

Innate Direct Anticancer Effector Function of Human Immature Dendritic Cells. II. Role of TNF, Lymphotoxin- $\alpha_1\beta_2$, Fas Ligand, and TNF-Related Apoptosis-Inducing Ligand¹

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Our recent studies have demonstrated that human immature dendritic cells (DCs) are able to directly and effectively mediate apoptotic killing against a wide array of cultured and freshly-isolated cancer cells without harming normal cells. In the present study, we demonstrate that this tumoricidal activity is mediated by multiple cytotoxic TNF family ligands. We determine that human immature DCs express on their cell surface four different cytotoxic TNF family ligands: TNF, lymphotoxin- $\alpha_1\beta_2$, Fas ligand, and TNF-related apoptosis inducing ligand; while cancer cells express the corresponding death receptors. Disruptions of interactions between the four ligands expressed on DCs and corresponding death-signaling receptors expressed on cancer cells using specific Abs or R:Fc fusion proteins block the cytotoxic activity of DCs directed against cancer cells. The novel findings suggest that DC killing of cancer cells is mediated by the concerted engagement of four TNF family ligands of DCs with corresponding death receptors of cancer cells. Overall, our data demonstrate that DCs are fully equipped for an efficient direct apoptotic killing of cancer cells and suggest that this mechanism may play a critical role in both afferent and efferent anti-tumor immunity. *The Journal of Immunology*, 2002, 168: 1831–1839.

Bone marrow-derived dendritic cells (DCs)⁴ are important APCs positioned in limiting numbers at strategic sites throughout the body. Thus, DCs and/or their precursors are present as a loose cellular network in the skin (1), mucosa (2), intestines (3), liver (4), lung (5), thymus (6), and secondary lymphoid tissues (7), as well as a minute circulating population in the peripheral blood (8). In addition to their presence in the normal tissues, where they are thought to serve as sentinel cells that interrogate invading pathogens and monitor cellular changes (7), DCs have been frequently found to infiltrate neoplastic tissues (9). Importantly, high natural or therapy-induced frequencies of tumor-infiltrating DCs have been associated with better clinical prognosis and/or regression of disease (9–11). These findings could be explained in the context of the recent demonstration that DCs mediate cross-priming of the anticancer adaptive immunity via the uptake of dead cancer cells and processing of tumor-associated Ags (12–18). Similar functions might be performed by tumor-infiltrating DCs in situ leading to specific anti-tumor therapeutic re-

sponses. This would most efficiently be accomplished if DCs both mediated the death of tumor cells and subsequently uptake and processed the byproduct of such cytotoxic interactions.

In a companion manuscript (47), we have demonstrated that human immature DCs may directly mediate apoptosis of a diverse array of tumor cells, but not of normal cells. We have also shown that both cell membrane-bound and -secreted proteins are mediators of this tumoricidal activity. In the current study, we have evaluated the molecular mechanisms by which tumor cell death results from interaction with DCs. Based on a limited series of publications indicating that mouse immature DCs and human-activated DCs may express functional Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL; Refs. 19–22), respectively, and our recent finding of the activation of TNF family death receptor signaling-specific caspase-8 in cancer cells exposed to DC killing (47), we presumed that the newly described tumoricidal activity associated with human immature DCs is mediated by cytotoxic TNF family ligands. In direct support of this hypothesis, we obtained novel information that human immature DCs express TNF, lymphotoxin (LT)- $\alpha_1\beta_2$, FasL, and TRAIL, and kill cancer cells by the concerted engagement of all four cytotoxic TNF family ligands. This direct effector function of immature DCs provides the sentinel APCs with the ability to generate a source of tumor Ags that may be subsequently processed and presented to specific T cells by the same or additional host DCs.

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⁴ Abbreviations used in this paper: DC, dendritic cell; LT, lymphotoxin; TRAIL, TNF-related apoptosis-inducing ligand; FasL, Fas ligand; PCA, polyclonal Abs; PBMNL, peripheral blood mononuclear leukocytes; SA-HRP, streptavidin HRP; int, intermediate; CD40L, CD40 ligand.

Materials and Methods

Reagents

The following Abs were used in this study: anti-human TNF (IgG1) mouse mAb (Endogen, Woburn, MA), AO.D12.10 anti-human LT- α (IgG1) mouse mAb (Biogen, Cambridge, MA), H398 anti-human TNFRp60 (IgG2a) mouse mAb (Alexis Biochemicals, San Diego, CA), anti-human TNFRp80 (IgG2b) rat mAb (Genzyme, Cambridge, MA), B9.C9 anti-human LT- β (IgG1) mouse mAb (Biogen), BDA8 anti-human LT- β R (IgG1) mouse mAb (Biogen), anti-human LT- β R goat polyclonal Abs (PCA; University of California, Riverside, CA), NOK-1 anti-human FasL

(IgG1) mouse mAb (Caltag Laboratories, Burlingame, CA), M3 anti-human Fas (IgG1) mouse mAb (Immunex, Seattle, WA), anti-human TRAIL (anti-Apo-2L) rabbit PCA (Calbiochem, San Diego, CA), 32A1380 anti-human TRAILR1 (IgG1) mouse mAb (Imgenex, San Diego, CA), 54B1005 anti-human TRAILR2 mouse mAb (Imgenex), isotype control mouse mAbs (Caltag Laboratories), normal rabbit serum (Endogen), biotin-conjugated anti-mouse IgG (H + L) goat PCA (Jackson ImmunoResearch, West Grove, PA), biotin-conjugated anti-rat IgG (H + L) goat PCA (Jackson ImmunoResearch), biotin-conjugated anti-goat IgG (H + L) rabbit PCA, (Vector Laboratories, Burlingame, CA), CyChrome-conjugated anti-human HLA-DR (IgG1) mouse mAb (BD PharMingen, San Diego, CA), and all previously described conjugated and unconjugated Abs used for determination of DC phenotype and purification of monocytes and DCs (47). PE-conjugated streptavidin was obtained from Jackson ImmunoResearch. Streptavidin-HRP (SA-HRP) was purchased from Vector Laboratories.

