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A Novel Role for IL-27 in Mediating the Survival of Activated Mouse CD4 T Lymphocytes

Gisen Kim,* Ryo Shinnakasu,*1 Christiaan J. M. Saris,† Hilde Cheroutre,* and Mitchell Kronenberg*

IL-27, an IL-12 family cytokine, has pleiotropic functions in the differentiation and expansion of CD4+ T cell subsets. In this study, we discovered a novel function of IL-27. CD4+CD45RBhigh T cells from mice deficient for the α-chain of IL-27 receptor failed to induce colitis in Rag2−/− recipients, because of an inability of activated donor cells to survive. Interestingly, IL-27 was indispensable for the prevention of colitis by regulatory T cells, also because of a defect in long-term cell survival. IL-27 affected the survival of activated T lymphocytes, rather than promoting cell proliferation, by inhibiting Fas-mediated activation-induced cell death, acting through the STAT3 signaling pathway. The addition of IL-27 during activation resulted in an increased cell number, which was correlated with decreased activation of both caspases 3 and 8. This prosurvival effect was attributed to downregulation of FasL and to the induction of the antiapoptotic protein cFLIP. Although activation induced cell death is an important mechanism for the maintenance of immunological homeostasis, protection of lymphocytes from excessive cell death is essential for effective immunity. Our data indicate that IL-27 has a crucial role in the inhibition of activation-induced cell death, thereby permitting Ag-driven T cell expansion. The Journal of Immunology, 2013, 190: 1510–1518.

P rimaling T lymphocytes through the TCR and CD28 elicits a series of events that drives cell proliferation. The induction of cell death is an important mechanism for limiting the expansion of activated T lymphocytes, a result not only of passive mechanisms, such as nutrient deprivation, but also resulting from the increased expression of molecules that trigger programmed cell death. Among the molecules involved in triggering cell death, the importance of the death receptor Fas (CD95) is demonstrated by the phenotype of the lymphoproliferation spontaneous mutation mouse strain (FasS99 mutant) (1), in which accumulated, activated T lymphocytes cause autoimmune disease. Because the activation of T cells induces both Fas and its ligand FasL (2, 3), the induction of Fas-dependent activation-induced cell death (AICD) occurs concomitantly with T cell activation. Therefore, protection of activated T cells from excessive Fas-mediated AICD is crucial for sustaining T lymphocyte expansion. The cellular form of FLICE inhibitory protein (cFLIP) is a direct inhibitor of Fas-mediated signaling (4). cFLIP is recruited to the Fas death-inducing signaling complex and inhibits the activation of caspase 8, thereby preventing Fas-mediated AICD. cFLIP protein is expressed by various cell types, including naive T cells (4), and as predicted from its biochemical function, cFLIP deficient T lymphocytes are more susceptible to Fas-mediated cell death (5). Furthermore, cFLIP overexpression is a proposed mechanism of the abnormal accumulation of CD4+ T cells in IL-2−/− mice (6). Collectively, these data indicate that the amount of cFLIP is a key element regulating the population size of activated T cells.

IL-27 is a heterodimeric IL-12 family cytokine composed of p28 and EBV-induced (EBI3) chains (7). It binds to a receptor, expressed by most hematopoietic cells, composed of IL-27Rx+ (also known as TCCR or WSX-1) and gp130 chain, which is shared by many cytokines (8–10). IL-27 has been reported to influence the differentiation of several functional CD4 T cell subtypes, including the stimulation of IL-10–producing cells and Th1 cells (7, 9, 11–14), and the inhibition of Th17 cells and Th2 cells (15–17), in addition to suppressing IL-2 expression (18). In addition to these IL-27 functions in Th cell differentiation, early studies demonstrated its function as a growth factor for CD4+ T lymphocytes (7). Because of these important and diverse effects, we initiated an investigation of the role of IL-27–mediated signals in a T cell–dependent colitis model. As a result, we discovered an indispensable role for IL-27 in the expansion of activated CD4+ T lymphocytes, predominantly by inhibiting AICD, which we attribute not only to the inhibition of FasL expression, but also to the induction of cFLIP expression through the activation of STAT3 pathway. Interestingly, the anti-AICD function of IL-27 was also required for the maintenance of the regulatory T cell population. Together these data suggest that a major role of IL-27 in vivo in T lymphocytes is related to the survival of activated cells.

Materials and Methods

Mice

IL-27Rα−/− mice reported previously were provided by Amgen (Thousand Oaks, CA) (19). STAT1−/−, STAT3flox/flox, and gp130flox/flox mice were used with the permission of Drs. David Levy (New York University), Kiyoshi Takeda (Osaka University, Japan), and Kirk Knowlton (University of California–San Diego), respectively. CD4 Cre recombinase transgenic mice were purchased from Taconic. All other mouse strains were pur-
chased from the Jackson Laboratory (Bar Harbor, ME). Triple knockout IL-2−/− B7-1−/− B7-2−/− mice were bred from single and double knockouts at the La Jolla Institute for Allergy and Immunology. Animal care and experimentation were consistent with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the La Jolla Institute for Allergy and Immunology.

