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Autocrine TNF Is Critical for the Survival of Human Dendritic Cells by Regulating BAK, BCL-2, and FLIP<sub>L</sub>

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The life span of dendritic cells (DCs) is determined by the balance of pro- and antiapoptotic proteins. In this study, we report that serum-free cultured human monocyte-derived DCs after TLR stimulation with polynosinic acid-polyuridylic acid or LPS underwent apoptosis, which was correlated with low TNF production. Apoptosis was prevented by the addition of exogenous TNF or by concomitant stimulation with R-848, which strongly amplified endogenous TNF production. Neutralization of TNF confirmed that DC survival was mediated by autocrine TNF induced either by stimulation with R-848 or by ligation of CD40. DCs stimulated by polynosinic acid-polyuridylic acid or IFN-β, another known inducer of DC apoptosis, were characterized by high levels and activation of the proapoptotic protein BAK. The ratio of antiapoptotic BCL-2 to BAK correlated best with the survival of activated DCs. Addition of TNF increased this ratio but had little effect on BAX and XIAP. Knockdown experiments using small interfering RNAs confirmed that the survival of activated and also of immature DCs was regulated by TNF and showed that TNF was protective only in the presence of FLIP<sub>L</sub>. Together, our data demonstrate that the survival of DCs during differentiation and activation depends on autocrine TNF and that the inhibition of BAK plays an important role in this process. *The Journal of Immunology, 2012, 188: 000–000.

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with either K63-linked or linear ubiquitin chains responsible for IKK complex recruitment and activation (19–21). At a later time point (>2 h) after stimulation of TNFR1, caspase-8 and FADD are recruited into complex II in which caspase-8 activation is blocked by presumably NF-kB–induced FLIP, providing one potential explanation for NF-kB’s ability to block cell death induced by TNF (17, 22).

In this study, we provide evidence that the levels of autocrine TNF secretion determine DC survival throughout differentiation and in particular after activation by TLR agonists and that the TNF secretion determines DC survival throughout differentiation and development.

**Materials and Methods**

**Culture of DCs**

Endotoxin-free isolation of monocytes from the blood of healthy donors (commissioned by the local ethic committees) and differentiation of DCs in serum-free AIM-V medium (Invitrogen) supplemented with IL-4 and GM-CSF was performed as previously described (23). The cells were cultured at a density of 0.5 × 10^6/ml2 cm^-2, and half of the medium was exchanged every 2 or 3 d. Immature DCs were stimulated on day 5 either by 1000 U/ml human IFN-β (Strathmann Biotec), 1 μg/ml LPS (Escherichia coli 055:B5; Sigma), 20 μg/ml endotoxin-free polyinosinic acid-polyribocytidylic acid [poly(I:C)] (Axxora), 10 μg/ml R-848 (Axxora), or a cytokine mixture containing 2000 U/ml recombinant human TNF (Bender Med Systems), 1 μg/ml PGE2 (Sigma), 10 ng/ml IL-1β (R&D Systems), and 1000 U/ml IL-6 (Strathmann Biotec) as indicated. In some experiments, immature DCs were stimulated with cells of a murine fibroblast cell line with or without stable expression of human CD40L (kindly provided by R.A. Kroczek, Robert-Koch Institute, Berlin, Germany). Where indicated, TNF was neutralized by 100 μg/ml soluble chimeric TNF receptor 2–IgG1–Fc protein (Enbrelcept, Wyeth) and/or 100 μg/ml of an anti-TNF Ab (R&D Systems), and propidium iodide according to the manufacturer's protocol. For determination of cell yields, adherent cells were detached by addition of 0.25% trypsin-EDTA (Invitrogen) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and in particular after activation by TLR agonists and that the TNF secretion determines DC survival throughout differentiation.