We also used the following fusion proteins in the performance of this study: dimeric human TNFRp60:Fc fragment of human IgG1 (Biogen), dimeric human TNFRp80:Fc fragment of human IgG1 (Immunex), dimeric human LT- β :Fc fragment of human IgG1 (Biogen), dimeric mouse Fas:Fc fragment of human IgG1 (R&D Systems, Minneapolis, MN), dimeric human TRAILR2:Fc fragment of human IgG1 (Alexis Biochemicals), and dimeric human IL-4R:Fc fragment of human IgG1 (Immunex).

The following cytokines and ligands were used: recombinant human GM-CSF (Schering-Plough Research Institute, Kenilworth, NJ), recombinant human IL-4 (Schering-Plough Research Institute), recombinant human trimeric CD40L (Immunex), recombinant human trimeric FasL (Alexis Biochemicals), recombinant human trimeric Flag-FasL (Alexis Biochemicals), recombinant human trimeric TNF (Endogen), recombinant human LT- $\alpha_1\beta_2$ (Biogen), and recombinant human trimeric Apo-2L/TRAIL (Genentech, South San Francisco, CA). All reagents from Immunex, Biogen, UC, Genentech, and Schering-Plough Research Institute were generous gifts.

Polymerase chain reaction

Two sets of PCR primers were used. Primers of the first set had structures as previously described (23). The second set of primers was prepared to perform nested RT-PCR. The primers were designed using the Primer Express version 1.0 (Applied Biosystems, Foster City, CA). The gene sequences were obtained from the National Center for Biotechnology Information database (Bethesda, MD). The PCR primers and their product lengths were: TNF sense 5'-GGCAGTCAGATCATCTTCTCGA-3' and antisense 5'-TCAGCTTGAGGGTTTGCTACAA-3' (72 bp); FasL sense 5'-TGGCCCATTTAACAGGCAA-3' and antisense 5'-ACAATTCCATAGGTGCTTCCCA-3' (70 bp); and TRAIL sense 5'-GGGCATTCATTCCTGAGCAA-3' and antisense 5'-CTCCTGAAATCGAAAGTATGTTTGG-3' (105 bp).

RT-PCR for detection of mRNA expression of β -actin, TNF, LT- α , LT- β , FasL, and TRAIL was performed using their previously constructed sense/antisense primers and a previously described, slightly modified technique (23). RNA was extracted from sorted, highly purified (>98%) populations of monocytes and DCs ($0.5-1 \times 10^6$ of each), using the acid-guanidinium phenol-chlorophorm method. RT-PCR was performed by using the SuperScript preamplification system for first strand cDNA synthesis (Life Technologies) and the AmpliTaq Gold (Applied Biosystems) for PCR.

Nested RT-PCR was performed as follows. The PCR products, obtained as previously described (23), were diluted 10-fold in H₂O. A total of 5 μ l of the diluted PCR products were used as template for the second round of PCR, using the above described second set of primers. The nested PCR mixture (50 μ l) contained 5.5 mM MgCl₂, 0.5 μ M of each primer, 0.3 mM dNTP, 5 μ l of the diluted corresponding first-round PCR product, and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The amplification was performed on a PerkinElmer GeneAmp PCR 2400 thermal cycler (PerkinElmer, Norwalk, CT). The reaction was started with a 12-min incubation at 95°C, followed by 40 cycles of 15 s denaturation at 95°C, and finished by 60 s annealing and extension at 60°C. Eight-microliter aliquots of the each amplified product were separated by electrophoresis using 15% polyacrylamide gel (Bio-Rad, Hercules, CA) in Tris-borate EDTA buffer. The gels were stained with SYBR Green I (Sigma Aldrich, St. Louis, MO) diluted 1/10,000. Finally, to prove the specificity of RT-PCR, the amplicons were extracted from the gels and purified with the Concert rapid gel extraction/purification system (Life Technologies) and the obtained DNA was sequenced in an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Isolation of monocytes and DCs

Monocytes and DCs were purified from normal human peripheral blood mononuclear leukocytes (PBMNL) as >95% pure populations of CD5⁻CD3⁻TCR- $\alpha\beta$ ⁻CD19⁻CD56⁻CD16⁻CD14⁺ and CD3⁻TCR- $\alpha\beta$ ⁻CD14⁻CD19⁻CD16⁻CD56⁻CD11b⁻CD4⁺HLA-DR⁺ cells, respectively, as described (47).

Generation of immature and mature DCs in culture

Immature and mature DC were generated as described (47). Briefly, immature DCs were produced by 5–7 day culturing of purified blood monocytes in the presence of GM-CSF + IL-4. Mature DCs were obtained by a 2-day incubation of immature DCs in the presence of CD40 ligand (CD40L).

Cell lines

Normal and cancer cell lines used in this study were of human origin and they were maintained in culture as previously described (24, 47).

Cytotoxicity assays

MTT cytotoxicity assays were performed as previously described (25, 47). Killing mediated by soluble TNF family ligands was assessed by addition of various dilutions of rTNF, LT- $\alpha_1\beta_2$ and FasL, TRAIL, and CD40L to target cells either individually or in various combinations, as previously described (23). Blocking of the interactions between the TNF family ligands and their receptors was performed as previously described (23).