Abs and reagents

mAbs directed against the following target proteins were purchased from BD Biosciences: CD4, CD45RB, TGF-β, FasL, CD45.1, CD45.2, CD25 (3C7, used for blocking), FasL (MF3, used for blocking), and CD28 (37.51, used for stimulation). Anti-Foxp3, anti–IL-2 neutralizing mAb (JES6-1A12), anti CD3ε mAb (145-2C11), and biotinylated mAbs for negative selection were purchased from eBioscences. Brilliant Violet 421 streptavidin was HRP-conjugated anti-rabbit polyclonal Ab, and recombinant mouse IL-27 purchased from BioLegend. A biotinylated anti-Armenian hamster polyclonal Ab was purchased from Jackson ImmunoResearch. Alexo Fluor 488-conjugated anti-cleaved caspase 3 and rabbit IgG isotype control were purchased from Cell Signaling Technology. The FLICA caspase 8 assay kit was purchased from Immunochemistry Technologies. Anti-FLIP mAb, HRP-conjugated anti-rabbit polyclonal Ab, and recombinant mouse IL-27 were purchased from R&D Systems.

Transfer of CD4+ T cells

CD4+ cells were purified from spleens using CD4 (L3T4) microbeads (Miltenyi Biotech) according to the manufacturer’s protocol. Purified CD4+ cells were further sorted for the CD4+CD45RB+ or CD4+CD25+ subsets on a FACSVantage SE with FACS DiVa option (BD Biosciences). Groups of recipients were injected i.v. with 5–10×10^6 donor lymphocytes.

Histologic scoring for colonic inflammation in the recipients

Samples of 2–3 mm tissue, obtained from the distal, middle, and proximal portions of the colon and cecum, were processed for H&E staining. Samples were coded and scored by a pathologist blinded to the conditions under which the experiment was conducted. A previously described scoring system (maximum score = 14) was used for the tissue sections (20). Scores (maximum = 14) from three parts were averaged to represent the severity of disease.

Ex vivo FACS analyses

Large intestinal lymphocytes were isolated as described previously (21), except that we used collagenase type VIII instead of type IV. For ex vivo FasL staining, cells were stained by serial incubation with anti-FasL mAb, biotinylated anti-hamster Ab, and aliphophycinin-conjugated streptavidin. For in vitro FasL staining, cells were incubated with biotinylated anti-FasL mAb followed by detection with brilliant violet dye-conjugated streptavidin.

In vitro induction of AICD

Naive CD4+ T lymphocytes were isolated from either B7-1−/− B7-2−/− or IL-2−/− B7-1−/− B7-2−/− mouse spleens by negative selection using anti-biotin MACS microbeads (Miltenyi Biotech), after incubation with an Ab mixture containing anti-CD8a (53-6,7), anti-CD11b (M1/70), anti-CD11c (N418), anti-NK.1.1 (PK136), anti-Ter119 (TER-119), anti-CD45R (RA3-6B2), and anti-CD25 (7D4). CD4+CD25 cells isolated from B7-2−/− mice were >97% CD45RB+high, containing <0.5% CD4+CD25 cells (data not shown). For isolating naive CD4+ cells from various gene-deficient mice, CD4+CD45RB+high cells were sorted, as described above. Isolated CD4+ T cells were stimulated in a high-bind microplate (Corning no. 3361) coated with 1.0 µg/ml anti-CD3ε mAb; 0.5 µg/ml soluble anti-CD28 mAb was added for costimulation. After the culture, cells were stained with 7-aminoactinomycin D (7-AAD; BD Biosciences) and they differentiate into pathogenic T helper cells (25, 26). Therefore, this model is useful for analyzing how naive T cells go through the process of activation, expansion, differentiation and migration, leading to inflammation in the large intestine. Using IL-27Ra+− donor CD4+CD45RB+high cells in this model, we tested the function of IL-27–mediated signals in the induction of T cell–mediated colitis. As reported previously, recipients that received wild type (WT) CD4+CD45RB+high cells developed chronic colitis by 8 wk after transfer, as demonstrated by body weight loss and high histology scores (Fig. 1A, 1B). Interestingly, the recipients of IL-27Ra+− donor cells did not exhibit symptoms of colitis, including body weight loss (Fig. 1A), diarrhea, and rectal prolapse (data not shown). Histologic analyses confirmed the absence of inflammation in recipients of IL-27Ra+− CD4+CD45RB+high cells (Fig. 1B). Separate transfer experiments revealed a profound defect in the accumulation of IL-27Ra+− donor cells. Donor IL-27Ra+− T lymphocytes, gated in the FACS analysis as TCR+B+ cells, were barely detectable either in the mesenteric lymph nodes (MLNs) or the large intestine at 4 wk after transfer (Fig. 1C, 1D). These data suggest that the deficiency of IL-27 signaling in donor T cells caused a defect in the proliferation or survival of the donor T cell population, which prevented the induction of colitis.

