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Dendritic Cell Maturation and Survival Are Differentially Regulated by TNFR1 and TNFR2

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The capacity of dendritic cells (DC) to regulate adaptive immunity is controlled by their maturation state and lifespan. Although TNF is a well-known maturation and survival factor for DC, the role of the two TNFR, TNFR1 and TNFR2, in mediating these effects is poorly understood. By using unique TNF variants that selectively signal through TNFR1 and/or TNFR2, we demonstrate differential functions of TNF in human monocyte-derived and blood CD1c+ DC. Activation of TNFR1, but not TNFR2, efficiently induced DC maturation, as defined by enhanced expression of surface maturation markers (CD83, CD86, and HLA-DR) as well as enhanced T cell stimulatory capacity. In contrast, both TNFR1 and TNFR2 significantly protected DC against cell death, indicating that innate signals can promote DC survival in the absence of DC maturation. We further show differential activation of NF-κB signaling pathways by the TNFR: TNFR1 activated both the p65 and p52 pathways, whereas TNFR2 triggered p52, but not p65, activation. Accordingly, the p65 NF-κB pathway only played a role in the prosurvival effect of TNFR1. However, cell death protection through both TNFR was mediated through the Bcl-2/Bcl-xL pathway. Taken together, our data show that TNFR1, but not TNFR2, signaling induces DC maturation, whereas DC survival can be mediated independently through both TNFR. These data indicate differential but partly overlapping responses through TNFR1 and TNFR2 in both inflammatory and conventional DC, and they demonstrate that DC maturation and DC survival can be regulated through independent signaling pathways. The Journal of Immunology, 2014, 193: 4914–4923.

Dendritic cells (DC) play a key role in both instigating effective immunity against pathogens and maintaining tolerance to self-Ags. The process of DC maturation is critical in determining their immunomodulatory role (1). Under steady-state conditions, DC typically remain in an immature state, unable to initiate effector T cell responses and instead induce T cell tolerance. Upon maturation in response to “danger signals” (e.g., pathogen-associated molecular patterns or tissue-derived factors), DC express enhanced levels of MHC class II–peptide complexes, costimulatory molecules, and cytokines. They are now equipped to drive distinct T cell responses (e.g., Th1, Th2) versus regulatory T cell responses, depending on the nature of the maturation signals they received (1–3). In addition to DC maturation, the survival of DC also determines their immunomodulatory role. Extending the lifespan of DC breaks immune tolerance, resulting in autoimmune manifestations (4), whereas shortening the DC lifespan inhibits autoimmune disease (5). Thus, both the maturation status and survival of DC play a key role in controlling DC function. Understanding the pathways that control these important aspects of DC biology will assist the development of novel immunotherapeutic strategies.

TNF is a pleiotropic proinflammatory cytokine that promotes both DC maturation and survival (6, 7). Mice deficient in TNF fail to induce full DC maturation in vivo in response to a viral challenge (8), and the ex vivo generation and maturation of DC from these mice are impaired but can be restored by exogenous TNF (9–11). For the human system, we and others have shown that neutralization of autocrine TNF during DC maturation impairs their survival, enhancement of costimulatory molecule expression, and T cell stimulatory capacity (12–14). Furthermore, DC derived from rheumatoid arthritis patients on anti-TNF therapy display an impaired CD80 and CD86 upregulation after LPS stimulation (13).

TNF is produced as a type II transmembrane protein (mTNF), which can be processed by various metalloproteases, resulting in the release of the soluble ligand (sTNF) (15). Both mTNF and sTNF exist as homotrimers and exert distinct but also overlapping functions. Studies with genetically modified mice have demonstrated that sTNF is required for the development of acute and chronic inflammation, whereas mTNF can protect against chronic inflammation and autoimmunity (16).

TNF signals through two transmembrane receptors, TNFR1 and TNFR2. TNFR1 is ubiquitously expressed at low levels, whereas the expression of TNFR2 is restricted and primarily found on subpopulations of immune cells, neuronal tissues, and endothelial cells (15). Notably, TNFR2 is only efficiently activated by mTNF, but not sTNF, whereas TNFR1 is equally well activated by both TNF forms (17). We have shown that this differential responsiveness of the TNFR to their cognate ligands is controlled by the stalk region of TNFR (18).

In this study, we made use of our previously developed TNFR-selective ligands and a TNF variant mimicking mTNF bioactivity (19, 20) to dissect the roles of both receptors, TNFR1 and TNFR2, as well as sTNF and mTNF in regulating human DC maturation and survival. We found that both TNFR were expressed by monocyte-derived DC (a model for inflammatory DC) and freshly

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Abbreviations used in this article: DC, dendritic cell; LRS, leukocyte reduction system; monDC, monocyte-derived DC; mTNF, membrane-bound TNF; sTNF, soluble TNF.