Flow cytometry

The analysis was performed using a FACScan (BD Biosciences, Mountain View, CA) flow cytometer. Phenotypes of monocytes and DCs were determined following their direct two-color staining with fluorochrome-conjugated mAbs specific for the lineage markers of T cells, B cells, NK cells, monocytes, and DCs as described (26). The cell surface or intracellular protein expression of TNF family ligands and their receptors was assessed using a previously described, highly sensitive, three-step flow cytometry technique (23). To assess expression of TNF family ligands on circulating DCs, three-color flow cytometry was performed with total PBMNL following their specific staining with Abs. First, the TNF family ligands were labeled with nonconjugated specific Abs, biotin-conjugated secondary Abs, and PE-conjugated streptavidin as described (23). Second, PBMNL were simultaneously labeled with CyChrome-conjugated anti-HLA-DR mAb and FITC-conjugated anti-CD3, anti-CD56, anti-CD14, and anti-CD19 mAbs in the presence of excess of nonconjugated normal mouse Ig (200 μ g/ml). Following the staining procedures, cells were fixed with 1% paraformaldehyde and analyzed. One- and two-color flow cytometry analyses were performed as previously described (23, 26). Three-color flow cytometry was performed by gating DCs (HLA-DR⁺/lineage marker⁻ PBMNL) and their analyzing for the expression of TNF family ligands. Specific reactivity of an Ab and levels of a cellular Ag expression were determined by comparative analyses of cell fluorescence and ratios between the mean fluorescence intensities of cells stained with specific Abs and isotype-matched control Abs, respectively.

Amplified immunocytofluorescence and confocal microscopy

DCs were resuspended in PBS containing 1% FCS and sequentially stained in cell suspensions with primary Abs against TNF family ligands (1 μ g/ml), biotin-conjugated goat anti-mouse Ig Abs (1 μ g/ml), SA-HRP (1/100 dilution), and cyanine 3-conjugated tyramide (NEN, Boston, MA). After each of these stainings, DCs were washed twice in PBS plus 1% FCS. Following the staining with tyramide and subsequent washing, DCs were resuspended in 25 μ l of PBS containing 5 μ g/ml of acridine orange (Sigma Aldrich), applied on poly-L-lysine-treated slides, and immediately examined by a Leica TCS-NT laser confocal microscope (Leica, Allendale, NJ). Two-color analysis was performed. The specimens were illuminated at 514 nm, the emitted image was split by a 565-nm beam splitter and collected simultaneously by two photomultipliers through a 535-nm (acridin orange) and 600-nm (cyanine 3) final barrier filter. Optical sections were cut with a 50 μ m confocal pinhole that ensures maximal Z resolution. In reconstructed series, images were collected every 1 μ m. Reconstruction was performed with ImageSpace Software (Molecular Dynamics).

Statistical analysis

Statistical analyses of the results were performed using the Wilcoxon's signed-rank pair tests. To determine whether data provide evidence for differences in profiles of percent killing as a function of E:T ratio or ligand

concentration, multivariate permutation methods were used. Differences were considered significant when $p < 0.05$.

Results

DCs express four cytotoxic TNF family ligands

In a companion manuscript (47), we have demonstrated that immature DCs can efficiently induce death of cancer cells *in vitro* using an apoptotic mechanism that involves both cell membrane-bound and -secreted cytotoxic molecules. Previous studies have shown that mouse and human DCs may express functional FasL and TRAIL (19–22), respectively. In addition, our recent findings have indicated that the TNF family death receptor signaling-specific caspase-8 is activated in cancer cells exposed to DC cytotoxic activity (47). Based on these findings, we hypothesized that human immature DCs might express and use to kill cancer cells cytotoxic TNF family ligands. Therefore, to determine the molecules which potentially mediate killing of cancer cells by DCs, we first examined whether freshly isolated blood immature DCs as well as *in vitro* generated, monocyte-derived, immature and mature DCs express mRNAs encoding TNF, LT- α , LT- β , FasL, and TRAIL. Freshly isolated blood monocytes, precursors of DCs, were also tested. Monocytes (Fig. 1A) and blood immature DCs (Fig. 1B) as well as both *in vitro*-generated immature (Fig. 1C) and mature (Fig. 1D) DCs consistently expressed mRNAs encoding each of these ligands. However, while the mRNAs could be detected in monocytes and in *in vitro*-generated monocyte-derived DCs using a standard RT-PCR, they could be found in blood DCs only by using a more sensitive nested RT-PCR. This finding indicates that blood DCs might express lower levels of the TNF family ligand mRNAs than monocytes and their *in vitro*-generated DC derivatives.

To determine whether transcription of the TNF family ligand genes was accompanied by specific protein expression in all four

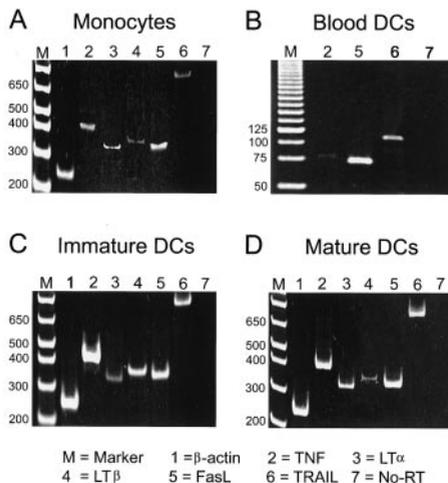


FIGURE 1. DCs express mRNAs of five TNF family ligands. All tested cell populations were $\geq 98\%$ pure. Monocytes and blood immature DCs were obtained by cell sorting of $CD14^+$ or $HLA-DR^+CD3^-CD56^-CD14^-CD19^-$ PBML, respectively. Immature and mature DCs were generated *in vitro* by culturing of purified blood monocytes in the presence of GM-CSF + IL-4 or GM-CSF + IL-4 + CD40L, respectively. Expression of the mRNAs in monocytes and monocyte-derived DCs was assessed by a standard RT-PCR, while the mRNA expression in blood DCs was examined using a nested RT-PCR. The data are from a representative experiment of five performed. The presented results are amplified cDNA products separated by PAGE and detected by SYBR Green fluorescent dye. A, Monocytes; B, freshly-isolated blood immature DCs; C, *in vitro* generated, monocyte-derived, immature DCs; D, *in vitro* generated, monocyte-derived, mature DCs.

