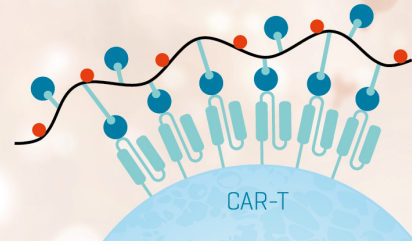


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Virus or TLR Agonists Induce TRAIL-Mediated Cytotoxic Activity of Plasmacytoid Dendritic Cells¹

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Among dendritic cells, plasmacytoid dendritic cells (PDC) represent a functionally distinct lineage. Regarding innate immunity, PDC secrete large amounts of type I IFN upon viral exposure or stimulation by microbial products such as unmethylated CpG-motif containing oligo-DNA due to their selective expression of TLR7 and TLR9. We asked whether they could acquire cytotoxic functions during the early phases of infection or after activation with TLR7 or TLR9 agonists. In the present study, we describe a human PDC cell line called GEN2.2, derived from leukemic PDC, that shares most of the phenotypic and functional features of normal PDC. We show that after contact with the influenza virus, GEN2.2, as well as normal PDC, acquires TRAIL and killer activity against TRAIL-sensitive target cells. Moreover, we show that activation of GEN2.2 cells by CpG-motif containing oligo-DNA or R848 also induces TRAIL and endows them with the ability to kill melanoma cells. Therefore, PDC may represent a major component of innate immunity that could participate to the clearance of infected cells and tumor cells. This phenomenon could be relevant for the efficacy of TLR7 or TLR9 agonists in the therapy of infectious disease and cancer. *The Journal of Immunology*, 2006, 176: 248–255.

Plasmacytoid dendritic cells (PDC)⁴ are thought to link innate and adaptive immunity during microbial infection (1). PDC found as precursors in blood are the natural IFN-producing cells; they are also located in the T cell zone of lymphoid tissue, in tonsils, and in the thymus. Under pathological conditions, they infiltrate cutaneous lesions of lupus erythematosus (2) or mucosa during allergy (3) or infection (4). Regarding human cancers, PDC have been found associated with melanoma (5, 6), head and neck carcinoma (7), ovarian carcinoma (8), and breast cancer (9), but little is known about their role in tumor biology. In response to the virus, PDC secrete inflammatory cytokines and high levels of type I IFN (IFN- $\alpha\beta$) (10, 11), which regulates a diverse set of cytokines and their receptors, and directly inhibits the intracellular-viral life cycle. Regarding adaptive immune responses, type I IFN activates dendritic cell (DC) maturation (12), allows their licensing for cross-priming (13), and favors the priming of CD4⁺ T cells toward the Th1 pathway (14).

PDC express TLR7 and TLR9 (15, 16), both triggering type I IFN production (17, 18). Antitumor activity of CpG-motif containing oligo-DNA (CpG ODN) (19–22) (TLR9 agonist) and of imiquimod (23, 24) (TLR7 agonist) has been demonstrated in dif-

ferent murine tumor models. In man, topical treatment with imiquimod is used for enhancing immune response to treat external genital warts, diseases caused by papillomavirus, as well as basal cell carcinoma (25). It may also induce the regression of melanoma lesions (26, 27). The mechanisms responsible for antitumor or antiviral effects of these immune modifiers are poorly understood but might involve the recruitment and activation of PDC. Indeed, in a murine model of melanoma, the antitumor effect of imiquimod could be mediated in part by the recruitment of PDC to the treated skin (28).

By producing large amounts of type I IFN, PDC could also be important in stimulating the host innate immune response. Indeed, IFN- $\alpha\beta$ has been described in particular to orchestrate the elimination of infected cells and tumor cells by inducing TRAIL expression on NK cells (29) and T lymphocytes (30). In the present study, we asked whether activated PDC could play a direct role in the clearance of infected cells or tumor cells by acquiring killer functions via TRAIL expression, hence becoming cytotoxic effector cells.