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isolated blood-derived myeloid CD1c+ DC (representing steady-state conventional DC) and demonstrate that sTNF signals (primarily) through TNFR1 whereas mTNF triggers both TNFR. Phenotypical maturation was selectively induced by TNFR1 signaling, whereas the main action of TNFR2 was to prolong survival of DC. These findings suggest that human DC maturation and survival are controlled differentially by TNFR1 and TNFR2, and that regulation of these critical DC features can be segregated. As therapies directed at modulating DC function gain promise in the clinic (21, 22), an understanding of how TNF influences DC biology will help to develop new strategies for the design of novel DC-modulating therapeutics.

Materials and Methods

Monocyte-derived DC and blood CD1c+ DC

All DC cultures were carried out at 37°C, 5% CO₂ in RPMI 1640 (Sigma-Aldrich) supplemented with 10% (v/v) FBS (Life Technologies BRL), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (all Sigma-Aldrich). The use of leukocyte separation system (LRS) comes from platelet donations from healthy volunteers was approved by the Newcastle and North Tyneside Research Ethics Committee 2. PBMC were isolated from LRS cones by density gradient centrifugation on Lymphoprep (Axis-Shield Diagnostics). To generate immature monocyte-derived DC (moDC) as previously described (23, 24), CD14+ monocytes were isolated from PBMC by positive magnetic selection using anti-CD14 magnetic microbeads (Miltenyi Biotec) and cultured at 0.5 × 10⁶ cells/ml in a 24-well plate in the presence of IL-4 and GM-CSF (50 ng/ml each; Immunotools, Friesoythe, Germany) for 6 d. Medium supplemented with cytokines was refreshed at day 3. CD1c+ DC were separated from PBMC using an immunomagnetic negative selection kit (EasySep human myeloid DC enrichment kit; StemCell Technologies) and were cultured in a 96-well plate at 6.5 × 10⁵ cells/200 µl.

Stimulation of DC with TNF

Immature moDC were harvested on ice, washed three times in HBSS (Sigma-Aldrich) containing 1% FBS, and replated in a 24-well plate at either 2.5 × 10⁵ cells/ml (for flow cytometric analyses) or 5 × 10⁵ cells/ml (for all other assays). MoDC or blood CD1c+ DC were left untreated or were stimulated at 37°C with recombinant sTNF (50 ng/ml; 2 g total protein from lysed moDC samples according to the manufacturer’s instructions (R&D Systems) using g-nitroaniline–coupled caspase-3–specific tetrapeptides Asp-Glu-Val-Asp (DEVD) as substrate. Absorbance was measured at 405 nm on a microplate reader (Sunrise; Tecan).

Western blotting

moDC were harvested, pelleted, and lysed in high salt buffer (250 mM Tris-HCl [pH 7.5], 375 mM NaCl, 2.5% [w/v] sodium deoxycholate, 1% [v/v] Triton X-100) supplemented with protease inhibitors (Roche Applied Science) and 0.5 mM PMSF. Cell lysates were sonicated and centrifuged at [v/v] Triton X-100) supplemented with protease inhibitors (Roche Applied Science) and 0.5 mM PMSF. Cell lysates were sonicated and centrifuged at 405 nm on a microplate reader (Sunrise; Tecan).

Normal K-B activation assay

The oligonucleotide binding capability of p65 and p52 NF-kB was assessed using TransAM transcription factor assay kits specific for p65 or p52 (Active Motif). Nuclear extracts were prepared from moDC stimulated with TNF, variants thereof, or LPS for 15 min (for p65) or 24 h (for p52). Preparations of nuclear solutions were run on a nondenaturing gel, and NF-kB binding to oligonucleotide-coated 96-well plates was determined at 450 nm in a colorimetric reaction using p65- or p52-specific and anti-IgG-HRP Abs.

Statistical analysis

Statistics were performed using Prism 5.0 (GraphPad Software), and a general linear model was performed using SPSS (version 19) software. Differences between the treatment groups were tested after adjusting for

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subject-to-subject variability, and the assumptions underlying the model were confirmed by residual analysis. An F test was performed to test for differences between the treatment groups and contrasts formed to compare the groups stimulated with TNFR-selective ligands with and without inhibitors.

