, Reinhold U, Kukel S, Abken H, Müller R, Oltermann I and Kreysel HW (1994) CD7-negative helper T cells accumulate in inflammatory skin lesions. *J. Invest. Dermatol.* 102: 328–332
5. Lazarovits AI, White MJ and Karsh J (1992) CD7⁺ T cells in rheumatoid arthritis. *Arthritis Rheum.* 35: 615–624
6. Schmidt D, Goronzy JJ and Weyand CM (1996) CD4⁺ CD7⁺ CD28⁺ T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J. Clin. Invest.* 97: 2027–2037
7. Barrou B, Legac E, Blanc C, Bitker MO, Luciani J, Chatelain C, Debré P and Autran B (1995) Expansion of CD4+CD7⁺ T helper cells with a Th0-Th2 function in kidney transplant recipients. *Transplant. Proc.* 27: 1676–1677
8. Liu L, Abken H, Pföhler C, Rappl G, Tilgen W and Reinhold U (2000) Accumulation of CD4+CD7⁺ T cells in inflammatory skin lesions: evidence for preferential adhesion to vascular endothelial cells. *Clin. Exp. Immunol.* 121: 94–99
9. Rappl G, Kapsokafalou A, Heuser C, Rößler M, Ugurel S, Tilgen W, Reinhold U and Abken H (2000) Dermal fibroblasts sustain proliferation of activated T cells via membrane-bound IL-15 upon long-term stimulation with TNF- α . *J. Invest. Dermatol.* (in press)
10. Tsudo M, Kitamura F and Miyasaka M (1989) Characterization of the interleukin 2 receptor β chain using three distinct monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* 86: 1982–1986
11. Tinhofel I, Marschitz I, Henn T, Egle A and Greil R (2000) Expression of functional interleukin-15 receptor and autocrine production of interleukin-15 as mechanisms of tumor propagation in multiple myeloma. *Blood* 95: 610–618
12. Anderson DM, Kumaki S, Ahdieh M, Bertles J, Tometsko M, Loomis A, Giri J, Copeland NG, Gilbert DJ, Jenkins NA, Valentine V, Shapiro DN, Morris SW, Park LS and Cosman D (1995) Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL-15R α and IL-2R α genes. *J. Biol. Chem.* 270: 29862–29869
13. Rückert R, Asadullah K, Seifert M, Budagian VM, Arnold R, Trombotto C, Paus R and Bulfone-Paus S (2000) Inhibition of keratinocyte apoptosis by IL-15: A new parameter in the pathogenesis of psoriasis. *J. Immunol.* 165: 2240–2250.
14. Musso T, Calosso L, Zucca M, Millesimo M, Ravarino D, Giovarelli M, Malavasi F, Ponzi AN, Paus R and Bulfone-Paus S (1999) Human monocytes constitutively express membrane-bound, biologically active, and IFN- γ upregulated interleukin-15. *Blood* 93: 3531–3539.
15. Waldmann TA and Tagaya Y (1999) The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Ann. Rev. Immunol.* 17: 19–49.
16. Aggarwal S and Gupta S (1998) Increased apoptosis of T cell subsets in aging humans: altered expression of Fas (CD95), Fas ligand, Bcl-2, and Bax. *J. Immunol.* 160: 1627–1637.
17. Lorenz HM, Grünke M, Hieronymus T, Herrmann M, Kühnel A, Manger B and Kalden JR (1997) In vitro apoptosis and expression of apoptosis-related molecules in lymphocytes from patients with systemic lupus erythematosus and other autoimmune diseases. *Arthritis Rheum.* 40: 306–317.
18. Uehara T, Miyawaki T, Ohta K, Tamaru Y, Yokoi T, Nakamura S and Taniguchi N (1992) Apoptotic cell death of primed CD45RO⁺ T lymphocytes in Epstein-Barr virus-induced infectious mononucleosis. *Blood* 80: 452–458.
19. Akbar AN, Borthwick N, Salmon M, Gombert W, Bofill M, Shamsadeen N, Pilling D, Pett S, Grundy JE and Janossy G (1993) The significance of low bcl-2 expression by CD45RO⁺ T cells in normal individuals and patients with acute viral infections. The role of apoptosis in T cell memory. *J. Exp. Med.* 178: 427–438.
20. Naora H and Gougeon ML (1999) Interleukin-15 is a potent survival factor in the prevention of spontaneous but not CD95-induced apoptosis in CD4 and CD8 T lymphocytes of HIV-infected individuals. Correlation with its ability to increase Bcl-2 expression. *Cell Death Differ.* 10: 1002–1011.