APC types, or whether differential expression might be associated with the observed variations in their effector function (47), a flow cytometry was performed. Intact and permeabilized monocytes, intact blood immature DCs, and intact and permeabilized monocyte-derived immature and mature DCs were stained using Abs specific for the five TNF family ligands. Freshly-isolated intact monocytes consistently demonstrated cell surface expression of TNF only (Fig. 2, intact). In addition, monocytes of some individuals exhibited low levels of cell surface FasL. In contrast, both *in vivo*- (Fig. 3) and *in vitro*- (Fig. 4, intact) generated immature DCs consistently expressed all five TNF family ligands on their cell surface. Furthermore, based on different levels of expression of HLA-DR, blood-circulating DCs could be classified into two subsets, $HLA-DR^{low}$ (“immature”) and $HLA-DR^{int}$ (“mature”) DCs, which also displayed different levels of cell membrane-bound TNF family ligands. Thus, the former subset expressed higher levels of the ligands than the latter (Fig. 3). Monocyte-derived mature DCs, like monocytes, expressed only low levels of TNF on their cell surface (Fig. 5, intact). The cell surface levels of TNF family ligands were relatively low, even on immature DCs, and could be consistently detected only by using an amplified flow cytometry method. In contrast, when the cells were permeabilized, both types of DCs as well as monocytes were strongly and equivalently stained with the five ligand-specific probes (Figs. 2, 4, and 5, permeabilized). This indicates that the proteins of five cytotoxic TNF

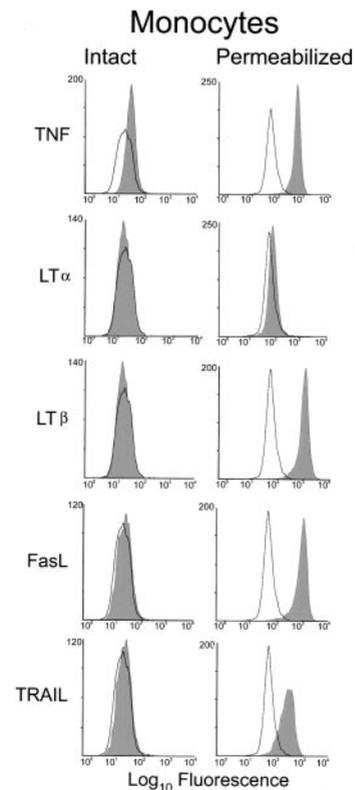


FIGURE 2. Monocytes express plasma membrane-bound TNF and cytoplasmic TNF, LT- α , LT- β , FasL, and TRAIL proteins. Monocytes were purified from PBML by negative immunoselection of $CD14^+CD3^-CD56^-CD19^-$ cells using immunomagnetic beads and were stained intact or permeabilized with specific mAbs for TNF family ligands. Single-color flow cytometry analysis was then performed, as described in *Materials and Methods*. The data are from a representative experiment of five performed. The results are presented as overlays of single-color histograms of \log_{10} fluorescence intensity obtained with isotype-matched control (open histograms) and specific mAbs (filled histograms).

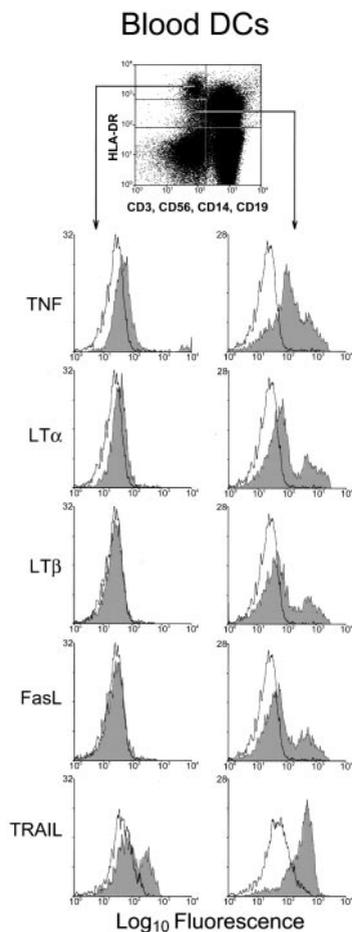


FIGURE 3. Immature peripheral blood DCs express the TNF, LT- α , LT- β , FasL, and TRAIL proteins on their cell surface. PBMDL were simultaneously stained with PE-conjugated Abs specific for TNF family ligands, CyChrome-conjugated anti-HLA-DR mAb and FITC-conjugated anti-CD3, anti-CD56, anti-CD14, and anti-CD19 mAbs. Three-color flow cytometry analysis was performed. First, HLA-DR⁺ lineage marker⁻ cells (DCs) were defined by two-color analysis. Then, HLA-DR^{low} and HLA-DR^{int} DCs were electronically gated and analyzed for expression of TNF family ligands. Definition and gating of DCs was depicted in the two-color dot plot, while expression of TNF family ligands was presented by single-color histograms of log₁₀ fluorescence intensity, obtained with isotype-matched control (open histograms) and specific mAbs (filled histograms). The data are from a representative experiment of five performed.

family ligands were expressed at high and similar quantities in the cytoplasm of all three types of APCs. The finding of coexpression of LT- α and - β on the cell surface of DCs indicates that the membrane-bound LT- $\alpha_1\beta_2$ heterotrimer might be present on these cells. These data are in a full accordance with those for mRNA expression (Fig. 1, *B* and *C*), and provide clear evidence that human immature DCs express on their cell surface at least four cytotoxic TNF family ligands, including TNF, LT- $\alpha_1\beta_2$, FasL, and TRAIL.