**Small interfering RNA transfection of DCs by electroporation**

Small interfering RNAs (siRNAs) specific for BAK, BCL-2, and FLIP, were obtained from Thermo Scientific (‘‘ON-TARGETplus SMARTpool’’ from Dharmacon), and siRNA targeting EGFP (target sequence GAAGCUCCAGAGUUGCUUCAU; Sigma) was used as a control. Electroporation was performed as previously described (24). Briefly, <6 × 10^6 cells were electroporated (square wave, 500 V, 1 ms) in 4-mm cuvettes with a GenePulser Xcell (Bio-Rad) in 100 μl Dulbecco’s modified Eagle’s medium (DMEM) containing 1% fetal bovine serum with or without BAK, BCL-2, and FLIP antagonists. Where indicated, poly(I:C) was added 5 h after the electroporation procedure on day 5.

**Flow cytometric analysis**

For immunophenotypic analysis of the DCs, the following mAbs were used: PE-labeled anti-CD80 (clone MAB104; Immunotech), alphacyclopin, labeled anti-CD83 (clone HB15e; BD Biosciences), FITC-labeled anti-CD86 (clone 233I; BD Biosciences), anti-CD14–alphacyclopin (clone RMO52; Beckman Coulter), and IgG1 (clone DAK-GO1; Dako) labeled with FITC, PE, or alphacyclopin. Flow cytometric detection of mitochondrial cytochrome c was performed using a kit from Calbiochem according to the manufacturer’s instructions. Activated BAK was detected in DCs fixed with formaldehyde and permeabilized by saponine using the mAb clone 3C52 (Ab-1; Calbiochem) recognizing the N-terminal peptide epitope aa 1–52, which is accessible under nondenaturing conditions only in active/activated BAK (25). A PE-conjugated anti-mouse F(ab’’)_2 (Dako) was used as secondary Ab. Quantification of apoptosis was performed by staining the cells with annexin V–FITC (Bender Med Systems) and propidium iodide according to the manufacturer’s protocol. For determination of cell yields, adherent cells were detached by addition of EDTA (5 mM final concentration, 15–20 min at 37°C). After addition of propidium iodide, viable cells were counted on a FACSCalibur flow cytometer (BD Biosciences) by using Counting Beads (Caltag). Data analysis was performed with CellQuest software (BD Biosciences).

**Statistical analysis**

Statistical significance was estimated with the paired two-tailed Student t test.

**Results**

**Poly(I:C) or LPS, but not R-848, induces cell death in human monocyte-derived DCs and is associated with low TNF production**

We have previously observed enhanced survival of serum-free cultured human monocyte-derived dendritic cells (Mo-DCs) after maturation with R-848, which triggers TLR8, when compared with stimulation with poly(I:C) (mimic for dsRNA, TLR3 ligand) or LPS (TLR4 ligand) (23). In the current work, we studied cell survival after the combined stimulation of poly(I:C) plus R-848 or LPS plus R-848 and found that the addition of R-848 rescued Mo-DCs from poly(I:C)- or LPS-induced cell death (Fig. 1A). The same response pattern was also seen when cord blood-derived monocytes were used for the DC culture (Fig. 1B). In congruence with our previously reported study (23), all TLR stimulation conditions yielded mature DCs characterized by the upregulated expression of CD83, CD80, and CD86 and the absence of CD14 (data not shown).

Cell death after stimulation with poly(I:C) or LPS has been proposed to be the result of apoptosis (12). Accordingly, we found phosphatidylserine exposure, detectable by annexin V staining and a decrease in the forward scatter profile of the DC population after stimulation with poly(I:C). Addition of R-848 dramatically protected against poly(I:C)-induced cell death (Fig. 1C). Cytokine quantification in the supernatants revealed that the DCs secreted high levels of TNF in response to R-848. Also the combination of R-848 with poly(I:C) or LPS resulted in enhanced secretion of TNF, and particularly of IL-12 (Fig. 1D). In contrast, only low levels of cytokines were produced after poly(I:C) or LPS stimu-
lation alone, in agreement with earlier reported data (28). These data indicated that protection of DCs by R-848 correlated with the amount of their TNF secretion.