Because of the low frequency of PDC in human blood, we tried and succeeded to generate a PDC line established from the new leukemic entity derived from PDC that we described recently (31). Leukemic PDC (LPDC) are characterized by their phenotype: CD4⁺, CD56⁺, CD123⁺, CD3⁻, CD13⁻, and CD19⁻. Clinically, this aggressive pathology presents extranodal and skin lesions and frequently progresses to leukemia (32). Moreover, LPDC overexpress mRNA encoding the lymphoid B cell characteristic J chain and Ig λ and κ light chains (33); they express BDCA-2 and BDCA-4 and polarize naive T cells toward Th1 or Th2 pathways (34). Because malignant cells share phenotypic and functional features with their normal counterpart, these LPDC and the GEN2.2 cell line we describe here are of particular interest to study PDC functions.

In the present study, we show that activation by microbial products turns GEN2.2 and normal PDC into powerful killer cells against TRAIL-sensitive target cells. After contact with the influenza virus, CpG ODN, or R848, PDC express TRAIL and become

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⁴ Abbreviations used in this paper: PDC, plasmacytoid DC; DC, dendritic cell; LPDC, leukemic PDC; CpG ODN, CpG-motif containing oligo-DNA; MoDC, myeloid DC; h, human; s, soluble.

able to kill infected and tumor target cells. Induction of TRAIL on GEN2.2 by the virus is regulated by an autocrine IFN- α loop. Therefore, PDC may represent in vivo a major component of innate immunity participating in the early clearance of TRAIL-sensitive infected cells and tumor cells.

Materials and Methods

Cells

Malignant LPDC were obtained from invaded peripheral blood of one patient, previously described as GEN, LPDC#7 (31, 34). Mononuclear cells were cryopreserved and they contained >98% tumor cells, as determined by flow cytometry.

Normal PDC were isolated from PBMC with a BDCA-4 cell isolation kit (Miltenyi Biotec). Their purity, checked with anti BDCA-2 and CD123 mAbs, was >90%.

The murine stromal cell line MS-5 (provided by R. Berthier, Commissariat à l'Énergie Atomique, Grenoble, France), A549 lung carcinoma (a gift from J.-L. Coll, INSERM U578, Grenoble, France), and Mel (a gift from M.-J. Richard, Unité Mixte de Thérapie Cellulaire et Tissulaire, Grenoble, France) were grown in RPMI 1640 Glutamax (Invitrogen Life Technologies) supplemented with sodium pyruvate, gentamicin, nonessential amino acids (referred to as complete medium), and 10% FCS (Invitrogen Life Technologies) and passaged weekly.

Myeloid DC (MoDC) were generated from blood monocytes and purified from fresh blood by the Rosette Sep isolation kit (StemCell Technologies) by a 6-day culture in complete medium and 10% of deplete complemented FCS added to 500 U/ml GM-CSF (Leucomax) and 10 ng/ml IL-4 (PeproTech). At the end of the culture, these DC (MoDC) were 100% CD1a⁺, CD14⁻, and CD83⁻.

Establishment of long-term LPDC culture

The MS-5 cell line was chosen as feeder for its ability to allow the proliferation of human progenitor cells (35). Five million LPDC from patient GEN were seeded in a flask (25 cm²) precoated with confluent irradiated (60 Gy) MS-5. During the first 5 wk of the culture, Flt3-L (50 ng/ml) and stem cell factor (10 ng/ml) were added to complete 10% FCS medium, and the cells were counted and diluted weekly. At this stage of the culture, a sustained proliferation was observed, and then, over 4 wk, 0.6 million tumor cells were transferred with 1 million irradiated MS-5 every week to a new flask (25 cm²) in 6 ml of fresh medium with cytokines. The cell line established was called GEN2. We then stopped stem cell factor and Flt3-L, and the cell line went on proliferating on the MS-5 feeder cell line. This cell line was called GEN2.2 and was maintained in culture during >5 mo.