To determine the topography of the ligands on cell surface of immature DCs, we performed confocal microscopy using specific Abs against TNF family ligands and an amplified immunocytofluorometric method. As can be seen in Fig. 6, FasL was not evenly distributed on the cell surface, but it appeared to be principally arrayed on the protrusions and dendrites of the cell membrane. Similar data were obtained in analyses of the other cell-surface-expressed TNF family ligands (data not shown). The selective expression of such ligands on DC dendrites might increase the focal

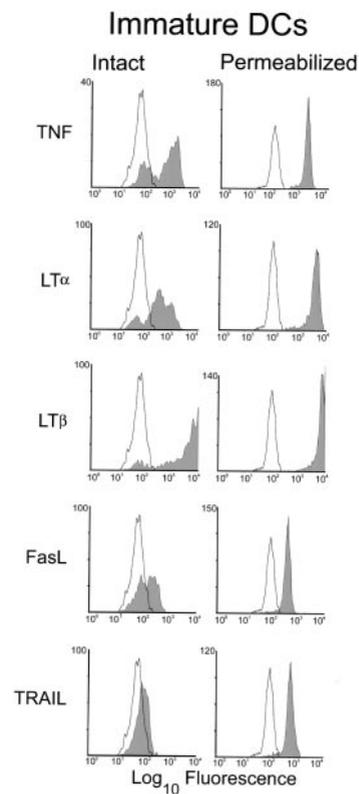


FIGURE 4. In vitro-generated, monocyte-derived immature DCs express cell surface and cytoplasmic TNF, LT- α , LT- β , FasL, and TRAIL proteins. Immature DCs were generated by 7-day culturing purified blood monocytes in the presence of GM-CSF + IL-4. Single-color flow cytometry analysis for the expression of TNF family ligands by immature DCs was performed exactly as described in Fig. 2. The data are from a representative experiment of seven performed.

density and lethality of these molecules in intimate contact points of DC-tumor cell conjugates.

DCs produce soluble forms of the TNF family ligands

Based on our previous finding that DC conditioned media contain tumoricidal proteins (47), we analyzed such media for the presence of soluble TNF and FasL, using specific ELISAs. We observed the presence of substantial levels of both ligands in the supernatants of both immature and mature DCs (Table I). However, significantly higher levels of these ligands were present in the conditioned culture media of mature vs immature DCs.

Differential expression of TNF family receptors on tumor cells and normal cells

TNF family ligands mediate death in susceptible target cells by interacting with their cell surface-bound TNF family death receptors. Therefore, if this ligand-receptor interaction is a physiologically relevant molecular mechanism used by DCs to kill cancer cells, then cancer cells susceptible to killing by DCs should express at least a subset of these TNF family receptors. In support of this model, we have previously demonstrated (23), using flow cytometry, that cells of a variety of human cancer cell lines express multiple TNF family receptors, including TNFR1, TNFR2, LT- β R, and Fas. In the present study, we extended this investigation and demonstrated that cancer cell lines also expressed TRAILR1 and TRAILR2 (Fig. 7). However, while TNFR1, LT- β R, Fas, and TRAILR1 were consistently expressed on all 20 cancer cell lines tested, TNFR2 and TRAILR2 were significantly expressed only on

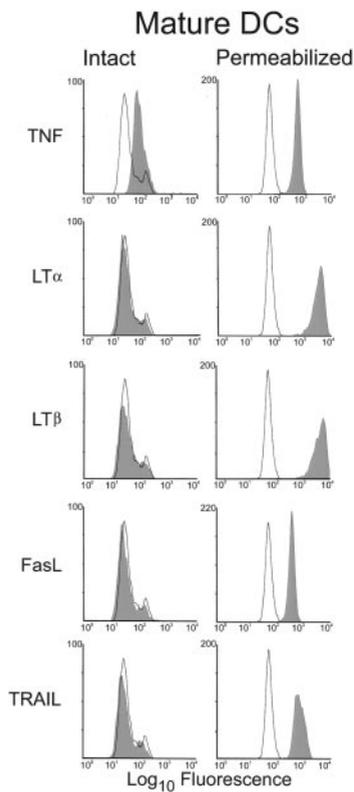


FIGURE 5. In vitro-generated, monocyte-derived mature DCs express only TNF on the cell surface, but contain the TNF, LT- α , LT- β , FasL, and TRAIL proteins in the cytoplasm. Mature DCs were generated by culturing purified blood monocytes in the presence of GM-CSF + IL-4 for 5 days and in the presence of GM-CSF + IL-4 + CD40L for an additional 2 days. Single-color flow cytometry analysis for the expression of TNF family ligands by mature DCs was performed as described in Fig. 2. The data are from a representative experiment of seven performed.

a proportion of these lines. Interestingly, cancer cell lines, which did not significantly express TNFR2 and TRAILR2 on their cell surface, generally showed lower susceptibility to killing by DCs than those which significantly expressed these receptors. Such examples were lung cancer 226 and Daudi compared with lung cancer 596 and Jurkat, respectively (47) (data not shown). These data indicate that TNFR2 and TRAILR2 may have a significant role in DC-mediated killing of cancer cells. In addition, we determined that freshly isolated noncultured tumor cells (e.g., squamous cell carcinoma of head and neck) expressed all the TNF family receptors found on cultured cancer cells. The pattern of expression of the death receptors on freshly-isolated cancer cells was similar to that described above for cultured cancer cells (data not shown). In contrast to the large majority of cancer cell lines and freshly isolated tumor cells, which consistently showed both susceptibility to DC-mediated killing (47) and cell surface expression of multiple TNF family death receptors, normal cells such as DCs, T cell blasts, fibroblasts, mammary epithelial cells, melanocytes, and keratinocytes were resistant to killing by DCs (47) and consistently expressed only LT- β R and Fas, but either did not express or expressed at very low levels, TNFR1, TNFR2, TRAILR1, and TRAILR2 on their cell surfaces (Fig. 8, intact). However, significant levels of these molecules were observed in the permeabilized cells (Fig. 8, permeabilized). Among several cancer cell lines tested, only the Pittsburgh Cancer Institute-30 SCCHN line showed a pattern of expression of TNF family receptors (data not shown) and resistance to killing by DCs (47), which was similar to that of normal

