Cutting Edge: Resistance to Apoptosis and Continuous Proliferation of Dendritic Cells Deficient for TNF Receptor-1

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Dendritic cells (DC) are a specialized subset of highly potent APCs of the adaptive immune system that can be activated by pathogens and inflammatory signals (1). Several cytokines have been described to influence DC development or function (2). The proinflammatory cytokine TNF-α mediates different signals for proliferation, functional activation, and apoptosis of many cell types, including DC (3). Early in hematopoiesis, TNF-α, together with GM-CSF, promotes the generation of DC from CD34+ bone marrow precursors (4–6). TNF-α also plays a role for the migration of DC from peripheral tissues to lymphoid organs (7, 8). The best known effect, however, is the induction of DC maturation characterized by the down-regulation of endocytosis mechanisms and the increased surface expression of MHC and costimulatory molecules for Ag presentation (9–11).

Two receptors bind the soluble and membrane form of TNF-α. TNFR1 (p55, CD120a) is expressed on almost every cell type, whereas TNFR2 (p75, CD120b) expression is restricted to endothelial and hematopoietic cells. The two TNFR bind different sets of adapter proteins inducing distinct signaling cascades (3, 12, 13). The most obvious consequences of TNFR deficiency in mice are impairment of inflammatory responses (14). Whereas TNFR1−/− mice especially show defects in the defense of intracellular pathogens (15, 16), the defects to transmit inflammation in TNFR2−/− mice are more general (17). Little is known about the individual functions of the two TNFR on DC. TNFR1 is expressed on epidermal Langerhans cells; however, TNFR2 appears to be the major receptor because it promotes Langerhans cell survival in vitro (18) and mediates their migration (8, 19). In contrast, TNFR1 down-regulates endocytosis in human monocye-derived DC as a sign of maturation (20).

**Materials and Methods**

**Mice**

Male or female mice were used at the ages of 1–4 mo. C57BL/6 mice were kept and bred in our animal facilities. TNFR1−/− (H. Blüthmann, Roche, Basel, Switzerland, and K. Pfeffer, Technical University of Munich, Munich, Germany), TNFR2−/− mice (M. W. Moore, Genentech, South San Francisco, CA), and TNFR1/2−/− mice (H. Blüthmann) were bred under specific pathogen-free conditions and kept on a mixed C57BL/6 × 129 genetic background in our facilities.

**Bone marrow (BM)-DC culture**

The generation of BM-DC was performed as described in detail (21). RPMI 1640 (Life Technologies, Gaithersburg, MD) was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 50 μM 2-ME, 10% heat-inactivated filtered FCS. GM-CSF was used at 200...
U/ml (PeproTech/Tebu, Rocky Hill, NJ). Where indicated, LPS (Escherichia coli 026:B6, Sigma, St. Louis, MO) was used at 1 µg/ml, TNF-α (PeproTech/Tebu) at 500 U/ml, and camptothecin (Sigma), CD95 ligand (CD95L), or TNF-related apoptosis-inducing ligand (TRAIL)-leucine zipper trimers (22) at 1 µM.

**FACS analysis**

Cells were stained as described earlier (21) and analyzed on a FACSscan (Becton Dickinson, San Jose, CA). Abs (B7-1, B7-2, CD40, PharMingen, San Diego, CA; 2F8, Serotec, Oxford, U.K.) or hybridoma supernatants (ICAM-1, F4/80, N418, NLDC-145, 33D1, 2A1) with appropriate isotype controls, were used with FITC-conjugated secondary Abs followed by MHC II counterstaining (MS/114, PE-conjugated, PharMingen). Biotinylated CD95 (Jo2) was detected by a streptavidin-PE conjugate and double-stained with a directly FITC-conjugated MHC II Ab (2G9, all PharMingen). For intracellular staining (2A1 supernatant, caspase-3, Bcl-2, PharMingen), cells were permeabilized with 0.5% saponin. Endocytosis of FITC-conjugated dextran (m.w. 40,000, Molecular Probes, Eugene, OR) was conducted at 4°C (unspecific binding) and 37°C (binding and uptake) at 1 mg/ml for 30 min.