IL-27 signaling is required for the function of regulatory T cells

To investigate whether IL-27 also affects the population size of CD4+CD25+ Foxp3+ regulatory T cells (Tregs) after transfer, we compared the disease preventive effect of IL-27Ra− WT and IL-27Ra+− Tregs in the colitis model. Consistent with the previous experience of many groups, cotransfer of CD4+CD45RB+high cells with WT Tregs at a 4:1 ratio was sufficient for preventing colitis (27). In contrast, all the recipients that received IL-27Ra− Tregs, developed colitis by 5 wk after transfer, as manifested by increased weight loss (Fig. 2A) and analysis of tissue sections (Fig. 2B). The average histology scores demonstrated severe colonic mucosal inflammation in the recipients of IL-27Ra−/− Tregs, unlike the outcome when an equal number of WT Tregs was transferred (Fig. 2C). To determine whether there was a loss of regulatory function and Foxp3 expression following transfer, as has been reported previously in other contexts (28), we cotransferred CD45.1+ congenic CD4+CD45RB+high cells with CD45.2+
FIGURE 1. IL-27Ra−/−/CD4+CD45RBhigh T cells failed to induce colitis. CD4+CD45RBhigh T cells isolated from WT or IL-27Ra−/−/ spleens were transferred to Rag1−/− mice. The analyses of the recipients at 8 wk (A, B) and 4 wk (C, D) after transfer are shown. (A) The percentage bodyweight at each time point compared with day 0. Data from eight recipients of WT T cells (filled circles) and seven recipients of IL-27Ra−/− T cells (open circles), combined from two independent experiments, are shown. (B) Histologic scores of specimens obtained from the large intestine from the same transfers as in (A). Each dot represents an average score from four different parts of the large intestine from a single recipient (maximum score = 14). Horizontal lines indicate the mean. *p < 0.001 (two-tailed Student t test). (C) Representative dot plot analyses detecting TCR-β+ cells in the MLN (top) and large intestine (bottom) from recipients of either WT (left) or IL-27Ra−/− (right) donor cells. Numbers in each plot represent the percentage of TCR-β+ side scatter low (SSClow) cells from the total cells recovered. (D) Average total cell and T lymphocyte numbers in MLNs and large intestine. The CD4+ TCR-β+ cell numbers were calculated from the total cell count multiplied by the percentage of T lymphocytes, obtained from flow cytometric analyses. Averages from four recipients each transferred with WT or IL-27Ra−/− CD4+CD45RBhigh donor cells are shown (error bars indicate SD). *p < 0.005, two-tailed Student t test.

IL-27 inhibits AICD of CD4 T lymphocytes

To address how IL-27 acts to facilitate T cell accumulation after transfer, we performed in vitro culture experiments, in which purified naïve CD4 T lymphocytes were stimulated with a plate-bound anti-CD3ε mAb in the presence of a soluble anti-CD28 mAb. As shown in Fig. 2A, the addition of recombinant IL-27 (rIL-27) significantly enhanced the recovery of viable T cells 3 d after activation. To identify a mechanism for the increased number of activated T cells in the presence of rIL-27, CFSE-labeled cells were stimulated as above and stained with the DNA-binding dye 7-AAD. This method allowed us to analyze both proliferation and survival of the activated T lymphocytes simultaneously. The degree of AICD was determined by the percentage of 7-AAD+ cells in the population of cells that underwent at least one division. As shown in Fig. 3B, although the percentage of the cells that underwent AICD was increased between days 3 and 4 of the culture and reached 70%, the addition of rIL-27 greatly reduced this percentage. In contrast, the analysis of CFSE dilution for the 7-AAD+ viable cells demonstrated a moderate suppressive effect of rIL-27 on T cell proliferation (Fig. 3B, right panel). These data indicate that although IL-27 has a suppressive effect on T cell proliferation, it is able to support T cell expansion by inhibiting AICD. Consistent with the in vivo results, IL-27 also had a prosurvival effect on Tregs in vitro. When Tregs were cocultured with naïve CD4+ T cells and activated as described above, the percentage of 7-AAD− viable Tregs was increased when IL-27 was included in the culture medium (Supplemental Fig. 1).