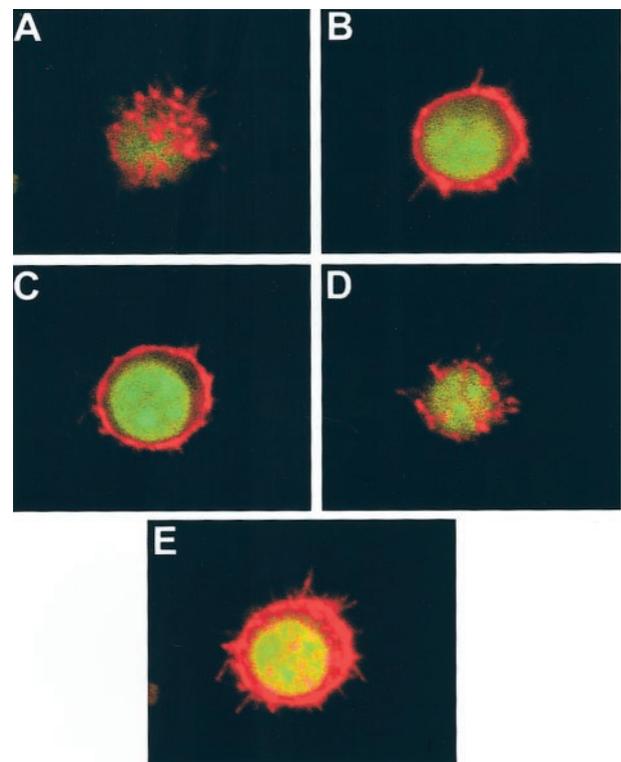


FIGURE 6. TNF family ligands are selectively expressed on the cell membrane processes and dendrites of DCs. Topography of the FasL protein on the cell surface of immature DCs was examined by confocal microscopy. *A–D* are optical sections. *E* is a reconstructed image of 16 0.25- μ m thick optical sections collected at 1 μ m intervals through a DC. *A* and *D* are two opposite spherical sectors of DCs with visible large cell surface areas containing several prominent, selectively stained cell membrane protrusions and dendrites. *B* and *C*, A few stained, laterally-extended cell membrane processes from two optical sections of DC are visible. *E*, Stained cell membrane processes are also clearly visible on the reconstructed image of DC.

cells. These data demonstrate that cancer cells express a more diverse set and/or higher levels of TNF family death receptors on their cell surface than normal cells. Therefore, DCs and cancer cells might be able to effectively interact via a range of complementary TNF family ligands and receptors, respectively, resulting in tumor cell apoptosis.

DCs mediate tumor cell apoptosis via a pathway involving multiple TNF family ligand-receptor interactions

To directly test the above possibility, we performed blocking experiments in which we attempted to interfere with the killing of cancer cells by DCs using blocking Abs or R:Fc fusion proteins

Table I. Immature and mature DCs produce different quantities of soluble TNF and FasL^a

Ligands	pg/ml/10 ⁶ Cells	
	Immature DCs	Mature DCs
TNF	84.5 \pm 3.5	706.5 \pm 7.5
FasL	180.4 \pm 2.1	341.4 \pm 0.6

^a Immature DCs were generated and cultured for 5 days in GM-CSF + IL-4, washed, and maintained in culture (1.0 \times 10⁶/ml) for an additional 48 h in the presence of GM-CSF + IL-4 or GM-CSF + IL-4 + CD40L. Cell-free supernatants were collected and assessed for the presence of the ligands using specific ELISA. The data from one representative experiment of five similar experiments performed with DCs obtained from different donors are reported. The results are means \pm SD of triplicates.

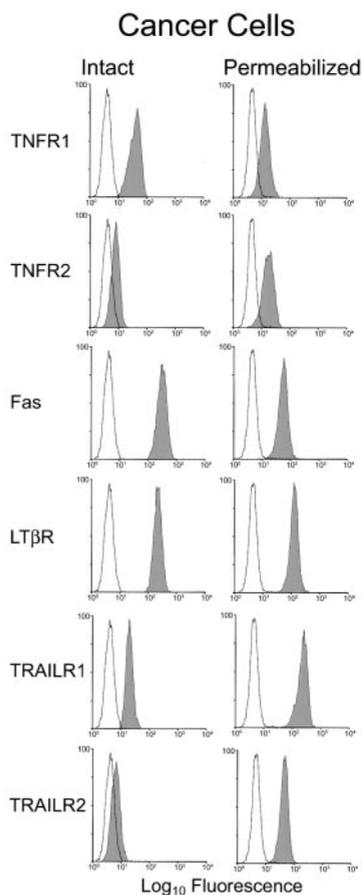


FIGURE 7. Cancer cells express multiple TNF family death receptors on their cell surfaces. PCI-13 SCCHN cells were stained intact or permeabilized, and then stained with specific mAbs for TNF family receptors. Single-color flow cytometry analysis was then performed, as described in *Materials and Methods*. The results are presented as overlays of single-color histograms of \log_{10} fluorescence intensity obtained with isotype control (open histograms) and specific mAbs (filled histograms).

specific for the TNF family ligands or their receptors. Immature DCs were preincubated with individual or combined Abs or R:Fc fusion proteins specific for TNF, LT- α , LT- β , LT- $\alpha_1\beta_2$, FasL, and TRAIL, while cancer cells were preincubated with individual or combined Abs specific for TNFR1, TNFR2, LT- β R, Fas, TRAILR1, and TRAILR2. The effects of these treatments on DC-mediated killing of cancer cells were assessed, using 3-h MTT cytotoxicity assays. Representative data of 1 of 10 similar experiments performed are shown in Fig. 9. We observed that each individual reagent which blocks interactions between either TNF-TNFRs, LT- $\alpha_1\beta_2$ -LT- β R, FasL-Fas, or TRAIL-TRAILRs consistently, but partially inhibited the induction of cell death in tumor cells mediated by immature DCs. The levels of inhibition were generally similar for different ligand-receptor pairs, and using optimal blocking reagents such as R:Fc fusion proteins, consistently ranged between 40 and 50% (Fig. 9B). Importantly, simultaneous disruption of the all four ligand-receptor pairs by the combined treatment of DCs with the TNFR1:Fc, LT- β R:Fc, Fas:Fc, and TRAILR2:Fc fusion proteins (Fig. 9B) or cancer cells with the anti-TNFR1, anti-TNFR2, anti-LT- β R, anti-Fas, anti-TRAILR1, and anti-TRAILR2 mAbs (Fig. 9C) more efficiently blocked DC-mediated killing of cancer cells than the individual disruption of the corresponding ligand-receptor pairs, and decreased this activity by $\sim 75\%$. Control isotype-matched (nonreactive) mAbs, normal human Ig, or IL-4R:Fc fusion protein failed to suppress DC-me-