Results

TNFR1, but not TNFR2, mediates TNF-induced maturation of moDC

To study the mechanism of TNF-mediated DC maturation and survival through the two TNFR, we treated moDC with sTNF and TNF variants with unique specificity: sTNF, which primarily activates TNFR1; CysTNFwt, an oligomerized TNF derivative featuring mTNF-like bioactivity, efficiently activating both TNFR; CysTNFR1, which selectively activates TNFR1; and CysTNFR2, showing TNFR2-selective stimulatory capacity (19, 20). CysTNFR2 was used in combination with the nonagonistic TNFR2-specific mAb 80M2, which facilitates efficient stimulation by the CysTNFR2 ligand through preclustering of TNFR2 (19). sTNF and all TNF variants had been demonstrated to possess similar bioactivities when used at saturating concentrations of 50 ng/ml, that is, at least 50-fold in excess of their ED50 of sTNF and ED50 of CysTNFR1 = 0.1–0.3 ng/ml; ED50 of CysTNFR2 plus mAb 80M2 and ED50 of CysTNFwt = 0.3–1 ng/ml) as determined by our previously established cellular in vitro system (18) (Supplemental Fig. 1).

Interestingly, all TNFR1-stimulating ligands (sTNF, CysTNFwt, and the TNFR1-selective CysTNFR1) but not the TNFR2-selective ligands (CysTNFR2 plus mAb 80M2) induced high-level cell surface expression of the maturation marker CD83, albeit to a lower degree than in LPS-stimulated DC (Fig. 1A, top). The expression level of this marker could be further enhanced by combining any of the TNFR1-stimulating agents with IL-4, which by itself did not affect DC maturation (Fig. 1A, bottom) but had been previously described to augment TNF-induced DC maturation in murine bone marrow–derived DC (25). However, even in the presence of IL-4, TNFR2-selective stimulation did not significantly enhance expression of CD83, CD86, or HLA-DR, whereas the TNFR1-stimulating ligands significantly enhanced expression of all these markers (Fig. 1 and data not shown). The less potent upregulation of CD83 by CysTNFR1 as compared with TNFR2 signaling did not inhibit TNFR1-mediated maturation, as coactivation of both TNFR by CysTNFwt resulted in similar DC maturation marker expression compared with TNFR1 activation alone (Fig. 1). As observed for TNF (24) TNFR signaling did not lead to the production of proinflammatory cytokines such as IL-12p70, IL-6, and IL-23, indicating that TNFR1

![FIGURE 1. TNFR1- but not TNFR2-mediated signaling results in enhanced expression of cell surface maturation markers in moDC.](http://www.jimmunol.org/)

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signaling results in partial rather than full moDC maturation (data not shown).

Next, we determined the T cell stimulatory capacity of TNF-stimulated moDC in an MLR. Immature moDC stimulated with TNFR1-stimulating ligands displayed a significantly increased ability to induce T cell proliferation but not IFN-γ production, whereas LPS-activated DC induced both T cell proliferation and IFN-γ production (Fig. 2). In contrast, TNFR2-stimulated DC did not have an enhanced T cell stimulatory capacity (Fig. 2), which is in keeping with the lack of a significant increase in maturation marker expression by these moDC (Fig. 1). Thus, TNF-induced moDC maturation as defined by enhanced expression of maturation markers and T cell proliferative capacity is mediated via TNFR1, but not TNFR2.

**DC express functional TNFR1 and TNFR2 on their surface that differentially activate the p65 and p52 NF-κB pathways**

The finding that only TNFR1 signaling induced DC maturation raised the question of whether signaling-competent TNFR2 is expressed by immature moDC. Flow cytometric analysis confirmed that these cells homogeneously expressed both TNFR1 and TNFR2 (Fig. 3A). To verify the signaling competence of, in particular, TNFR2, we analyzed the potency of our TNFR-selective ligands to activate p65 and p52 NF-κB pathways in colorimetric oligonucleotide binding assays. Surprisingly, p65 was only efficiently activated in immature moDC by ligands stimulating TNFR1 (sTNF and CysTNFR1), but not by TNFR2-selective stimulation (CysTNFR2 plus mAb 80M2; Fig. 3B). In contrast, stimulation of either TNFR1 or TNFR2 resulted in significant nuclear translocation and DNA binding of p52 (Fig. 3C). As expected, stimulation by mAb 80M2 alone did not induce p65 or p52 activation (Fig. 3B, 3C). The differential capacity of the two TNFR to activate p65 could be confirmed by flow cytometry using phospho-p65-specific Abs (data not shown). These data demonstrate that immature moDC express signaling-competent TNFR2 and indicate that NF-κB signaling mediated by the two TNFR in immature moDC differs in terms of both quality and quantity.