DC survival after TLR activation depends on autocrine TNF

We hypothesized that autocrine TNF secretion might counteract cell-intrinsic or proapoptotic autocrine signals after microbial stimulation. To test this hypothesis directly, we added exogenous TNF to LPS- or poly(I:C)-stimulated DCs or, conversely, neutralized endogenously produced TNF during the maturation step and subsequently measured the cell yield 5 d later (Fig. 2A). These experiments indeed confirmed a protective effect of exogenous TNF on the survival of poly(I:C)- or LPS-activated DCs. The saturating dose of this effect was found to be above 20 ng/ml (data not shown). In line with these data, a reduced cell yield was found in R-848-treated DC cultures when TNF was neutralized by an anti-TNF Ab (infliximab) and a soluble TNF receptor (100 μg/ml; Etanercept) (Fig. 2A). Stimulation with IFN-β was included in these experiments, as it was suggested that it contributes to poly(I:C)- or LPS-induced apoptosis in Mo-DCs (10, 11). Indeed, IFN-β induced massive cell death also in our experiments, and this proapoptotic effect was in part antagonized by exogenously added TNF (Fig. 2A). Fig. 2A also illustrates that the cytoprotective effects of TLR8 activation on poly(I:C)- or LPS-triggered apoptosis was attributed to TLR8-induced autocrine TNF secretion because neutralization of TNF reversed this effect. In line
The molecular basis of the observed TNF antagonism of cell death induced by microbial stimulation was studied in more detail with poly(I:C)-stimulated DCs. In these cells, a high rate of apoptosis is associated with low TNF production. For this purpose, we performed Western blot analysis of prototypic pro- and antiapoptotic proteins of the extrinsic and intrinsic apoptosis pathways, respectively. A typical result of these experiments is shown in Fig. 3A. To compensate for experimental and donor variations, we performed repeated experiments with four different donors, which, after densitometric analysis, allowed the calculation of mean values of relative protein expression (normalized to a housekeeping protein and relative to the expression level averaged from all experimental conditions, as detailed in Materials and Methods). Together, these mean values give an indication of upregulation and downregulation of the various proteins in response to different stimuli.

Fig. 3B shows that procaspase-8 was upregulated in response to poly(I:C) or IFN-β but appears to be not influenced by TNF treatment. In contrast, and in accordance with published data (31, 32), FLIP_L was induced by TNF and also after TLR stimulation. BAK was found in immature DCs at varying but frequently high levels. Its expression in DCs was elevated by poly(I:C) and also IFN-β but strongly reduced by R-848. In contrast, another major proapoptotic BCL-2 family protein, BAX, displayed only little regulation by the tested stimuli. In particular, there was no effect of TNF on BAX expression. The antiapoptotic counterpart BCL-2, however, in accordance with the literature, was clearly upregulated by TNF and also by TLR ligands. Notably, BCL-2 was also induced by poly(I:C), but then not further influenced by added TNF.

Finally, calculation of the relative ratios of the expression levels of antiapoptotic to proapoptotic proteins of the extrinsic and the intrinsic pathways, respectively, namely FLIP_L/caspase-8, BCL-2/BAX, and BCL-2/BAK, showed that the concerted regulation of BCL-2 and BAK by TNF indeed most closely paralleled the cell yields in the corresponding cultures (Figs. 2A, 3C). The obvious discrepancy with immature DCs (low BCL-2/BAK ratio together with good survival; Figs. 1A, 3C) might be explained by the absence of any proapoptotic stimulus triggering the activation of BAK in these cells, as indeed confirmed later in the experiment shown in Fig. 4A (see below).