21. Mugnaini EN, Haaheim LL, Sannes M and Brinchmann JE (1999) In vivo expansion coincident with excessive in vitro cell death within the memory subset of CD8⁺ T cells in HIV type 1 infection. *AIDS Res. Hum. Retroviruses* 15: 265–272.

22. Heib NC, Deas O, Rouleau M, Durrbach A, Charpentier B, Beaujean F, Vernant JP and Senik A (1999) Peripheral blood T cells generated after allogeneic bone marrow transplantation: lower levels of bcl-2 protein and enhanced sensitivity to spontaneous and CD95-mediated apoptosis in vitro. Abrogation of the apoptotic phenotype coincides with the recovery of normal naive/primed T-cell profiles. *Blood* 94: 1803–1813.
23. Maurice MM, van der Voort EA, Leow A, Levarht N, Breedveld FC and Verweij CL (1998) CD28 co-stimulation is intact and contributes to prolonged *ex vivo* survival of hyporesponsive synovial fluid T cells in rheumatoid arthritis. *Eur. J. Immunol.* 28: 1554–1562.
24. Salmon M, Scheel-Toellner D, Huissoon AP, Pilling D, Shamsadeen N, Hyde H, D'Angeac AD, Bacon PA, Emery P and Akbar AN (1997) Inhibition of T cell apoptosis in the rheumatoid synovium. *J. Clin. Invest.* 99: 439–446.
25. Akbar AN, Lord JM and Salmon M (2000) IFN- α and IFN- β : a link between immune memory and chronic inflammation. *Immunol. Today* 21: 337–342.
26. Akbar AN, Borthwick NJ, Wickremasinghe RG, Panayiotidis P, Pilling D, Boffill M, Krajewski S, Reed JC and Salmon M (1996) Interleukin-2 receptor common γ -chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (bcl-2, bcl-xL) but not pro-apoptotic (bax, bcl-xS) gene expression. *Eur. J. Immunol.* 26: 294–299.
27. Reinhold U, Abken H, Kukul S, Moll M, Müller R, Oltermann I and Kreysel HW (1993) CD7⁺ T cells represent a subset of normal human blood lymphocytes. *J. Immunol.* 150: 2081–2089.
28. Grundstrom S, Dohlsten M and Sundstedt A (2000) IL-2 unresponsiveness in anergic CD4⁺ T cells is due to defective signaling through the common γ -chain of the IL-2 receptor. *J. Immunol.* 164: 1175–1184.
29. Doms H, Van Belle T, Desmedt M, Rottiers P and Grooten J (2000) Interleukin-15 redirects the outcome of a tolerizing stimulus from apoptosis to anergy. *Blood* 96: 1006–1012.
30. Bulfone-Paus S, Ungureanu D, Pohl T, Lindner G, Paus R, Rückert R, Krause H and Kunzendorf U (1997) Interleukin-15 protects from lethal apoptosis in vivo. *Nature Med.* 3: 1124–1128.
31. Doms H, Desmedt M, Vancaeneghem S, Rottiers P, Goossens V, Fiers W and Grooten J (1998) Quiescence-inducing and antiapoptotic activities of IL-15 enhance secondary CD4⁺ T cell responsiveness to antigen. *J. Immunol.* 161: 2141–2150.
32. Chu CL, Chen SS, Wu TS, Kuo SC and Liao NS (1999) Differential effects of IL-2 and IL-15 on the death and survival of activated TCR gamma delta⁺ intestinal intraepithelial lymphocytes. *J. Immunol.* 162: 1896–1903.
33. Dobbeling U, Dummer R, Laine E, Potoczna N, Qin JZ and Burg G (1998) Interleukin-15 is an autocrine/paracrine viability factor for cutaneous T-cell lymphoma cells. *Blood* 92: 252–258.
34. Bulfone-Paus S, Bulanova E, Pohl T, Budagian V, Durkop H, Rückert R, Kunzendorf U, Paus R and Krause H (1999) Death deflected: IL-15 inhibits TNF- α -mediated apoptosis in fibroblasts by TRAF2 recruitment to the IL-15R α chain. *FASEB J.* 13: 1575–1585.
35. Perola O, Ripatti A and Pelkonen J (2000) T-lymphocyte subpopulations do not express identical combinations of interleukin-2 receptor chains in the early phase of their activation and proliferation. *Scand. J. Immunol.* 52: 123–130.