IL-27 inhibits Fas-mediated cell death through activation of STAT3

IL-27 has been reported to activate multiple STAT pathways (31). To determine which pathways are involved in the IL-27–mediated inhibition of AICD, we stimulated sorted CD4+CD45RBhigh T cells obtained from the indicated gene-deficient mice (Fig. 3C) in the presence or absence of IL-27, and we measured the percent that were 7-AAD−. Cells deficient for either subunit of the IL-27 receptor provided negative controls for the effect of IL-27, consistent with a specific, receptor-mediated effect of the cytokine. IL-27 absolutely required STAT3 for inhibiting AICD, as the prosurvival effect of rIL-27 was negligible when we stimulated CD4 Cre+ STAT3flox/flox (STAT3+/−) T cells. In contrast, STAT1 and STAT6 were dispensable for the prosurvival function of IL-27, although a lower percentage of 7-AAD− cells in the cultures of activated STAT1−/− cells without IL-27 suggested a role for STAT1 for the induction of AICD.

Importantly, there was decreased cell death in cultures of T cells carrying a Fastay mutation and no further decrease when rIL-27 was added (Fig. 3C), which is consistent with the hypothesis that IL-27 acts through the Fas pathway. In addition, we tested whether
FIGURE 2. IL-27 is required for the maintenance of the Treg population. (A–C) CD45.1+ WT CD4+CD45RB<sup>high</sup> cells (4 × 10<sup>5</sup>) were cojected to Rag2<sup>−/−</sup> mice with 1 × 10<sup>6</sup> CD45.2 CD4+CD25<sup>+</sup> cells sorted from WT (filled symbols) or IL-27Ra<sup>−/−</sup> (open) spleens. (A) Relative body weight compared with the original body weight at each time point, averaged from a total of seven recipients. Data shown are combined from two independent experiments. (B) Representative H&E staining of the large intestine of recipients that received CD4+CD45RB<sup>high</sup> cells and WT (top) or IL-27Ra<sup>−/−</sup> (bottom) Tregs at a 4:1 ratio. Original magnification ×100. (C) Histologic scores of large intestine. Each dot represents the average of four specimens obtained from a single recipient. Horizontal lines indicate the mean. *p < 0.001, two-tailed Student t test. (D and E) T lymphocytes were transferred as in (A). Four weeks after transfer, cells isolated from MLNs and large intestine were stained for Foxp3. A representative dot plot showing Foxp3 and CD45<br>Foxp3 and CD45.2+ Tregs. Numbers indicate the percentage of the cells in each quadrant. (F) Analysis at 3 d after transfer to Rag2<sup>−/−</sup> recipients of 8 × 10<sup>5</sup> CD45.1<sup>+</sup> CD4+CD45RB<sup>high</sup> cells with 2 × 10<sup>5</sup> WT or IL-27Ra<sup>−/−</sup> Tregs (CD45.1<sup>−</sup>). (F) Top panels represent CD4 and TCR-β<sup>+</sup> staining of total spleen cells at 3 d after transfer. Numbers in the plots indicate the percentage of CD4<sup>+</sup>TCR-β<sup>+</sup> cells gated as shown. CD4<sup>+</sup>TCR-β<sup>+</sup> splenic (middle panels) or MLN (bottom panels) cells gated as above were analyzed further for Foxp3 expression by CD45.1<sup>+</sup> or CD45.1<sup>−</sup> cells. Percentages are indicated in each quadrant. (G) Total number of spleen cells (top) or CD4<sup>+</sup> T cells (bottom) isolated from the recipients of either WT Tregs (black bar) or IL-27Ra<sup>−/−</sup> Tregs (blank bar) with WT CD45.1<sup>+</sup> naive CD4<sup>+</sup> T cells, or naive CD4<sup>+</sup> T cells only (gray bar). Each bar indicates the average of four recipients in each condition (error bars indicate SD). *p < 0.001, two-tailed Student t test. The prosurvival function of IL-27 is independent of IL-2 Autocrine or paracrine IL-2 signaling is a key factor for mediating AICD and for upregulating FasL expression by activated T lymphocytes. Because IL-27 suppresses IL-2 expression (18, 32), we hypothesized that IL-27 might downregulate FasL through inhibition of IL-2 synthesis by T cells. We tested for an IL-2–independent function of IL-27 by analyzing naive CD4 T cells from IL-2<sup>−/−</sup> mice that were also deficient for both the B7-1 and B7-2 costimulatory molecules. Deficiency for both B7 molecules prevents T cell hyperactivation in the IL-2<sup>−/−</sup> mice and therefore allows them to remain healthy. FasL induction was impaired in vitro in these activated IL-2<sup>−/−</sup> T lymphocytes compared with controls (Fig. 3E). However, the induction of AICD in these IL-2<sup>−/−</sup> cells still was reduced by rIL-27 (Fig. 3F). Furthermore, cell death when IL-2 signaling was blocked with mAbs was also Fas mediated, and the action of IL-27 in reducing it was STAT3 dependent (Supplemental Fig. 2). The data therefore indicate that although the inhibition of increased expression of FasL by IL-27 may contribute to its prosurvival effect, IL-27 has additional modes of action for preventing AICD, which are independent of IL-2 regulation.