The experiments shown in Figs. 1 and 2 had revealed that TNF was a survival factor for DCs activated by different TLR ligands or IFN-β. Together, the data shown in Fig. 3B now suggested that TNF might act by inhibiting both the extrinsic as well as the intrinsic apoptosis pathway through the regulation of FLIP_L, BCL-2, and BAK. Although further proteins such as BAX, XIAP, death receptors, and apical caspas might also contribute to the regulation of DC survival, none of them appeared to be markedly affected by TNF (Fig. 3B, Supplemental Fig. 1, and data not shown). Taking a closer look, our data indicate that the prosurvival effect of TNF under conditions of poly(I:C) or R-848 activation did not correlate with the expression levels of FLIP_L and/or procaspase-8. Therefore, we considered the intrinsic pathway a central target of TNF acting by either directly lowering BAK expression or by

with this model, we found that induction of cell death through poly(I:C) progressed slowly and that exogenous TNF was almost fully protective when added up to 2 d after poly(I:C) stimulation (Fig. 2B).

The ligation of CD40 has been reported to rescue DCs from CD95-induced apoptosis (29, 30). Although CD40L could not antagonize TLR-induced death of murine DCs (2), we hypothesized that this could be a species-specific effect and thus tested human DCs. Indeed, the presence of CD40L-expressing fibroblasts during the stimulation of DCs with poly(I:C) or LPS improved cell survival (Fig. 2C). This effect was mediated by enhanced autocrine TNF production based on the major impact of neutralization of TNF.

The ratio of BAK to BCL-2 and the fraction of DCs with activated BAK correlates with apoptosis and is regulated by TNF

FIGURE 2. DC survival after microbial or CD40L stimulation depends on autocrine TNF. (A) The diagram shows the cell yield on day 10 of culture (i.e., 5 d after stimulation of the immature DCs by the indicated stimuli). Shown are the effects of either exogenous 50 ng/ml (2000 U/ml) TNF or 100 μg/ml of a monoclonal neutralizing anti-TNF Ab plus a soluble TNF receptor (100 μg/ml; Etanercept), which were added to the immature DCs together with the indicated stimuli (mean ± SD; n = 4). An anti-CD20 Ab (100 μg/ml; MabThera) served as isotypic control Ab (isotype ctrl). The cell numbers are given as percentage values relative to the numbers obtained with the culture in which TNF was added either concomitantly or 1, 2, 3, or 4 d later (one representative experiment). The cell numbers on day 11 of culture are given as percentage relative to the cell numbers obtained with the culture in which TNF was added concomitantly with poly(I:C). (C) Shown are the cell yields on day 11, that is, 6 d after stimulation of the immature DCs by poly(I:C) or LPS. TNF (50 ng/ml), a monoclonal neutralizing anti-TNF Ab (100 μg/ml; infliximab), or 100,000 fibroblasts with or without stable expression of human CD40L (“CD40L-cells” or “CTRL-cells”) were added as indicated. The cell numbers are given as percentage values (mean ± SD; n = 3) relative to the cell numbers after activation with the cytokine mixture, which was set to 100%. *p < 0.05, **p < 0.01, n.s. (p > 0.05).
inhibiting BAK activation, for example by inducing BCL-2. This was based on our speculation that BAK could well mediate proapoptotic signals triggered by poly(I:C) or IFN-β. To this end, we analyzed the activation of BAK on a single-cell level by intracellular staining with a conformation-specific anti-BAK Ab. Fig. 3D demonstrates that poly(I:C) indeed triggered BAK activation in a considerable proportion of cells (likely corresponding to the fraction of cells slowly entering apoptosis at the snapshot moment of the analysis). As hypothesized, the addition of TNF substantially reduced the fraction of poly(I:C)-treated DCs containing activated BAK (Fig. 3D), complementing the data of tendentially lowered total BAK levels in the bulk cultures (Fig. 3B). Only a small fraction of cells with activated BAK was seen after R-848 stimulation (Fig. 3D), again corresponding to the extent of cell survival and low/absent levels of BAK in bulk cultures (Figs. 1A, 3B).