**Ag presentation and proliferation assays**

Titrated numbers of BM-DC were added to 1 x 10⁵ purified T cells/well and plated as triplicates in 96-well flat-bottom plates (Falcon, Becton Dickinson, Heidelberg, Germany). After labeling with 1 µCi/well (methyl-³²P)thymidine (Amersham) for 16 h, cells were harvested with an Inotech harvester (Dottikon, Switzerland) on a glass fiber mat, dried, and measured in a Microbeta 1450 counter (Wallac, Gaithersburg, MD). For cell counting and thymidine incorporation, 1 x 10⁵ BM-DC were plated as triplicates in 96-well plates and then counted under trypan blue exclusion or pulsed for 24 h. Cell cycle analysis by 5-bromo-2'-deoxyuridine labeling was performed as described (23).

**Electromobility shift assays and immunoblotting**

The activity of NF-κB was analyzed as described previously (24), NF-κB consensus oligonucleotides (Promega, Madison, WI) were labeled using γ-³²P-ATP (3000 Ci/mmol, Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (New England BioLabs, Beverly, MA). The specificity of the NF-κB complexes was also determined by Ab supershift. Immunoblotting was performed as described in detail (23). Whole cell lysates were analyzed by standard SDS-PAGE techniques with anti-p27 (Onco- gene Research Products, Cambridge, MA), anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-CDK2 (PharMingen) Abs.

**Results and Discussion**

**Phenotypical and functional characterization of LTC**

While studying the biology of DC derived from different TNFR-deficient mice, we observed, unexpectedly, that TNFR1⁻/⁻ BM-DC survived well beyond the point when WT, TNFR2⁻/⁻, or TNFR1/2⁻/⁻ BM-DC had died and only adherent macrophages remained in the cultures. The life span of such standard MHC II⁺ BM-DC cultures was maximally 3–4 wk. In sharp contrast, BM-DC cultures from two independently generated strains of TNFR1⁻/⁻ mice (15, 16) survived and proliferated for 6–9 mo in a GM-CSF-dependent manner. More than 20 independent long term cultures (LTC; i.e., >4 wk in culture) had been set up, and all showed continuous cluster formation as well as morphologically veiled DC in suspension (not shown).

FACS analysis of LTC showed a largely homogeneous population of cells with a mixed immature/mature DC phenotype. They expressed markers of mature DC (MHC II⁺high, B7-2high, ICAM-1high, intracellular 2A1⁺) and also markers of immature DC (B7-1weak, N418weak, F4/80weak, 2F8⁺) (Fig. 1a). Surprisingly, the LTC completely lacked the mature DC markers NLDC145 and CD40 and expressed high 33D1 levels, something we had never observed on WT DC (Fig. 1a). Other early myeloid differentiation markers such as Gr-1, 3D6, and CD14 were not expressed (not shown).

Functionally, immature DC are efficient in Ag uptake but weak Ag presenters, whereas mature DC down-regulate endocytosis and drastically enhance Ag presentation (1). The LTC were weak in uptake of FITC-conjugated dextran (Fig. 1b) and at the same time weak in allogenic Ag presentation to T cells (Fig. 1c, left). However, after maturation with LPS, the Ag-presenting capacity of the LTC was increased, although not reaching the level of WT BM-DC (Fig. 1c, right). This LTC maturation was also observed phenotypically after treatment with LPS, but not with TNF-α or
CD40 ligation (not shown). Importantly, TNFR2\(^{-/-}\) or TNFR1/2\(^{-/-}\) BM-DC also did not mature on stimulation with TNF-α (not shown), indicating that both TNFR chains are required to induce DC maturation by TNF-α. The inability of TNFR1\(^{-/-}\) DC to respond to TNF-α was further confirmed by lack of NF-κB induction as judged by gel shift experiments (Fig. 1d). Activation of NF-κB by TNF, but not LPS, seems to be dependent on TNFR1, as also observed in total spleen cells (16), which underscores the importance of TNFR1 for NF-κB activation as a critical signal for DC maturation (25).