T cell intrinsic prosurvival function of IL-27 We explored whether Fas-mediated signals leading to AICD also could be downregulated by IL-27 via a T cell intrinsic mechanism. To test whether IL-27 regulates the Fas-mediated pathway in a cell-intrinsic manner, we performed cotransfer experiments using a mixture of WT (CD45.1<sup>+</sup>) and IL-27Ra<sup>−/−</sup> (CD45.2<sup>+</sup>) donor cells. As shown in Fig. 4A (left panels), the ratios of the cotransferred populations in each organ were maintained at ∼1:1 in the spleen.
FIGURE 3. IL-27 inhibits AICD through STAT3 signaling upstream of FasL-Fas. CFSE-labeled naïve CD4+ T cells (1 × 10^4) were stimulated with plate-bound anti-CD3ε and soluble anti-CD28 mAbs in the absence or presence of 10 ng/ml rIL-27, as indicated in each panel. (A) Viable cell numbers identified with trypan blue, averaged from three culture wells at each time point. The figure represents data compiled from three independent experiments. *p < 0.01, Student t test. (B) Representative contour plots at day 3 (top) and day 4 (bottom) analyzing the proliferation, measured by CFSE dilution, and cell death, by staining with 7-AAD, of the activated T cells. Numbers in each plot represent the percentage of 7-AAD+ cells that underwent at least one division. Representative histograms (right) show the CFSE dilution of 7-AAD+ viable cells gated in the contour plots. The numbers in the histograms are the averaged proliferation indexes of activated cells in the absence (top number in both panels) and presence (bottom numbers) of rIL-27. **p < 0.001, Student t test. (C) Sorted CD4+CD45RBhigh cells from the indicated mouse spleens were stimulated as above. The percentages of 7-AAD+ cells, identified with trypan blue, averaged from three culture wells at each time point. The figure represents data compiled from three independent experiments. (D) Flow cytometric analyses showing FasL expression by WT (top) and IL-27R−/− (bottom) cells at day 2 of stimulation. Numbers represent the percentage of FasL+ cells in the absence (top) and presence (bottom) of rIL-27, averaged from three independent experiments. **p < 0.001, Student t test. (E) FasL expression by WT and IL-27−/− cells, which was determined as (D). (F) 7-AAD staining of activated IL-27−/−CD4+ T cells in the presence (solid line) and absence (shaded contour, no line) of rIL-27. Numbers represent the percentage of 7-AAD+ cells among the stimulated cells.

and MLNs by 2 d after transfer. FasL was expressed by both WT and IL-27Ra−/− cells, although to an even greater extent by WT cells in MLNs. By day 7, however, the numbers of TCR-β+ cells were significantly skewed toward the CD45.1+ WT population in the spleen and MLNs (Fig. 4A, right panels), and at 2 wk after transfer, IL-27Ra−/− donor cells were barely detectable in the MLNs and the large intestine (data not shown). The difference was due to the accumulation of FasL−/−-activated T cells only in the IL-27Ra WT (CD45.1+) compartment, constituting 50–70% of the expanded WT cells (Fig. 4B). FasL−/− cells were barely detectable in the surviving cells in the IL-27Ra−/− population, although FasL expressed by the WT donor cells should bind equally to the Fas receptor on cells from either population to initiate a death signal. Therefore, we conclude that activation of IL-27Ra−/− T cells, leading to induction of FasL expression, puts these cells at a strong survival disadvantage because of the absence of IL-27 signals. Interestingly, because activation of donor T cells in the Rag1−/− recipients mostly depends upon Ags originating from the intestinal microflora, T cells of either genotype that migrated to the nondondraining peripheral lymph nodes (PLNs) did not express FasL (Fig. 4B), consistent with the notion that FasL expression reflected the Ag-dependent activation of donor T cells. Furthermore, at least up to day 7, the ratio between the IL-27Ra−/− and IL-27Ra WT populations did not deviate far from 1:1 in the PLNs, where T cell activation was not sufficient for upregulating FasL (Fig. 4B). These data suggest that IL-27–mediated signals are involved in a cell-intrinsic fashion in the inhibition of AICD, but not in the maintenance of a steady-state T cell population.