BAK is a central mediator of TLR-induced DC apoptosis

To test directly the hypothesis that BAK plays a central role in poly(I:C)-induced cell death, immature DCs were electroporated

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**FIGURE 3.** TNF regulates pro- and antiapoptotic proteins. (A) Immature DCs were stimulated as indicated in presence or absence of either TNF (50 ng/ml) or a monoclonal neutralizing anti-TNF Ab (100 μg/ml; infliximab) with or without a soluble TNF-receptor (100 μg/ml; Etanercept). The expression of procaspase-8 p55/p53, FLIP_L, BCL-2, BAK, and actin was determined by Western blot analysis 48 h later. (B) The diagrams illustrate the results of the densitometric analysis of the Western blots. Shown are the mean values of relative expression levels ± SD. The data were obtained from repeated experiments (as indicated) using cells from four different donors. (C) These diagrams show the relative ratios of expression levels of antiapoptotic to proapoptotic proteins. The ratios of the individual relative expression levels are displayed after normalization to the average ratio of all 10 conditions. The normalized individual ratios were then related to this mean ratio (set to 100%), which thus allowed pooling of the data and calculation of mean values shown in the diagrams. (D) The dot plots illustrate the expression of activated BAK analyzed by flow cytometry 2 d after activation of the immature DCs (one of three independent experiments). The percentages of cells containing activated BAK are displayed.
Apoptosis in DCs is mediated by BAK and inhibited by BCL-2 and FLIP L. DCs were transfected with 2 μM siRNA as indicated by electroporation on day 4 and day 5. (A) siRNA-transfected immature DCs were then stimulated on day 5 with 2 μg/ml poly(I:C) or medium, and expression of activated BAK was determined by flow cytometry on day 7. (B) Cell viability was determined on day 8 by flow cytometric analysis of the expression of cytochrome c contained within intact mitochondria. The numbers in the dot plots illustrate the percentage values of the gated cytochrome c+ cells. (C) Shown is the Western blot analysis of BCL-2 and FLIP L expression 2 d after stimulation of siRNA-transfected immature DCs with 2 μg/ml poly(I:C) plus 50 ng/ml TNF. GAPDH was used as a loading control in these experiments. (D) Cell viability was determined on day 8 by flow cytometric analysis of the cytochrome c expression as described in (B).

with BAK-siRNA before TLR stimulation. BAK knockdown was confirmed by flow cytometry 2 d after activation with poly(I:C) (Fig. 4A). BAK-siRNA transfected cells showed only low levels of activated BAK after poly(I:C) stimulation compared with those of DCs transfected with a control siRNA. Moreover, BAK-siRNA mitigated poly(I:C)-induced apoptosis and increased the proportion of non-apoptotic DCs (as defined by high mitochondrial cytochrome c content) (Fig. 4B). Furthermore, BAK-siRNA reduced cytochrome c release to some extent also in nonactivated (immature) DCs, which were characterized by a small but detectable fraction of cells with activated BAK (Fig. 4A; electroporation with control siRNA). The prosurvival effect of BAK-siRNA on nonactivated DCs is particularly obvious with donor B, a cell donor with a relatively high rate of spontaneous cell death in cultures without poly(I:C) (survival data not shown). As a consequence of our hypothesis, we expected that knockdown of BCL-2 would abolish the improved survival of DCs stimulated by poly(I:C) plus TNF. Indeed, BCL-2 knockdown (confirmed by Western blot analysis; Fig. 4C) resulted in decreased DC survival (Fig. 4D).

When we tested the effect of FLIP knockdown, we observed a strongly reduced viability of DCs 2 d after activation with poly(I:C) plus TNF (Fig. 4D). The observation, however, does not mean that TNF is protective in DCs stimulated by poly(I:C) or R-848 by upregulating FLIP L, which is not the case (shown in Fig. 3B). Rather, the results of these knockdown experiments confirm that TNF can be protective only in the presence of FLIP. We furthermore suggest that low levels of FLIP after siRNA knockdown allow rapid TNF-mediated apoptosis in DCs, explaining the pronounced loss of cell viability. Contrary, cell death purely mediated by intrinsic apoptosis pathways is slower and thus will result in only moderate effects as detected in our knockdown experiments.