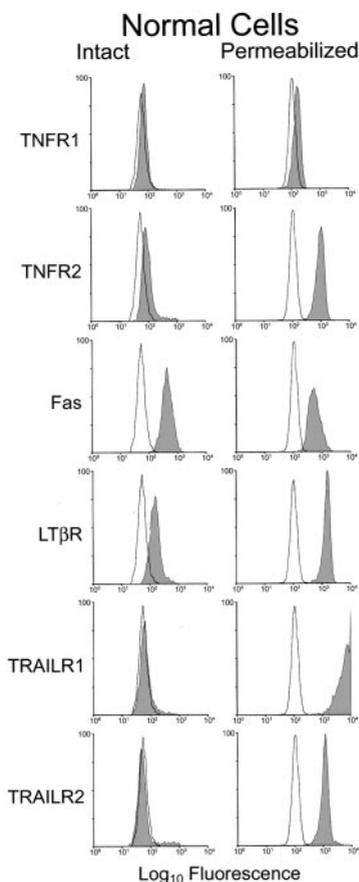


FIGURE 8. Monocyte-derived immature DCs express cell surface TNFR2, Fas, and LT- β R, but not TNFR1, TRAILR1, and TRAILR2. Immature DCs were stained intact or permeabilized, and then stained with mAbs specific for TNF family receptors and analyzed using flow cytometry as described in Fig. 7. Similar data were obtained with normal T cell blasts, fibroblasts, melanocytes, and keratinocytes.

diated killing of cancer cells. These data show that most of DC tumoricidal activity is mediated by a coordinate engagement of TNF, LT- $\alpha_1\beta_2$, FasL, and TRAIL, and that all four cytotoxic TNF family ligands are critical mediators and equal contributors in this activity. The results may also indicate a minor participation of additional, yet unknown, cytotoxic molecules in DC tumoricidal activity.

Of note, additional assays involving soluble mediators suggested that the death of cancer cells could be efficiently induced by recombinant soluble TNF family ligands used in combinations of two (TNF and Apo-2L/TRAIL, FasL and Apo-2L/TRAIL, or LT- $\alpha_1\beta_2$ and Apo-2L/TRAIL) or three (i.e., TNF, LT- $\alpha_1\beta_2$, and FasL; TNF, LT- $\alpha_1\beta_2$, and Apo-2L/TRAIL; or TNF, FasL, and Apo-2L/TRAIL), able to engage at least three death receptors. However, tumor cell death was not effectively induced when these reagents were applied individually or in combinations of two (TNF and FasL, TNF and LT- $\alpha_1\beta_2$, or FasL and LT- $\alpha_1\beta_2$), capable of engaging only one or two death receptors (Ref. 23, data not shown). The ligands showed similar activity in a wide range of concentrations (from 0.01 to 50.00 ng/ml). These data show that cancer cells express functional TNFR1, TNFR2, LT- β R, Fas, TRAILR1, and TRAILR2, which are able to signal apoptosis when they are simultaneously and coordinately engaged. They also complement the above results of blocking experiments and additionally support the possibility that DCs mediate the apoptotic killing of cancer

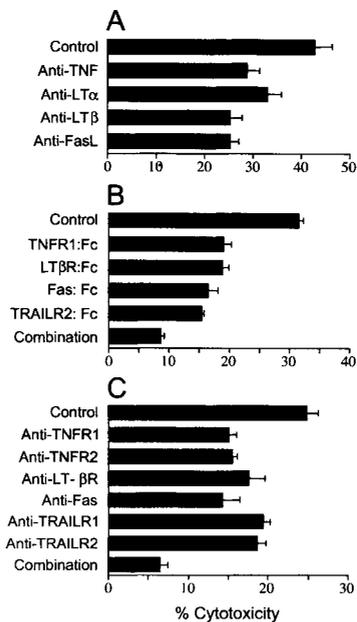


FIGURE 9. DCs induce apoptosis in tumor cells by simultaneously engaging multiple TNF family death receptors. Blocking experiments were performed using 3 h MTT cytotoxicity assays with immature DCs as effectors and PCI-13 SCCHN cells as targets at a 1:1 E:T ratio. Where indicated, effector cells were preincubated with individual Abs, or individual or combined (combination) R:Fc fusion proteins specific for TNF family ligands, while target cells were preincubated with individual or combined (combination) Abs specific for TNF family receptors. Following this preincubation, the cytotoxic activity of DCs was assessed. *A*, Blocking of DC-mediated killing by Abs specific for ligands. *B*, Blocking of DC-mediated killing by R:Fc fusion proteins specific for TNF family ligands. *C*, Blocking of DC-mediated killing by Abs specific for TNF family receptors. Data represent the mean percentages of cytotoxicity \pm SD. Controls include isotype-matched nonreactive mAbs, normal human Ig, and IL-4R:Fc fusion protein.

cells via a simultaneous and coordinate engagement of their multiple TNF family ligands with corresponding death receptors of cancer cells. The findings also indicate that normal cells are spared from killing by DCs, because they express only one or two TNF family death receptors which engagement is not sufficient to efficiently signal apoptosis.

Discussion

The results obtained in this study provide novel, biologically significant information that human immature DCs express at least four different cytotoxic TNF family ligands and use these deadly molecules in a coordinated and synchronized manner to effectively destroy cancer cells by inducing apoptosis.