To prove that the IL-27–mediated survival of activated T cells is not a phenomenon confined to immune deficient recipients, we performed similar experiments monitoring Ag-specific OTII TCR transgenic T cells in immune-competent mice. OVA-specific IL-27Ra WT (CD45.1+CD45.2+) and IL-27Ra−/− (CD45.1−CD45.2+) OTII T cells were cotransferred to WT recipients (CD45.1+CD45.2−). OVA protein was injected together with donor cells, and 1 wk after transfer, the ratio of the cotransferred donor cell populations was skewed toward CD45.1+ IL-27Ra WT donor cells in the spleen. Following a reinjection of OVA Ag, the IL-27Ra−/− cells were even more greatly outnumbered by the WT donor cells (Fig. 4C), because of a failure of the IL-27Ra−/− cells to expand (data not shown). Interestingly, the injection of LPS at the same time with donor cells partially supported the expansion of IL-27Ra−/− cells (Fig. 4D), which suggests that IL-27 function might be compensated for by other molecules that are induced by a strong activation of the innate immune system. In the absence of OVA injection, the deficiency in IL-27 signaling did not cause a difference in the maintenance of the IL-27Ra−/− OTII T cells.
FIGURE 4. IL-27 mediated enhancement of CD4 T cell survival involves a cell intrinsic mechanism. (A-C) WT (CD45.1+) and IL-27RaΔ/Δ (CD45.1−) cells (5 × 10^5 each) were cojected i.v. to Rag1−/− mice. Cells were harvested from spleen, MLN, and PLN at days 2, 7, and 14. (A) Representative contour plots of CD4+TCR− cells stained for FasL and CD45.1. Numbers represent the percentage of the cells in each quadrant. Data are representative of one of two independent experiments with three recipients each time. (B) Skewed expansion of the donor cell populations by 7 d after transfer. Bars represent the total (100%) of CD45.1+ WT (above 0) and CD45.1− IL-27RaΔ/Δ (below 0) cells averaged from three recipients. The percentage of FasL+ cells in each cell population is shown as the shaded portion of the bars. (C, D) An equal number of sorted, naive CD45.1+CD45.2+ WT and CD45.1−CD45.2− IL-27RaΔ/Δ OTII T cells (5 × 10^5 each) were cojected to CD45.1+CD45.2− recipients in the absence and presence of OVA as indicated. (C) Bars represent the ratios of WT and IL-27RaΔ/Δ cells in splenic CD4+CD45.2+ donor T cells analyzed 1 wk (left and middle) and 2 wk (right) post transfer (error bars indicate SD). Mice analyzed 2 wk after transfer received an additional i.p. OVA injection at day 7. Numbers represent the percentage of WT cells in the CD4+CD45.2− donor population. (D) Histograms represent CD45.1 staining of CD45.2+ TCR− donor cells. Some recipients (right panel) received LPS at the same time as the donor cells. Numbers represent the percentage of CD45.1+ (WT) and CD45.1− (IL-27RaΔ/Δ) cells, averaged from three mice.

IL-27 inhibits Fas-downstream signaling mediated through caspases

Interestingly, the addition of anti-FasL Ab on day 2 of an in vitro activation culture still inhibited AICD as effectively as the addition of IL-27 (Fig. 5A), indicating that Fas-mediated signaling is still required at this time. To demonstrate which aspects of the cell death process downstream of Fas are inhibited by IL-27, we first analyzed the activation of caspase 3, the general executioner caspase for programmed cell death. Both death receptor–mediated and the mitochondrial apoptotic pathways lead to activation of caspase 3 by proteolytic cleavage (33). Intracellular staining experiments demonstrated an increased amount of the activated form of caspase 3 in CD4 T cells in the absence of IL-27 (Fig. 5B). This increase was completely inhibited by the addition of an anti-FasL mAb. As we expected from the previous results, rIL-27 dramatically inhibited the activation of caspase 3, providing direct evidence for IL-27–mediated inhibition of caspase-mediated apoptotic cell death. We further analyzed the activation of caspase 8, an initiator caspase for Fas-mediated cell death, by FACS analyses using a cell-permeable compound that specifically binds to activated caspase 8. As shown in Fig. 5C, CD4 T cells that were activated in vitro for 3 d exhibited an increased percentage of activated caspase 8+ cells, but this increase was significantly inhibited by the addition of either rIL-27 or anti-FasL blocking mAb. These data clearly indicate that IL-27 inhibits the Fas-mediated signaling cascade for inducing AICD at or before the activation of caspase 8.