**moDC can be rescued from cell death by both TNFR1 and TNFR2**

We have previously shown that a large proportion of immature moDC die within 48 h of cytokine withdrawal (i.e., after removing exogenous IL-4 and GM-CSF) and that LPS-induced autocrine TNF can rescue these cytokine-deprived immature moDC from cell death (13). We therefore investigated whether TNF increased immature moDC survival through TNFR1 and/or TNFR2 by treating cytokine-deprived moDC with our TNF variants and measuring the level of caspase-3 activation as an indicator of cell death. Interestingly, both TNFR1 and TNFR2 signaling significantly promoted the survival of immature moDC as shown by a reduction in the levels of intracellular active caspase-3 (Fig. 4A, 4B), caspase-3 enzymatic activity (Fig. 4C), and annexin V/7-aminoactinomycin D staining (data not shown). As expected, CysTNFR2 or mAb 80M2 alone had no effect on moDC survival (Fig. 4). We also addressed the question of whether the TNF ligands could rescue moDC from cell death after they had been matured with LPS for 24 h. We found that fully LPS-matured moDC had downregulated their expression of TNFR1 and TNFR2 and were not protected from cell death by any of the TNF ligands (data not shown). Thus, TNF plays a role in promoting the survival of immature but not mature DC.

To investigate the possibility that TNFR2-selective stimulation prolonged moDC survival indirectly, through TNFR1, for example, by inducing the production of sTNF or by co-operating with TNFR1, we used TNFR1-specific antagonistic Abs. The CysTNFR1-mediated rescue of moDC apoptosis was completely blocked by anti-TNFR1, whereas it did not affect the TNFR2-mediated response (Fig. 5), indicating that TNF exerts its effect in a TNFR1-independent manner. Furthermore, we addressed the question of whether the TNF ligands sTNF and CysTNFR2 mediate their cell death protective effect. The sTNF-mediated rescue of apoptosis was almost fully inhibited by blocking TNFR1 but not TNFR2, indicating that sTNF primarily acts through TNFR1 in this respect (Fig. 5), as previously seen for other cell types and cellular systems (17, 19). In contrast, the prosurvival effect of CysTNFR2 was only partially alleviated by blocking either TNFR1 or TNFR2, which is most likely due to the strong avidity effect of preoligomerized CysTNFR2 and the ability of this ligand to signal through both TNFR. Indeed, the combination of antagonistic TNFR1- and TNFR2-specific Abs almost completely inhibited CysTNFR2-mediated rescue of apoptosis, indicating that membrane-bound TNF enhances moDC survival through both TNFR1- and TNFR2-mediated signaling pathways (Fig. 5). These data not only indicate an important role for TNFR2 in DC survival, but they also demonstrate that TNFR1- and TNFR2-mediated rescue is independent of each other and that sTNF is not
capable of efficiently activating TNFR2 in moDC, whereas membrane-bound TNF potently activates both TNFR1 and TNFR2 in these cells.

The Bcl-2/Bcl-x<sub>L</sub> pathway mediates prosurvival effects of both TNFR1 and TNFR2, whereas p65 NF-κB is only involved in the TNFR1-mediated antiapoptotic effect.

To address the question of whether TNFR1 and TNFR2 regulated the lifespan of DC through similar or different pathways, we used specific small molecule inhibitors to target either the p65 NF-κB or the Bcl-2/Bcl-x<sub>L</sub> pathways (Fig. 6A, 6B), with both pathways having been shown to be critical for DC survival (14, 26–29). The BH3 mimetic ABT-737, which inhibits the heterodimerization of Bcl-x<sub>L</sub> and Bcl-2 with members of the proapoptotic machinery such as Bax and Bak (30), reversed the TNFR1- and TNFR2-mediated rescue of moDC cell death (Fig. 6A), indicating that both TNFR can act through the Bcl-2/Bcl-x<sub>L</sub> pathway. Indeed, ligation of either TNFR1 or TNFR2 efficiently induced Bcl-x<sub>L</sub> in moDC (Fig. 6C). Expression of Bcl-2 in moDC could not be consistently detected in response to the TNFR-selective ligands (data not shown). In contrast, inhibition of IκB kinase β, that is, the classical NF-κB signaling pathway, with the BAY 11-7082 compound impaired the cell death rescue by TNFR1- but not by TNFR2-mediated signaling (Fig. 6B). These data confirm a prosurvival role of the classical NF-κB signaling pathway in moDC and are in line with our observation that activation of the p65 NF-κB pathway could only be detected after TNFR1- but not TNFR2-selective stimulation (Fig. 3A). Taken together, our data suggest that TNFR1 and TNFR2 activate distinct, but overlapping, survival pathways.

**FIGURE 3.** Immature moDC express signaling-competent TNFR1 and TNFR2. (A) Cell surface expression of TNFR1 and TNFR2 on immature moDC. Cells were stained using a biotin/streptavidin-based amplification protocol and TNFR-specific Abs [TNFR1, black line in (A), left; TNFR2, black line in (A), right] and analyzed by flow cytometry. Cells stained only with biotin-conjugated anti-human IgG Abs and PE-coupled streptavidin are also shown (gray shaded). Data are representative of three independent donors. (B and C) Assessment of TNF-induced p65 (B) and p52 (C) NF-κB activation by oligonucleotide-binding assays. Immature moDC were stimulated for 30 min (B) or 24 h (C) as indicated, with 50 ng/ml TNF ligands or mAb 80M2. Nuclear proteins were extracted and the translocation and DNA-binding capability of p65 (B) and p52 (C) was determined in a colorimetric oligonucleotide-binding assay using anti-human p65- and p52-specific Abs, respectively. The means ± SEM from three to six independent donors are shown. Significance is shown in relationship to unstimulated (immature) DC and is determined using a Student t test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