Abs, flow cytometry, and microscopy

Immunophenotype was analyzed by flow cytometry on a FACScan (BD Biosciences), using direct or indirect labeling. The following mAbs were obtained from Immunotech: CD1a (BL6), CD1c (L161), CD3 (UCHT-1), CD4 (13B8.2), CD7 (M-T701), CD8 (B9.11), CD11c (BU15), CD14 (RMO52), CD16 (3G8), CD19 (J1.119), CD40 (mAb89), CD45RA (J33), CD56 (N901), CD62L (DREG56), CD64 (22), CD80 (MAB104), CD83 (HB15a), CD86 (HA5-2B7), anti-HLA ABC (B9.12.1), and HLA DR (B8.12.2). Anti-BDCA2 (AC144) and BDCA4 (AD5-17F6) were purchased from Miltenyi Biotec, CD123 (9F5) and CLA (HECA-452) were purchased from BD Pharmingen, and CD13 (WM-47) and CD33 (WM-54) were purchased from DakoCytomation. Unconjugated anti-TRAIL (2E5) was obtained from Alexis, and PE-conjugated anti-TRAIL (RIK-2) was obtained from eBioscience. Anti-IFN- γ R was purchased from Genzyme, and anti-IFN- α BR (MMHAR-2) and blocking polyclonal anti-IFN- α and anti-IFN- β were purchased from PBL Laboratories. Cytospins of cell suspensions were stained with May-Grünwald Giemsa (Kit Ral 555; Ral) for microscopic examination.

RT-PCR

Total RNAs isolated from GEN, GEN2.2, MoDC, and MS-5 cells using RNeasy kit (Qiagen) were converted to cDNA by standard methods using reverse transcriptase (Roche) and dNTP (Roche). These cDNA were amplified with specific primers (pre-T α , λ like, SpiB, and actine) as described previously (36). Primers used for TLR were as follows: human (h)TLR6, forward, 5'-CCC TCA ACC ACA TAG AAA CG-3', and reverse, 5'-GAG ATA TTC CAC AGG TTT GG-3'; hTLR7, forward, 5'-TTA CCT GGA TGG AAA CCA GCT ACT-3', and reverse, 5'-TCA AGG CTG AGA AGC TGT AAG CTA-3'; hTLR8, forward, 5'-AAC TTT CTA TGA TGC TTA CAT TTC TTA TGA C-3', and reverse, 5'-GGT GGT AGC GCA

GCT CAT TT-3'; hTLR9, forward, 5'-TGA AGA CTT CAG GCC CAA CTG-3', and reverse, 5'-TGC ACG GTC ACC AGG TTG T-3'; and hTLR10, forward, 5'-TTT GAT CTG CCC TGG TAT CTC A-3', and reverse, 5'-AGT TGT TCT TGG GTT GTC CTA AC-3'. After amplification, PCR products were separated by electrophoresis on agarose gel containing ethidium bromide and visualized by UV light illumination.

Activation/differentiation of GEN2.2 cells

Cells were cultured at 10⁶ cells/ml in complete 10% FCS medium, without MS-5 cells. Initially, two culture conditions were analyzed: 1) "virus" by stimulation with 137 ng/ml hemagglutinating formaldehyde-inactivated influenza virus strain A/New Caledonia/20/99 IVR116 (Aventis Pasteur); and 2) "IL-3+CD40L" by addition of IL-3 (10 ng/ml; PeproTech), in the presence of soluble human rCD40L (soluble (s)CD40L; Alexis). After 24 or 48 h, phenotypic and functional analyses were performed. Culture supernatants were cryopreserved for cytokine measurements. These supernatants were tested for IFN- α content by ELISA (Immunotech) and for IFN- γ , IL-1 β , IL-6, IL-8, IL-10, IL-12, and TNF- α by Cytometric Bead Array kit (BD Biosciences).

Naive T lymphocyte activation and polarization

Proliferation of naive T lymphocytes was evaluated in response to GEN2.2 cells precultured 24 h in medium, virus, or IL-3+sCD40L. CD4⁺CD45RA⁺ lymphocytes were isolated from cord blood by negative immunomagnetic depletion (StemCell Technologies), resulting in >97% purity. Mixed lymphocyte cultures were conducted in quadruplicate in 200- μ l 96-well flat-bottom plates (Falcon) by mixing 25 \times 10³ responding purified CD4⁺CD45RA⁺ cells and 5–25 \times 10³ irradiated (30 Gy) GEN2.2 cells. Six-day cultures were performed in complete medium supplemented with 15% heat inactivated human AB serum. A total of 37 \times 10³ Bq of [³H]thymidine was added to each well and harvested 18 h later.