Thus, the continuously proliferating LTC 1) are impaired in both functional characteristics of immature (endocytosis) and mature (Ag presentation) DC, typical for proliferating tumor cells, and 2) express a unique set of markers similar to other DC long term cultures (26).

Proliferative characteristics of LTC

Both early WT and TNFR1\(^{-/-}\) BM-DC expanded readily in the presence of GM-CSF but not in the absence of GM-CSF or after LPS treatment (Fig. 2a, top). GM-CSF promotes the growth of neutrophilic granulocytes, macrophages, and DC from bone marrow (21, 27). A 3-wk-old WT BM-DC culture consisted mainly of macrophages, and the remaining suspension cells did not expand further in the presence of GM-CSF. In the absence of GM-CSF, the cells died rapidly (Fig. 2a, bottom). In contrast, the LTC did not die in the absence of GM-CSF or after LPS treatment and proliferated on GM-CSF (Fig. 2a, bottom). By removal of GM-CSF, the LTC survive, whereas the fraction of adherent macrophages died. The LTC retained ~20–25% S-phase cells, as judged by 5-bromo-2’-deoxyuridine incorporation (not shown). Exogenous TNF-α had no substantial influence on the growth of all cultures (Fig. 2a) but increased the survival of all but TNFR1/2\(^{-/-}\) BM-DC (not shown).

Because negative cell cycle signals might be down-regulated in LTC, we analyzed the expression of cyclin-dependent kinase (CDK) inhibitors. CDK/cyclin complexes phosphorylate cellular substrates, thereby controlling progression through the cell cycle. The CDK inhibitors, particularly the family members p27\(^{kip1}\) and p21\(^{cip1}\), are prominent downstream mediators of exogenous stimuli including cytokine-signaling pathways (28). Interestingly, p27 and p21 protein levels were down-regulated; 4- to 6-fold in LTC but not in the other cell types including early (day 10) TNFR1/2 BM-DC; the level of CDK2, the kinase regulating G1-S phase, remained unaltered in all cultures (Fig. 2b).

In many cell types, p27 protein level is down-regulated on productive stimulation of cells and is a prerequisite for the passage of the cells through G1-S (28). Importantly, p27 acts as a haplo-insufficient tumor suppressor in mice (29), and reduced p27 protein expression in tumors correlates well with survival of cancer patients. Therefore, the down-regulation of p27 and p21 in LTC likely points to an alteration of the p27/p21-regulatory mechanisms and might contribute to their long-lasting proliferation. Whether this may further contribute to DC tumorigenesis is currently under investigation.

Apoptosis signaling in LTC

The continuous proliferation of the LTC may explain their expansion but not their prolonged survival. The two major pathways of mitochondrial apoptosis (Bcl-2 family) and death receptor-mediated apoptosis (TNFR1, TRAILR, CD95) were therefore investigated. No differences were found in Bcl-2 family mRNA and Bcl-2 protein levels of LTC and early WT or TNFR1/2 BM-DC, whereas the induction of MHC II (Fig. 3a) and CD95 (Fig. 3b) was down-regulated in LTC as compared to WT controls. The CD95 death receptor was highly expressed on WT BM-DC, whereas LTC cells expressed a lower level of CD95 (Fig. 3b). In addition, LTC were resistant to apoptosis induced by Fas ligand, TRAIL, or TNF-α (Fig. 3b). The continuous proliferation of the LTC may explain their expansion but not their prolonged survival. The two major pathways of mitochondrial apoptosis (Bcl-2 family) and death receptor-mediated apoptosis (TNFR1, TRAILR, CD95) were therefore investigated. No differences were found in Bcl-2 family mRNA and Bcl-2 protein levels of LTC and early WT or TNFR1/2 BM-DC, whereas the induction of MHC II (Fig. 3a) and CD95 (Fig. 3b) was down-regulated in LTC as compared to WT controls. The CD95 death receptor was highly expressed on WT BM-DC, whereas LTC cells expressed a lower level of CD95 (Fig. 3b). In addition, LTC were resistant to apoptosis induced by Fas ligand, TRAIL, or TNF-α (Fig. 3b).
protein levels (not shown). The LTC were equally susceptible to mitochondrial cell death induced by camptothecin, as judged by active caspase-3 induction (not shown) or membrane permeability for propidium iodide (Fig. 3b). This indicates that the mitochondrial apoptosis cascade is intact and that antiapoptotic effects mediated via Bcl-2 may not contribute to the extended survival of LTC.