steady-state CD1c<sup>+</sup> DC respond differently to TNFR1- and TNFR2-mediated signals

moDC are regarded as a model for inflammatory DC, that is, DC derived from monocytes under inflammatory conditions (reviewed in Ref. 31). To investigate whether TNFR signaling differs in inflammatory DC versus steady-state DC, we examined the TNF response in myeloid CD11c<sup>+</sup>CD1c<sup>+</sup> DC from peripheral blood (hereafter referred to as CD1c<sup>+</sup> DC) as an example of a steady-state, conventional DC subset. We confirmed expression of both TNFR on CD1c<sup>+</sup> DC as well as other DC subsets: myeloid CD141<sup>+</sup> and CD16<sup>+</sup> DC (the latter also referred to as “nonclassical monocytes” by other investigators) (32) and plasmacytoid DC (Fig. 7A). Isolation of CD1c<sup>+</sup> DC generally obtained purities of 75–90% (Fig. 7B). Interestingly, when analyzing their response to TNFR1 and TNFR2 ligation, sTNF and CysTNF<sub>wt</sub> induced phenotypical maturation and protected CD1c<sup>+</sup> DC against cell death. This was very similar to the response of moDC; however, sTNF was less effective in promoting CD1c<sup>+</sup> DC survival when compared with moDC (Figs. 1, 4, 7C, 7D). The same tendency was observed for TNFR1-selective treatment; that is, CysTNF<sub>wt</sub> induced phenotypical maturation and protected CD1c<sup>+</sup> DC against cell death. This was very similar to the response of moDC; however, sTNF was less effective in promoting CD1c<sup>+</sup> DC survival when compared with moDC (Figs. 1, 4, 7C, 7D). The same tendency was observed for TNFR1-selective treatment; that is, CysTNF<sub>wt</sub> induced phenotypical maturation and protected CD1c<sup>+</sup> DC against cell death. This was very similar to the response of moDC; however, sTNF was less effective in promoting CD1c<sup>+</sup> DC survival when compared with moDC (Figs. 1, 4, 7C, 7D). The same tendency was observed for TNFR1-selective treatment; that is, CysTNF<sub>wt</sub> induced phenotypical maturation and protected CD1c<sup>+</sup> DC against cell death. This was very similar to the response of moDC; however, sTNF was less effective in promoting CD1c<sup>+</sup> DC survival when compared with moDC (Figs. 1, 4, 7C, 7D). The same tendency was observed for TNFR1-selective treatment; that is, CysTNF<sub>wt</sub> induced phenotypical maturation and protected CD1c<sup>+</sup> DC against cell death. This was very similar to the response of moDC; however, sTNF was less effective in promoting CD1c<sup>+</sup> DC survival when compared with moDC (Figs. 1, 4, 7C, 7D). The same tendency was observed for TNFR1-selective treatment; that is, CysTNF<sub>wt</sub> induced phenotypical maturation and protected CD1c<sup>+</sup> DC against cell death. This was very similar to the response of moDC; however, sTNF was less effective in promoting CD1c<sup>+</sup> DC survival when compared with moDC (Figs. 1, 4, 7C, 7D). The same tendency was observed for TNFR1-selective treatment; that is, CysTNF<sub>wt</sub> induced phenotypical maturation and protected CD1c<sup>+</sup> DC against cell death. This was very similar to the response of moDC; however, sTNF was less effective in promoting CD1c<sup>+</sup> DC survival when compared with moDC (Figs. 1, 4, 7C, 7D). The same tendency was observed for TNFR1-selective treatment; that is, CysTNF<sub>wt</sub> induced phenotypical maturation and protected CD1c<sup>+</sup> DC against cell death. This was very similar to the response of moDC; however, sTNF was less effective in promoting CD1c<sup>+</sup> DC survival when compared with moDC (Figs. 1, 4, 7C, 7D).
a much lower level than observed in response to sTNF (Fig. 7C).
Thus, although the TNF response of moDC and CD1c+ DC is largely the same, there are subtle differences in the extent that these DC respond to TNFR1 and TNFR2 agonists; however, the reason for these differences remains unclear at present. Moreover, experiments with antagonistic TNFR2-specific Abs prevented the protection from cell death by CysTNF<sub>wt</sub>, whereas antagonistic TNFR1-specific Abs had no effect, demonstrating that this protection is mediated through TNFR2 and is independent of TNFR1 (Fig. 7D). Furthermore, as expected, sTNF-mediated survival could be blocked only by antagonistic TNFR1-specific Abs, but not by TNFR2 Abs, indicating that sTNF acts through TNFR1 and not through TNFR2 (Fig. 7D). In contrast, we experienced again that it is difficult to antagonize the activity of the strong mTNF mimick, CysTNF<sub>wt</sub>, most likely due to avidity effects caused by its wild-type preoligomerized configuration. However, CysTNF<sub>wt</sub>-mediated survival was most efficiently blocked by a combination of antagonistic TNFR1- and TNFR2-specific Abs, indicating that both TNFR contribute to mTNF-mediated survival (Fig. 7D).