Together, the data support our hypothesis that poly(I:C) triggers apoptosis in DCs via BAK and that TNF mediates its protective function by a concerted regulation of the ratio of BAK and BCL-2 expression levels. Furthermore, our data show that at the same time, high FLIP levels are an essential prerequisite for the protective function of TNF.

Autocrine TNF controls DC survival during differentiation from monocytes

In the context of manufacturing clinical-grade DCs under serum-containing culture conditions, we had independently observed low DC numbers recovering from GM-CSF– and IL-4–supplemented monocyte cultures with cells from certain blood donors. This was associated with particularly low concentration (<50 pg/ml) of endogenously produced TNF. Similarly, neutralization of TNF during culture by etanercept reduced the DC yield in experiments with monocytes from other donors (data not shown). Fig. 5 demonstrates that the addition of already only low amounts of TNF during the differentiation phase now under serum-free conditions strongly enhanced the final yield of DCs generated from monocytes with very low autocrine TNF production. This illustrates the importance of TNF for the survival of DCs during the differentiation step from monocytes.

Discussion

This study was prompted by our initial observation that the combined stimulation of serum-free cultured DCs with the TLR8 ligand R-848 and with poly(I:C) or LPS not only increased the cytokine production, as reported before (33), but also enhanced the survival of DCs (Fig. 1A, 1B). Further experiments revealed
a remarkably low capacity of these DCs to produce TNF in response to those stimuli [poly(I:C) and LPS], which also induced apoptosis (Fig. 1D). On the basis of this finding, we hypothesized that the survival of DCs activated via TLRs or other receptors could critically be determined by the levels of autocrine TNF. Our study not only confirmed this hypothesis but also identified the BCL-2/BAK ratio as a predictor of DC survival (Fig. 3C) and BAK as an important mediator of DC apoptosis, which is induced and activated by TLR ligands and inhibited by TNF (Figs. 3B, 3D, 4B). Finally, we show that BAK may also regulate the survival of immature DCs (Fig. 4B) and that the addition of low amounts of TNF can be used to improve the DC yield in clinical settings (Fig. 5).

Many published DC culture protocols still rely on the presence of either bovine or human serum, which have the potential to influence either DC phenotype or DC function. With the aim to standardize DC culture under the aspect of future clinical use, we had previously studied serum-free conditions for the differentiation of these cells and had found that serum-free cultured DCs after stimulation by various TLR agonists acquired a mature phenotype, the capacity to migrate toward CCL19, T cell stimulatory capacity, and the capacity to produce IL-12, IL-23, and TNF. However, the amounts of secreted cytokines were reduced compared with those of serum-containing parallel cultures (23, 34). The latter proved to be important in the current context because it enabled us to see the link between differentially induced amounts of TNF in response to various TLR ligands and the regulation of cell survival. Also, these conditions allowed us to study in more detail the underlying mechanism of TNF counteraction of apoptosis induction. In contrast, culture of DCs in the presence of human serum resulted in increased TNF production and improved survival also after poly(I:C) stimulation (data not shown). Regardless of putative serum factors responsible for this phenomenon, these initial results were in line with the hypothesis that autocrine TNF is important for the survival of DCs.