IL-27 induces cFLIP expression through the activation of STAT3

Upon ligation by FasL, the Fas receptor forms a death-inducing signaling complex with an adaptor molecule, Fas-associated protein with death domain, which recruits the inactivated form of caspase 8 (procaspase 8 or FLICE) (34). cFLIP is a homolog of caspase 8 that inhibits caspase 8 activation by competing for the binding by procaspase 8 to Fas-associated protein with death domain (35). Based upon the data demonstrating suppressed activation of caspase 8 in the presence of rIL-27, despite a comparable expression of Fas receptor in the stimulated T cells, we analyzed the expression of cFLIP. Consistent with previous reports (3), cFLIP mRNA was expressed by CD4 T cells before activation and was quickly downregulated (data not shown). Interestingly, by day 3 when FAS-mediated AICD was occurring, the addition of rIL-27 significantly restored the expression of cFLIP protein, which was detectable by Western blot as two forms: cFLIPL (58 kDa) and cFLIPS (30 kDa; Fig. 6A). The amount of cFLIP protein at day 3 in the presence of IL-27 was even higher than the amount before activation. To determine whether the enhanced induction of cFLIP by IL-27 requires STAT3, we activated rIL-27−/− naive CD4+CD45RBhigh cells in the presence or absence of rIL-27. As shown in Fig. 6B, the addition of rIL-27 did not restore the cFLIP protein level by 72 h of activation in STAT3−/− T cells.

It has been reported that IL-2 has a role in the degradation of cFLIP protein (6); therefore, we analyzed the relationship between the expression of cFLIP and IL-2 in activated IL-2−/− CD4 T cells. In the absence of IL-2, the level of cFLIP protein at day 3 in activated IL-2−/− CD4 T cells was only slightly higher than those in WT cells (Fig. 6B). However, the presence of rIL-27 caused a significantly higher level of cFLIP protein at day 3 of the culture of IL-2−/− T lymphocytes. Finally, to prove that the lack of cFLIP induction by IL-27 was the major cause of atten-
CD4+ T lymphocytes. This function was demonstrated mostly in differentiation, IL-27 has been reported as a growth factor for differentiation (7, 9, 11–16). Besides the functions influencing T helper rIL-27. (mulation and hyperactivation of CD4+ T lymphocytes (36). mouse strain that overexpresses IL-27R

Discussion

IL-27 is a unique IL-12 family cytokine, in part because naive CD4 T cells constitutively express IL-27R, and because downstream of the receptor multiple STAT transcription factors are activated. Perhaps because of its expression pattern, or its ability to activate multiple signaling pathways, IL-27 has been reported to influence several types of CD4 T cell responses, including enhancement of IFN-γ and IL-10 production and inhibition of Th17 cell differentiation (7, 9, 11–16). Besides the functions influencing T helper differentiation, IL-27 has been reported as a growth factor for CD4+ T lymphocytes. This function was demonstrated mostly in two different settings. Pflanz et al. (7) showed that IL-27 promotes naive CD4+ T lymphocyte proliferation when cells were stimulated in the absence of IL-2. Another study demonstrated that a mouse strain that overexpresses IL-27R-α-chain exhibited accumulation and hyperactivation of CD4+ T lymphocytes (36). However, neither study determined whether the expansion of the CD4+ population was due to enhanced proliferation or survival. In this study, we have reported a novel function of IL-27 in promoting the survival of activated CD4 T lymphocytes while it moderately suppressed proliferation. We observed an effect of IL-27 on activated CD4 T lymphocyte survival using in vitro cultures, and in vivo using a colitis model initiated by T cell transfer into lymphopenic mice, in which IL-27Rα−/− CD4+ CD45RBhigh T cells were unable to cause disease. IL-27Rα−/− CD4 T cells also were at a disadvantage following immunization of immune competent mice. Our experiments clearly demonstrated the importance of IL-27 for the expansion of Foxp3+ Treg. Although at early times after transfer IL-27Ra−/− Treg functioned in vivo to inhibit effector cell expansion, the Treg population was not maintained in the absence of IL-27–mediated signals. Because the efficiency of Treg-mediated suppression of bystander T cell expansion and effector cell differentiation are highly dependent on the frequency of Tregs in the microenvironment, recipients of IL-27Ra−/− Tregs were not protected from colitis induction in the weeks after cell transfer.

While this article was in preparation, another group also reported that IL-27Ra−/− CD4+ CD45RBhigh T cells were deficient in their ability to induce colitis and effectors accumulated to a reduced extent (37). In this report, Cox et al. (37) concluded that the failure of colitis induction was attributed to the increased percentage of Foxp3+ cells converted from the naive donor cell compartment, consistent with studies indicating that IL-27 signals inhibit expression of Foxp3. Although they also demonstrated significantly less expansion of IL-27Ra−/− donor cells in the large intestine, we found a more profound effect of IL-27 on cell survival, such that donor cells were barely detectable, especially in the large intestine and regardless of Foxp3 expression. We further demonstrated that the IL-27–mediated survival effect also was required for Foxp3+ Treg expansion, consistent with other reports indicating that activated Tregs can be subject to Fas-mediated AICD (38, 39). In contrast, surprisingly Cox et al. (37) found that IL-27Ra−/− Tregs were unimpaired after transfer to Rag−/− mice, for regulatory function and in terms of expansion in the hosts. The reason for this discrepancy is uncertain, but a strain difference could be one important factor, as our experiments were carried out in C57BL/6 mice, whereas the earlier studies were performed with BALB/c animals. Differences in the microflora in different animal colonies could be another factor, and these could relate to the production of IL-27 or other stimulatory cytokines by the innate immune system.