36. Chae DW, Nosaka Y, Strom TB and Maslinski W (1996) Distribution of IL-15 receptor α -chains on human peripheral blood mononuclear cells and effect of immunosuppressive drugs on receptor expression. *J. Immunol.* 157: 2813–2819.
37. Naora H and Gougeon ML (1999) Activation, survival and apoptosis of CD45RO⁺ and CD45RO[−] T cells of human immunodeficiency virus-infected individuals: effects of interleukin-15 and comparison with interleukin-2. *Immunology* 97: 181–187.
38. Carson W and Caligiuri MA (1998) Interleukin-15 as a potential regulator of the innate immune response. *Braz. J. Med. Biol. Res.* 31: 1–9.
39. Zhang X, Sun S, Hwang I, Tough DF and Sprent J (1998) Potent and selective stimulation of memory-phenotype CD8⁺ T cells in vivo by IL-15. *Immunity* 8: 591–599.
40. Kumaki S, Armitage R, Ahdieh M, Park L and Cosman D (1996) Interleukin-15 up-regulates interleukin-2 receptor α chain but downregulates its own high-affinity binding sites on human T and B cells. *Eur. J. Immunol.* 26: 1235–1239.
41. Agrawal S, Marquet J, Delfau-Larue MH, Copie-Bergman C, Jouault H, Reyes F, Bensussan A and Farcet JP (1998) CD3 hyporesponsiveness and in vitro apoptosis are features of T cells from both malignant and nonmalignant secondary lymphoid organs. *J. Clin. Invest.* 102: 1715–1723.
42. Legac E, Autran B, Merle-Beral H, Katlama C and Debré P (1992) CD4⁺CD7[−]CD57⁺ T cells: a new T-lymphocyte subset expanded during human immunodeficiency virus infection. *Blood* 79: 1746–1753.
43. Chang KH, Kim JM, Kim HY, Song YG, Choi YH, Park YS, Cho JH and Hong SK (2000) Spontaneous programmed cell death of peripheral blood mononuclear cells from HIV-infected persons is decreased with interleukin-15. *Yonsei Med. J.* 41: 112–118.
44. Inagaki-Ohara K, Nishimura H, Mitani A and Yoshikai Y (1997) Interleukin-15 preferentially promotes the growth of intestinal intraepithelial lymphocytes bearing gamma delta T cell receptor in mice. *Eur. J. Immunol.* 27: 2885–2891.
45. Tsujino S, Di Santo JP, Takaoka A, McKernan TL, Noguchi S, Taya C, Yonekawa H, Saito T, Taniguchi T and Fujii H (2000) Differential requirement of the cytoplasmic subregions of gamma c chain in T cell development and function. *Proc. Natl. Acad. Sci. USA* 97: 10514–10519.
46. Yu CR, Young HA and Ortaldo JR (1998) Characterization of cytokine differential induction of STAT complexes in primary human T and NK cells. *J. Leukoc. Biol.* 64: 245–258.
47. Nielsen M, Kaestel CG, Eriksen KW, Woetmann A, Stokkedal T, Kaltoft K, Geisler C, Ropke C and Odum N (1999) Inhibition of constitutively activated STAT3 correlates with altered Bcl-2/Bax expression and induction of apoptosis in mycosis fungoides tumor cells. *Leukemia* 13: 735–738.
48. Gombert W, Borthwick NJ, Wallace DL, Hyde H, Boffill M, Pilling D, Beverley PC, Janossy G, Salmon M and Akbar AN (1996) Fibroblasts prevent apoptosis of IL-2-deprived T cells without inducing proliferation: a selective effect on Bcl-xL expression. *Immunology* 89: 397–404.
49. Barker JN (1995) Adhesion molecules in cutaneous inflammation. *Ciba Found. Symp.* 189: 91–106.
50. Reinhold U, Liu L, Sesterhenn J, Schnautz S and Abken H (1996) The CD7⁺ T cell subset represents the majority of IL-5-secreting cells within CD4⁺CD45RA[−] T cells. *Clin. Exp. Immunol.* 106: 555–559.
51. Yagi H, Tokura Y, Matsushita K, Hanaoka K, Furukawa F and Takigawa M (1997) Wells' syndrome: a pathogenic role for circulating CD4⁺CD7[−] T cells expressing interleukin-5 mRNA. *Br. J. Dermatol.* 136: 918–923.