The exogenous addition of TNF has previously been found to improve the survival of dermal Langerhans cells and to increase the viability of immature and of mature Mo-DCs cultured under serum-free conditions (35–37). In other reports, improved DC viability has been attributed to the differentiation and maturation process rather than to a direct antiapoptotic effect of TNF (31, 32, 38). We now suggest that DC survival after TLR stimulation is regulated independently from the process of maturation, as we observed reduced DC viability after TLR stimulation by poly(I:C) or LPS (Figs. 1A, 1B), which induced important markers of cell maturation (23). In contrast, TNF alone is not a complete DC maturation factor (39, 40). Our study rather shows that TLR-induced maturation of DCs triggers substantial proapoptotic signaling, which results in cell death, unless the same TLR stimulation simultaneously also induces strong autocrine TNF production or TNF is supplied exogenously (Fig. 2A). This was also observed after TLR8 stimulation when TNF was efficiently neutralized. Mechanistically, we show that TNF counteracts the activation of BAK and strongly influences the balance of proapoptotic BAK and antiapoptotic BCL-2 in TLR-activated and also in immature DCs (Figs. 3C, 3D). Our data also support a report on the reduced survival of LPS-stimulated DCs after TNF neutralization (41). Similar data obtained with human plasmacytoid pre-DCs as well as murine macrophages highlight the general relevance of TLR-induced autocrine TNF for the survival of APCs (42, 43).

We provide evidence that not only TLRs but also other receptors upon costimulation may mediate their survival-supporting function via the induction of autocrine TNF. In particular, CD40 and other receptors of the TNF receptor superfamily have been reported to increase DC survival in certain situations, but the possible role of autocrine TNF had not been investigated in this context (16, 29, 30, 44). In our experiments, we show that it is the endogenously released TNF triggered by CD40L that mediated protection against poly(I:C)-induced cell death (Fig. 2C).

In search for the molecular mechanism of the survival signals responsible for the protective role of TNF in TLR-activated DCs, we studied the expression of a panel of pro- and antiapoptotic proteins in human DCs by Western blot analysis. We found that TNF acts on both the extrinsic and intrinsic apoptosis pathway by regulating proteins such as FLIPL, BCL-2, and BAK. Although our knockdown experiments using BAK-siRNA, BCL-2-siRNA, and FLIP-siRNA clearly showed that DC survival is controlled by both cell death pathways (Figs. 4B, 4D), we speculate that the pro-survival effect of TNF for TLR-activated DCs could be mediated by the regulation of BAK activity, supported by our FACS analysis of BAK activation (Fig. 3D). Further, Fig. 3C illustrates that a low ratio of BCL-2/BAK expression most closely correlated with conditions of extensive apoptosis and that these ratios were elevated by the addition of TNF by concerted regulation of BAK and BCL-2. Mechanistic evidence that BAK mediates DC apoptosis in response to microbial stimulation is based on our knockdown experiments in which BAK-siRNA clearly reduced poly(I:C)-induced apoptosis (Fig. 4B). A role for BAK in DC apoptosis triggered by microbial stimuli as well as by IFN-β has not been described before, but a central role for BAK in IFN-α–mediated and Semliki Forest virus-mediated apoptosis has been reported in other cellular systems (45, 46). Extending these notions, BAK may also regulate apoptosis in immature DCs, as suggested by our Western blot analysis (Fig. 3B, 3C) and BAK knockdown experiments (Fig. 4B).

Previous reports on the effects of exogenously added TNF on DCs have already described an upregulation of the antiapoptotic proteins BCL-2, BCL-XL, and FLIPL (31, 32, 38), which we could reproduce in our experimental system with regard to BCL-2 and FLIPL (Fig. 3B). Likely, upregulated BCL-2 contributes to the protective effect of TNF because BCL-2 may inhibit BAK oligomerization and mitochondrial outer membrane permeabilization (47). In line with such a model, Lepelletier et al. (42) recently established a central role for BCL-2 in protecting plasmacytoid DCs from glucocorticoid-induced cell death. Also, BCL-2 knockdown in our experiments with Mo-DCs stimulated with poly(I:C) plus TNF reduced the viability of the cells (Fig. 4D). With regard to a possible role of FLIPL, it may well be that this protein dampens possible receptor-mediated cell death in poly(I:C)-activated DCs, which indeed expressed procaspase-8 at high levels. Whether this occurs directly at the activated receptor or is initiated at the recently described Ripoptosome (48, 49) remains to be determined in future studies in human DCs. However, TNF neither elevated FLIPL nor reduced procaspase-8 levels in poly(I:C)-activated DCs and thus acts independently of FLIPL. Congruently, a high FLIPL/caspase-8 ratio was not sufficient to protect DCs from microbial stimulation-induced apoptosis, as observed under the conditions of TNF blockade with R-848–activated DCs (Figs. 2A, 3C), further pointing to a dominant role of the intrinsic pathway of apoptosis or caspase-independent forms of cell death. Still, probing the extrinsic death receptor pathway by FLIP knockdown strongly reduced the viability of DCs stimulated by poly(I:C) plus TNF. Such a pronounced effect of FLIP knockdown confirms the requirement of FLIPL to protect from TNF complex II-induced cell death (17, 22).