To evaluate T lymphocyte polarization, 1 \times 10⁴ irradiated GEN2.2 cells were cocultured with 5 \times 10⁴ CD4⁺CD45RA⁺ cord blood T cells during 6 days. The supernatant were then tested for their IL-2, IL-4, IL-5, IL-10, IFN- γ , and TNF- α contents by flow cytometry (CBA kit; BD Biosciences).

TRAIL detection

GEN2.2 cells were cultured for 4–24 h in the presence of inactivated influenza virus (as previously described), then cells were harvested and analyzed for TRAIL expression either by flow cytometry (2E5 anti-TRAIL mAb detected with PE-conjugated goat anti-mouse mAb) or by Western blot on protein extract. Briefly, cells (10⁶), infected or not, were washed in PBS, lysed in 100 μ l of sample buffer and heated at 100°C for 5 min. Twenty microliters of the whole extract was loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad). After blocking nonspecific binding sites with 5% nonfat milk in PBS-Tween 20 0.1%, the membranes were incubated with primary Abs: anti-TRAIL (2E5) and anti-actin (Sigma-Aldrich). Ab labeling was revealed using goat anti-mouse or anti-rabbit HRP conjugated secondary Abs (DakoCytomation) and was visualized using ECL (Amersham Biosciences).

TRAIL expression was also analyzed after activation of GEN2.2 by TLR ligands. Cells were cultured for 24 h (10⁶/ml) in the presence of the virus, CpG ODN 2336 (12.5 μ g/ml TLR9 ligand; Coley Pharmaceuticals), or R848 (1 μ g/ml TLR7/8 ligand; InvivoGen) and were analyzed for TRAIL expression. These concentrations were set up in preliminary dose-response experiments (data not shown). To evaluate TRAIL expression on purified normal PDC, the PE-conjugated anti-TRAIL mAb (RIK-2) was used.

In some experiments, IFN- α (50,000 U/ml; PeproTech), IFN- β (200,000 U/ml), IFN- γ (10 ng/ml; Boehringer Ingelheim), or blocking anti-IFN- α (50,000 U/ml) and anti-IFN- β (25,000 U/ml) were added.

Cytotoxicity assays

Cytotoxicity was evaluated by a ⁵¹Cr release assay, as described previously (37–39). Because loss of membrane integrity is a late event during apoptosis, a 20-h incubation was necessary to detect this kind of cell death; death induced by soluble TRAIL was used to design this assay. The cytotoxicity of GEN2.2 cells or isolated normal PDC, either fresh or virus-activated (18 h before the test), was tested against either fresh or virus-activated (48 h before the test) human lung epithelial carcinoma A549 cells in triplicates. The percentage of specific lysis was calculated according to the following formula: percentage of lysis = 100 \times (ES – SR)/(MR – SR), where ER, SR, and MR represent experimental, spontaneous, and maximum ⁵¹Cr release, respectively. The ratio SR:MR was always <30%. For inhibition experiments, azide-free anti-TRAIL (5 μ g/ml 2E5; Alexis)

Table I. *GEN2.2 phenotype*^a

Specific Markers						
T/B cells						
CD3	CD4	CD7	CD8	CD19	CD20	
0	100	95	2	0	0	
Myeloid/monocyte						
CD13	CD33	CD11b	CD11c	CD14	CD64	CD116
2	66	2	10	0	1	25
NK cells						
CD16	CD56	CD57	CD25			
0	51	0	0			
DC						
CD1a	CD1c	CD83	BDCA2	BDCA4	CD123	ILT-3
4	5	2	70	88	100	100
APC						
CD40	CD80	CD86	HLA ABC	HLA DR		
27	2	100	100	100		
Miscellaneous						
CD34	CD36	CD38	CD45RA	CD45RO	CD65	CD117
0	89	74	90	21	17	0
Chemokine receptors/homing						
CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7
0	1	5	2	6	8	16
CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	CLA	CD62L
0	6	44	73	1	0	94

^a Percentages of positive cells were determined by flow cytometry. These values are representative of two (CD7, CD19, CD20, CD8, CD16, CD57, CD34, CD36, CD38, CD65, and CD11) to several experiments (for other markers).

was added to effector cells 30 min before addition of target cells. As a control, TRAIL-induced lysis was measured in the same assay by addition of killer TRAIL (10–100 ng/ml).