Importantly, both early TNFR1−/− and LTC DC had shown reduced CD95 levels (Fig. 3a). Accordingly, these cells were resistant to apoptosis induced via CD95 cross-linking by a CD95L-leucine zipper trimer, whereas a large proportion of all other BM-DC died (Fig. 3b). Other stimuli, such as LPS and a TRAIL-leucine zipper trimer, did not significantly induce apoptosis in any culture (Fig. 3b). Taken together, TNFR1 deficiency in BM-DC leads to decreased CD95 expression, which results in a block of CD95-mediated apoptosis. This may be an important prerequisite for long term survival of DC.

Little is known about TNFR signaling in DC during development. After sorting of lineage− c-kit+ cells from mouse bone marrow, no TNFR1−/− DC could be generated with GM-CSF and human TNF-α, the latter binding the mouse TNFR1 only (6). Using whole BM cultures supplemented with GM-CSF, BM-DC grew equally well from WT, TNFR1−/−, TNFR2−/−, and TNFR1/2−/− mice. However, all but WT BM-DC remained phenotypically and functionally immature (our unpublished observation) because they were unable to respond to paracrine TNF-α production by macrophages in the culture (30). Thus, both TNF receptors are required to transmit TNF-α signals for DC maturation (Fig. 4). Importantly, both the absence of TNFR1 and the presence of TNFR2 are required to enable long term survival of DC, as DC lacking both TNFR or only TNFR2 did not survive (Fig. 4). The molecular antiapoptotic pathways involved are under current investigation.

CD95 and its coreceptor CD95L (mRNA and protein) are both expressed on murine BM-DC (26, 31, 32). However, DC were found to be resistant to apoptosis mediated by incubation with either the anti-CD95 Ab Jo2 or CD95L expressed on T cells (32). We found CD95 to be functionally active because apoptosis was induced in a substantial proportion of WT (Fig. 3b), TNFR2−/−, and TNFR1/2−/− DC (not shown) after treatment with a trimeric CD95L-leucine zipper. In contrast, apoptosis was not induced in early TNFR1−/− or LTC DC, both of which had down-regulated CD95 (Fig. 3). The expression of CD95 is maintained only when TNFR1 is present on DC, indicating a functional relationship. A similar relationship has been found only in microglia, in which CD95 up-regulation by TNF-α led to a susceptibility to apoptosis (33). Our data indicate that apoptosis of DC can occur via CD95 under certain conditions. DC seem to rapidly disappear from lymphoid tissues after successful Ag presentation to T cells (34). This might be due to apoptosis, possibly induced by activated T cells to terminate immune responses at the DC level.

Conclusions

Signal transduction via members of the TNF receptor superfamily is pivotal for the balance between survival and cell death. We show that BM-DC derived from two independently generated strains of TNFR1−/− mice (15, 16) survived for 6–9 mo in culture with GM-CSF. These novel long term TNFR1−/− DC may not only serve as a stable source of DC but also as a useful tool for the analysis of apoptosis signaling pathways in DC (Fig. 4).

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