In summary, our data demonstrate that TNFR1-mediated signaling is capable of inducing phenotypical maturation and survival of both moDC and blood CD1c<sup>+</sup> DC, whereas the main role of

**FIGURE 4.** TNFR1- or TNFR2-mediated signaling protects moDC from cytokine deprivation–induced cell death. Immature moDC were left untreated or were treated as indicated with sTNF, CysTNF<sub>wt</sub>, CysTNF<sub>R1</sub>, or CysTNF<sub>R2</sub> (50 ng/ml each) in presence of mAb 80M2 or mAb 80M2 alone for 48 h. Cell viability was assessed by the presence of intracellular active caspase-3 determined by flow cytometry (A and B) and a colorimetric caspase-3 activity assay (C). (A) Cells were stained using anti-human active caspase-3 Abs. The percentages of cells gated positive for active caspase-3 are indicated. The data shown are representative of six independent experiments. (B) Data of at least five independent experiments with different donors are shown (means ± SEM). (C) Colorimetric assessment of caspase-3 activity in whole-cell lysates using p-nitroaniline–coupled DEVD as substrate. The means ± SEM of three independent experiments are shown. (B and C) Statistical differences were determined in relationship to unstimulated (immature) moDC using a Student t test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

**FIGURE 5.** TNFR1 antagonism blocks TNFR1-mediated but not TNFR2-mediated protection from cell death. Immature moDC were left untreated or were treated with antagonistic TNFR1- or TNFR2-specific Abs or a combination of both prior to stimulation with sTNF, CysTNF<sub>wt</sub>, or TNFR-selective variants. Cell viability was assessed by the presence of intracellular active caspase-3 determined by flow cytometry. The percentages of cells gated positive for active caspase-3 are indicated. The data shown are representative of at least four independent experiments with different donors. Statistical differences were determined using a Student t test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
TNFR2-mediated signaling is to prolong DC survival. These data indicate differential but partly overlapping responses through TNFR1 and TNFR2 in DC, and they demonstrate that DC maturation and survival can be regulated through independent signaling pathways (Fig. 8).

Discussion

The immunogenicity of DC is determined both by their maturation state and their lifespan. Despite the central role of TNF in promoting DC maturation and survival, the functional role of the underlying signaling receptors exerting these effects remains poorly understood, especially for human DC. We have explored the mechanism by which TNF controls the maturation and survival of human DC (“inflammatory” moDC and steady-state blood CD1c+ DC) through its two receptors, TNFR1 and TNFR2. We have shown that TNF-mediated phenotypical maturation of human DC is, overall, most potently regulated by TNFR1, whereas DC survival can be significantly enhanced by both TNFR, most likely through upregulation of the antiapoptotic factor Bcl-xL (Fig. 8). Thus, these data show that TNF promotes DC maturation and survival through distinct signaling pathways, demonstrating that innate signals enhancing DC survival do not necessarily result in enhanced DC maturation.

We used selective TNFR ligands to dissect the roles of TNFR1 and TNFR2 in regulating human DC maturation and survival. Previous studies demonstrated that maturation of DC is impaired in TNFR1 knockout mice in response to pathogenic challenges (33–35). However, it remains unclear whether this is due to an insufficient expression of mTNF, which also efficiently activates TNFR2, a lack of cross-talk between the two TNFR, or the inability of TNFR2 to induce DC maturation. We have therefore chosen a different approach, that is, adequate activators for TNFR1, TNFR2, or both TNFR, allowing us to directly investigate the functional roles of TNFR1 and TNFR2 as well as their cross-talk in DC biology.

One of our key results is that immature human DC express signaling-competent TNFR1 and TNFR2, but that TNFR1 is the major TNFR controlling maturation of both moDC and blood CD1c+ DC. Although TNFR2 activation also resulted in a weak, but significant, upregulation of maturation markers in CD1c+ DC (Fig. 7C), TNFR2 failed to enhance expression of costimulatory molecules and HLA-DR in moDC (Fig. 1). Importantly, TNFR2 signaling does not seem to suppress TNFR1-mediated DC maturation, as CysTNFω, mimicking mTNF action and thereby triggering both TNFR, potently induced DC maturation to a similar extent as did the TNFR1 ligands sTNF and CysTNFR1. These data indicate that DC maturation is independent of TNFR cross-talk and/or mixed TNFR1/2 receptor complex formation, indicating opposing functions for the two TNFR as described previously (16, 36).