Apoptosis of DCs in response to LPS and poly(I:C) previously has been attributed to a cytotoxic effect of IFN-β release (10, 11).
However, we could not find evidence for a role of type I IFN in our experimental system, as the addition of a blocking Ab against CD118 (IFN-γ receptor chain 2) together with the TLR ligands had no effects on DC survival (data not shown). In contrast, as illustrated in Fig. 2A, IFN-β clearly induced cell death in immature DCs. Importantly, TNF prevented also IFN-β–induced apoptosis underscoring the central role of TNF for regulating DC apoptosis in response to various receptor-coupled pathways. IFN-β can be triggered by additional stimuli such as cytosolic microbial DNA (50, 51). Notably, TNF itself has recently been found to trigger a partial IFN response in Mo-DCs or even a release of type I IFN in macrophages, although counterregulation has been observed as well, at least in plasmacytoid DCs (52–54). The net effect of TNF on DC apoptosis in our experiments, in any case, was clearly protective. This indicates that TNF protects the cells also from the potentially cytotoxic effects of an IFN response possibly induced by itself. It should be noted that within the panel of reagents included in our study, stimuli known also to trigger larger amounts of IFN-β such as poly(I:C) and LPS (28, 55) were ineffective with respect to the induction of TNF production (Fig. 1D). The molecular basis of this pattern of cytokine production has recently been explained by a reciprocal regulation of TLR signal transduction by the phosphatase SHP-1 (56). In this case, low TNF production is a result of the inhibition of NF-κB activation by SHP-1. Combined activation of multiple TLRs can change such a response, which is illustrated in our current study by the activation of DCs with poly(I:C) or LPS together with the TLR8 ligand R-848. Although it is known that R-848 induces an enhanced and more persistent TNF production (33), the effect on DC survival revealed in our study adds a new important aspect to the synergistic TLR cross-talk.

The critical dependency of DC survival on the amounts of autocrine TNF throughout differentiation has relevance to clinical programs that use the ex vivo generation of DCs for tumor therapies and to treatment strategies using TNF antagonists. We have been intrigued by the observation that certain patient donors reproducibly displayed a poor DC yield within a clinical-grade cell manufacturing process, even when exogenous TNF was provided (M.A. Schmid and E. Kämpgen, unpublished observations and Fig. 5). In contrast, anti-TNF therapy reduces DC density in vivo, also supporting the model in which TNF protects the cell to produce its autocrine survival factor to the regulation of the intrinsic pathway of apoptosis by the balance of pro- and antiapoptotic members of the BCL-2 family. This model also explains how combined TLR stimulation resulting in enhanced TNF production ultimately translates into prolonged DC survival. The high levels of FLIP-induced during maturation of human DCs may prove to be critical to protect at the same time against TNFR1- or TLR3–induced cell death (31). This assumption needs to be investigated in more detail in the future. Microrna stimuli as well as cells of the immune system supplying paracrine TNF or inducing autocrine TNF through, for example, CD40 ligation can thus impact the life span of professional APCs and thus the extent of the immune response.

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**Disclosures**

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**References**


SUPPLEMENTARY FIGURE 1. TNF does not affect the expression of XIAP. The diagram shows the relative expression levels of the anti-apoptotic protein XIAP in DCs activated on day 5 by the indicated stimuli. The data were obtained with three different donors.