Our experiments show that the mechanism of IL-27 action depends to a large degree on the inhibition of Fas-mediated cell death. It has been well documented that the Fas-mediated induction of cell death is essential for the maintenance of immune homeostasis. We considered it possible that the prosurvival effect of IL-27 is due primarily to the inhibition of IL-2 synthesis, causing reduced FasL expression after activation. However, even the minimal induction of FasL in the absence of IL-2 was sufficient for Fas-dependent AICD, which could be completely inhibited by IL-27. These data therefore indicate a broader role for IL-27.
in promoting the survival of activated CD4 T lymphocytes, in addition to the inhibition of IL-2 synthesis. Our in vivo cotransfer experiments clearly indicated a T cell-intrinsic mechanism whereby IL-27 inhibits the Fas-mediated signaling pathway. In these cotransfers, we could not confirm a higher expression of Fasl by IL-27Ra−/− donor cells, perhaps because of the rapid induction of AICD leading to the selective depletion of Fasl+ IL-27Ra−/−-activated T cells. In this study, the accumulation of WT CD4+ T cells that expressed increased amounts of Fasl, particularly at the sites of T cell activation, indicated that the WT but not IL-27Ra−/− cells were protected from AICD, regardless of the expression level of Fasl or the mode of Fas ligation, in cis or in trans. This finding led us to explore alterations in the Fas signaling pathway dependent on IL-27 that could explain the cell-intrinsic defect in the accumulation of IL-27Ra−/− T cells.

In accordance with an AICD process inhibited by IL-27 in a T cell intrinsic manner, we found that IL-27 enhanced the expression of cFLIP protein, an inhibitor of death-receptor mediated AICD, acting at late times (>2 d) after activation. Consistent with this finding, the activation of both caspase 8 and caspase 3 was attenuated by the addition of IL-27. In agreement with an important biologic effect of IL-27 in augmenting cFLIP synthesis, even in the absence of IL-2-dependent cFLIP degradation, IL-27 still increased cFLIP protein, which occurred relatively late, at 72 h, consistent with the Fasl-blocking Ab results. Moreover, consistent with the findings on AICD, the effect of IL-27 on cFLIP protein was STAT3 dependent and independent of its effects on IL-2 synthesis.

In the earlier studies that explored the multivalent functions of IL-27, the survival promoting effect of IL-27 may have been missed because of the antiapoptotic functions of several other STAT activating cytokines. For example, IL-6, which shares the gp130 chain with the IL-27R complex, prevents the apoptotic death of hepatocytes induced by Fas ligation (40, 41). Furthermore, IL-12 inhibits Fas-mediated cell death in CD8+ T cells (42). In our in vivo models in the absence of strong stimulation of innate immunity, however, the selective loss of IL-27Ra−/− T cells was dramatic, despite the intact functions of other cytokine and cytokine receptor genes. This unique importance of IL-27 could be related to the constitutive expression of the IL-27R by naive T cells (7), such as in contrast to the IL-12R, which is induced during T cell differentiation. Compared with IL-6 that is induced during inflammation, IL-27 might be efficiently induced by T-DC contact through the ligation of CD40 (7). Therefore, the rapid coexpression of both IL-27 and IL-27R could be a key for the nonredundant function of IL-27 for preventing AICD in our models. In fact, the lack of Ag-induced expansion of transferred IL-27Ra−/− OTII T cells was greatly restored by the injection of LPS at the same time with OVA. This suggests that in infections providing strong activation of the innate immune system, the induction of cytokines such as IL-6 or IL-12 might provide a redundant function for IL-27-mediated survival of Ag-stimulated T lymphocytes. Correspondingly, IL-27 function for the expansion of T lymphocytes might be more critical when T cells are activated with a less vigorous stimulation of the innate immune system.

The critical role for IL-27-mediated signals for the survival of Ag-activated CD4 T lymphocytes, while having a minimal or no effect on the homeostasis of bulk CD4 T cell populations, suggests that this pathway could provide important targets for the manipulation of the immune response. For example, in the context of
chronic autoimmunity, blockade of IL-27–mediated signals could be beneficial, whereas augmenting IL-27 or IL-27R signals could enhance the potency of vaccines.

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Disclosures
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