DC activation through TNFR1 enhanced the expression of cell surface markers that are typically involved in T cell activation (e.g., CD86). Although phenotypically mature moDC displayed enhanced ability to induce CD4+ T cell proliferation, they lacked the ability to promote the differentiation of IFN-γ–producing T cells (Fig. 2). In contrast, LPS-matured moDC potently induced IFN-γ responses in T cells (Fig. 2B). These data are in agreement with the notion that inflammatory mediators (including cytokines such as TNF) by themselves are not sufficient for the full functional maturation of DC, as defined by the DC’s ability to induce effector T cell responses (37). Induction of effector T cell responses by DC minimally requires signal 1 (MHC class II–peptide complexes),

FIGURE 6. Cell death protection mediated via both TNFR is dependent on the Bcl-2/Bcl-xL signaling pathway, but p65 NF-κB is only required by TNFR1. (A and B) Inhibition of signaling pathways at the level or upstream of Bcl-2/Bcl-xL inhibits TNFR1- and TNFR2-mediated protection from cell death. Immature moDC were left untreated or treated with 50 ng/ml CysTNFR1 or CysTNFR2 plus mAb 80M2 in the presence or absence of the chemical compound ABT-737 (A) or BAY 11-7082 (B). Cell viability was assessed by the presence of intracellular active caspase-3 determined by flow cytometry. The percentages of cells gated positive for active caspase-3 are indicated. The means ± SEM of at least four independent experiments with different donors are shown. Statistics were performed as described in Materials and Methods. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. (C) TNFR1- and TNFR2-mediated signaling upregulates Bcl-xL. Immature moDC were harvested immediately (−, 0 h), after 48 h (−, 48 h), or were stimulated with 50 ng/ml CysTNFR1 or CysTNFR2 plus mAb 80M2 for 48 h. Whole-cell extracts were analyzed for the expression of Bcl-xL (top) and reprobed for GAPDH (bottom) by Western blot analysis. The blots shown are representative of two independent experiments.
signal 2 (costimulatory molecules, e.g., CD86), and signal 3 (proinflammatory cytokines, e.g., IL-12p70) (1). However, activation of moDC by TNFR1 (or TNFR2) agonists alone did not lead to the production of proinflammatory cytokines (e.g., IL-12p70, IL-6, IL-23; data not shown), explaining their inability to induce a Th1 response and suggesting a more contributory role for TNF in generating an effective T cell response to pathogenic challenge.

Another key finding is that the two TNFR differentially activate the canonical (p65) and noncanonical (p52) (38) NF-κB pathways in DC, which may explain their distinct roles in regulating maturation and survival (Fig. 8). Activation of the noncanonical pathway is required for DC maturation (39–42). However, we observed only very little DC maturation after triggering TNFR2 in CD1c+ DC (Fig. 7C) and no significant maturation in moDC (Fig. 1), despite significant activation of the p52 NF-κB pathway following TNFR2 activation (Fig. 3C). Because TNFR1-mediated activation of the canonical NF-κB pathway, initiated by the temporary depletion of IκBa, activates the noncanonical pathway (40), qualitative and quantitative differences between TNFR1- and TNFR2-mediated activation of the noncanonical pathway are expected. Thus, the lack of TNFR2-induced phenotypical maturation in moDC is most likely caused by the failure of this receptor to induce detectable activation of the canonical p65 NF-κB pathway (Fig. 3B), suggesting that DC maturation requires p65 activation (Fig. 8). In accordance, IκBa was found to restrain spontaneous DC maturation (40).

Both the canonical and noncanonical NF-κB pathways trigger prosurvival signals in APCs (41–43). Our data suggest that activation of the noncanonical p52 pathway, in the absence of apparent signal 2 (costimulatory molecules, e.g., CD86), and signal 3 (proinflammatory cytokines, e.g., IL-12p70) (1). However, activation of moDC by TNFR1 (or TNFR2) agonists alone did not lead to the production of proinflammatory cytokines (e.g., IL-12p70, IL-6, IL-23; data not shown), explaining their inability to induce a Th1 response and suggesting a more contributory role for TNF in generating an effective T cell response to pathogenic challenge.