TNF, LT- $\alpha_1\beta_2$, FasL, and TRAIL are potent cytotoxic TNF family ligands with the unique function of inducing apoptosis in susceptible cancer cells (27–29). All four ligands are expressed and used to kill cancer cells by professional cytotoxic cells, such as CD8⁺ CTLs and NK cells (23, 27–35). FasL, TNF, and TRAIL are also expressed by activated CD4⁺ T cells, B cells, and macrophages (27, 28, 31, 36–42). In addition, it has been shown that FasL may be expressed by mouse immature DCs (19, 20), while TRAIL and TNF by human-activated DCs (21, 22, 43). Here, we first demonstrate that human immature DCs (both freshly isolated from the blood and in vitro generated by GM-CSF plus IL-4 stimulation of monocytes) express TNF, LT- $\alpha_1\beta_2$, FasL, and TRAIL on their cell membrane. In contrast, monocytes and mature DCs (generated by CD40L stimulation of immature DCs) express only

significant levels of TNF on their cell surface. However, high levels of all four ligands were detected in the cytoplasm of each of these cell types. This observation suggests that appropriate stimulation of the APCs might rapidly induce the translocation to the cell surface and/or the release into extracellular microenvironment of these intracellular molecules. Similar to in vitro-generated immature and mature DCs, freshly isolated blood HLA-DR^{low} (immature) DCs express higher cell surface levels of the cytotoxic TNF family ligands than their HLA-DR^{int} (mature) counterparts. In addition, in vitro-generated mature DCs produced higher levels of soluble TNF and FasL than immature DCs. Therefore, the cell surface expression and secretion of these cytotoxic ligands appears to be tightly regulated, and differentiation stage-dependent within the DC “lineage”. The possible regulatory mechanism might include activities of cytokines and/or metalloproteases (27, 28). This pathway has been currently scrutinized in our laboratory.

Previous studies have indicated that FasL (19, 20) and TRAIL (21, 22) may be used by mouse immature DCs and IFN-activated human DCs, respectively, to kill rare susceptible target cells. Our study shows that a diverse array of human cancer cell lines as well as freshly isolated cancer cells express multiple TNF family death receptors (i.e., TNFR1 and/or TNFR2, LT- β R, Fas, TRAILR1 and/or TRAILR2) and are efficiently killed by DCs. However, the susceptibility of cancer cells to killing by DCs appears to be dependent on the multiplicity and levels of cell membrane expression of their deadly receptors. In contrast, normal cells either do not express or express very low levels of TRAILR1, TRAILR2, TNFR1, and/or TNFR2 and are resistant to killing mediated by DCs (47). This suggests that cell surface expression of multiple TNF family death receptors on target cells may define their susceptibility to killing by DCs. Individually applied rTNF, LT- $\alpha_1\beta_2$, FasL, or TRAIL are minimally cytotoxic to most cancer cell lines that we have evaluated (data not shown). In addition, monocytes, which express only TNF, are not discernibly cytotoxic against cancer cells (47), while immature DCs, which express multiple cell membrane-bound TNF family cytotoxic ligands, are cytotoxic for most cancer cell lines. This indicates that cell surface expression of multiple cytotoxic TNF family ligands may determine the tumoricidal activity of DCs. Individual and especially combined disruption of interactions between TNF, LT- $\alpha_1\beta_2$, FasL, or TRAIL and their receptors notably inhibits DC-mediated killing of cancer cells. In contrast, simultaneous application of two or more recombinant cytotoxic TNF family ligands, which can engage at least three death receptors, rapidly induces apoptosis of cancer cells. Taken together, these findings show that human immature DCs kill cancer cells (47) by the simultaneous and coordinate engagement of their multiple TNF family ligands with the corresponding multiple death receptors on cancer cells.

These findings also point out that cancer cells are relatively resistant to apoptosis mediated through individual TNF family ligands and suggest that tumors may use anti-apoptotic mechanisms as survival skills. These mechanisms may be related to the low levels of cell surface expression of the receptors and/or to the activity of intracellular anti-apoptotic molecules (27, 43). Indeed, we observed that the TNF family death receptors are expressed on the cell surface of cancer cells at low levels, which are unfavorable for their ligand-mediated homotrimerization necessary for generation of an effective apoptotic signal. Under such conditions, the coordinate engagement of at least three different death receptors, sharing a domain of homology involved in generation of common proapoptotic signals, may result in their heterotrimerization leading to triggering of tumor cell apoptosis (23). This hypothesis is circumstantially supported by the presence of common death domain in four of the six death receptors expressed on cancer cells

(TNFR1, Fas, TRAILR1, and TRAILR2; Refs. 27, 28). Cell membrane-bound forms of cytotoxic TNF family ligands have been observed to be more cytotoxic than their soluble forms, that are frequently nonfunctional and may serve as functional antagonists of their cell membrane-bound forms (27, 28). In our study, mature DCs produced higher levels of the secreted forms of TNF and FasL than immature DCs and expressed lower levels or lack the cell membrane-bound forms. Consistent with these findings, mature DCs were less effective at inducing apoptosis against cancer cells than immature DCs (47).

This maturation-dependent change in expression of cytotoxic TNF family ligands (in terms of forms and function) might be also relevant to the maturation-dependent changes of Ag-presenting function of DCs. In this way, immature DCs most effectively prepare (by inducing apoptosis of tumor cells), capture, and process exogenous Ags (such as dying tumor cells), while mature Ag-loaded DCs are most predisposed to present tumor Ags and to promote the activation and expansion of Ag-specific T cells via direct cell-to-cell contact. Depending on the signals they receive (complete or incomplete), Ag-specific T cells are believed to differentiate into two different cell populations, one that is relatively resistant, and one that is relatively sensitive to TNF family ligand-induced apoptosis, respectively (44–46). Therefore, the low apoptosis-inducing ability of mature DCs might serve as a safeguard against the inappropriate death of clinically-important Ag-specific T cells (39).

We have shown that not only in vitro generated, monocyte-derived, but also freshly isolated peripheral blood immature DCs express the four major cytotoxic TNF family ligands and exhibit a marked apoptosis-inducing anticancer activity (47). Thus, this cytotoxic activity might be operative in vivo and may be critically and directly involved in the elimination of evolving tumors as well as in the control of tumor growth in situ. In addition to these direct anticancer activities, the ability of immature DCs to promote tumor apoptosis clearly suggests their pivotal role in the initiation of processing and, subsequently, cross-presentation of cellular tumor Ags and generation of specific anti-tumor T effector cells believed to be critical to effective immunotherapy of cancer (7, 17).

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