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### FIGURE 7
Steady-state blood CD1c+ DC respond differently to TNFR1- and TNFR2-mediated signals. (A) Cell surface expression of TNFR1 and TNFR2 by peripheral blood myeloid CD1c+ DC (CD1c DC), myeloid CD16+ DC (CD16 DC), myeloid CD141+ DC (CD141 DC), and plasmacytoid DC (pDC), as determined by flow cytometry. TNFR expression is indicated by a black line (open), isotype-matched control is indicated by gray (filled). (B) Purity of CD1c+ DC isolated from peripheral blood as determined by CD1c and CD11c expression. (C and D) CD1c+ DC were cultured for 24 h in the absence or presence of TNFR ligands and in the absence or presence of neutralizing anti-TNFR Abs as indicated. Expression of CD83 and CD86 (C) and active caspase-3 (D) was determined by flow cytometry. The medians (C) or means (D) ± SEM from three to seven independent donors are shown. Statistical differences were determined using a Student t test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

### FIGURE 8
TNF promotes DC maturation and survival through distinct signaling pathways in human moDC and CD1c+ DC. TNF-mediated phenotypical maturation of human DC is controlled by sTNF- or mTNF-activated TNFR1, but not TNFR2 and independent of TNFR cross-talk. In contrast, signaling by either TNFR augments DC survival, which can be enhanced by mTNF through either TNFR1 or TNFR2, and by sTNF through TNFR1, but not TNFR2. In contrast to TNFR1, TNFR2-mediated DC survival is independent on the activation of p65; however, both TNFR control DC survival through the Bcl-2/Bcl-xL survival pathway resulting in an upregulation of Bcl-xL.
p65 activation, is sufficient to promote DC survival. Furthermore, our inhibitory studies and Western blot analyses suggest Bcl-xL as one of the major antiapoptotic molecules by which both TNFR promote DC survival (Fig. 6A, 6C). Both p65 and p52 can bind to the Bcl-xL promoter, have been implicated in the regulation of its expression, and protect against intrinsic and extrinsic induced cell death (44–47). Thus, the differential and independent functions of TNFR1 and TNFR2 in DC may converge at the level of Bcl-2/ Bcl-xL, underlining the central role of Bcl-2 family members in DC function (14, 29).

The survival of DC is regulated by several TNF superfamily members that can either work independently, cross-react, and/or cooperate with TNFR signaling (24, 48). In particular, 41BB, CD40, and RANK show functional similarities to TNFR1 in DC survival and maturation (49–51), which may be at least partly due to the secretion of TNF (52–54). However, qualitative and quantitative differences exist. Whereas signaling through 41BB, RANK, and the two TNFR is particularly potent in promoting survival through upregulation of Bcl-2 and/or Bcl-xL (Fig. 6A, 6C) (49, 50, 54, 55), CD40 signaling results primarily in full DC maturation and to a lesser extent DC survival (24). In contrast, RANK signaling, similarly to TNFR1 signaling, leads to functional but not full maturation of human mDC, but has high capacity to prolong DC survival (Figs. 1, 2, 4) (24). This seems to leave TNFR2 with an exceptional role among the TNFR superfamily members, as TNFR2 signaling significantly extends the lifespan of DC whereas DC maturation remains nearly unaffected (Figs. 1, 2, 4, 7C, 7D).

What might be the role of TNFR2-induced extended lifespan of DC in the absence of DC maturation? Studies manipulating DC lifespan demonstrated that the regulation of DC survival is in itself an important factor determining DC function: prolonging the DC lifespan can break immune tolerance (4), whereas reducing DC survival can inhibit autoimmune disease (5), proposing the assistance of immune and/or proinflammatory responses by TNFR2. Thus, specific activation of TNFR2, proposed as a new immunotherapeutic approach to kill autoreactive T cells (56), may counteract tolerance induction through prolonging the lifespan of DC. Alternatively, development of a therapeutic TNFR2 agonist may be a useful and convenient tool for the ex vivo generation of tolerogenic DC-based immunotherapies, as it may prolong the survival of these tolerogenic DC without inducing their maturation. Additionally, and in contrast to above the noncanonical NF-kB pathway, RelB:p52 has been implicated in the control of the immunoregulatory phenotype of DC (57–59). Because TNFR2 activates primarily the noncanonical p52 NF-kB pathway, it is tempting to speculate that TNFR2 is involved in the induction/maintenance of self-tolerance by inducing tolerogenic DC. Accordingly, TNFR2 has been attributed an anti-inflammatory function in several models of inflammation (36, 60). However, our initial attempt to support this idea by investigating whether TNFR2 signaling in mDC promoted IL-10–producing T cells revealed inconclusive results, and further work is needed to explore this hypothesis.

In conclusion, the results of this study demonstrate that DC maturation and survival are differentially and independently regulated through TNFR1 and TNFR2 and point to the canonical NF-kB pathway controlling DC maturation and the noncanonical NF-kB pathway regulating DC survival (Fig. 8). Thus, this study contributes to a better understanding of TNFR-mediated immune regulation, which is particularly important for the development of novel immunotherapeutics designed to specifically target TNFR1 or TNFR2.
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