The cytotoxicity of GEN2.2 cells against melanoma cell line Mel was analyzed slightly differently, with TRAIL-inducers kept during the test. Briefly, GEN2.2 cells were preincubated 4 h with virus, CpG2336, or R848, before addition of ⁵¹Cr-labeled Mel-1 either fresh or presensitized 24 h with IFN- β (2000 U/ml; Tebu) in triplicates. After 20 h, supernatants were harvested and counted for their ⁵¹Cr content. Neither SR nor sensitivity to TRAIL were modified in the presence of the virus, R848, or CpG2336 (data not shown). Percentages of lysis were calculated as described previously. TRAIL expression on GEN2.2 cells cultured in the same conditions was verified.

Results

GEN2.2 cell line

We generated a human PDC cell line GEN2.2 from tumor blood cells from LPDC of patient GEN or LPDC#7 (31, 34). Cells proliferated rapidly, as a single cell suspension, with both nonadherent and weakly adherent cells. This proliferation was strictly dependent on the presence of the MS-5 feeder cell line (data not shown). The HLA typing of GEN2.2 was the following: A*0201, B*07, *44; C*05, *07; DRB1*0103, *08; DQB1*0501, *0402; and DPB1*0201, *0401. GEN2.2 cells, such as normal PDC, were

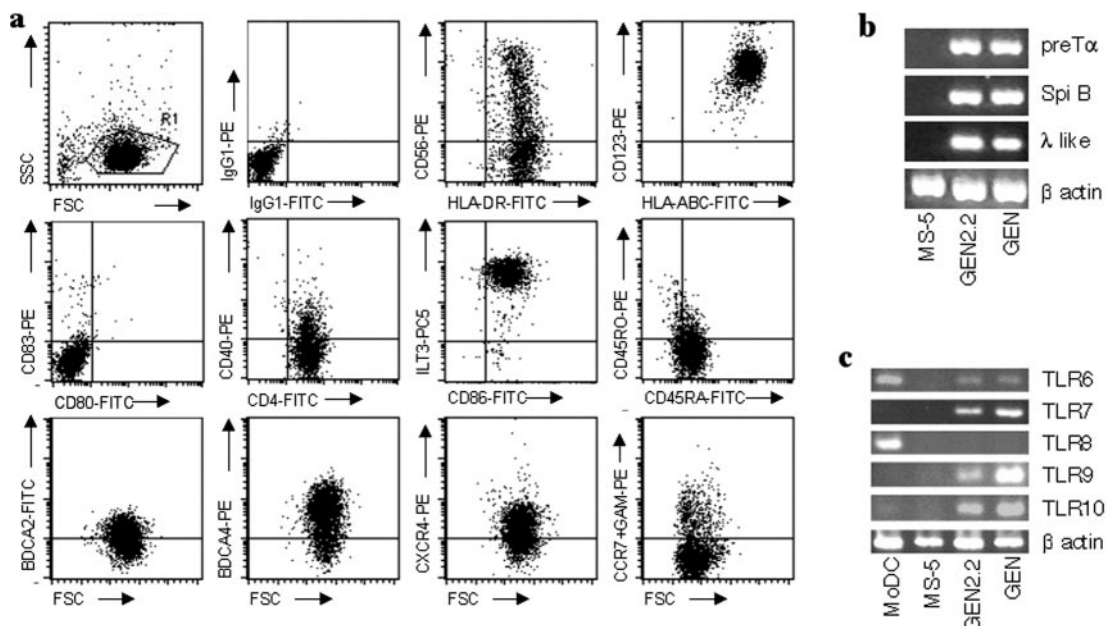


FIGURE 1. Description of the GEN2.2 plasmacytoid dendritic cell line under resting conditions. *a*, Phenotypic characteristics of fresh GEN2.2 cells analyzed by flow cytometry. Dot plot in upper left panel shows the forward light scatter/SSC profile of the cells that were further analyzed on the gate R1. These data are representative of at least three independent experiments. *b*, RT-PCR analysis of pre-T α , SpiB, and λ like in GEN2.2, GEN, and MS-5 cells. *c*, RT-PCR analysis of TLR6, 7, 8, 9, and 10 in GEN2.2, GEN, and MS-5 cells and MoDC.

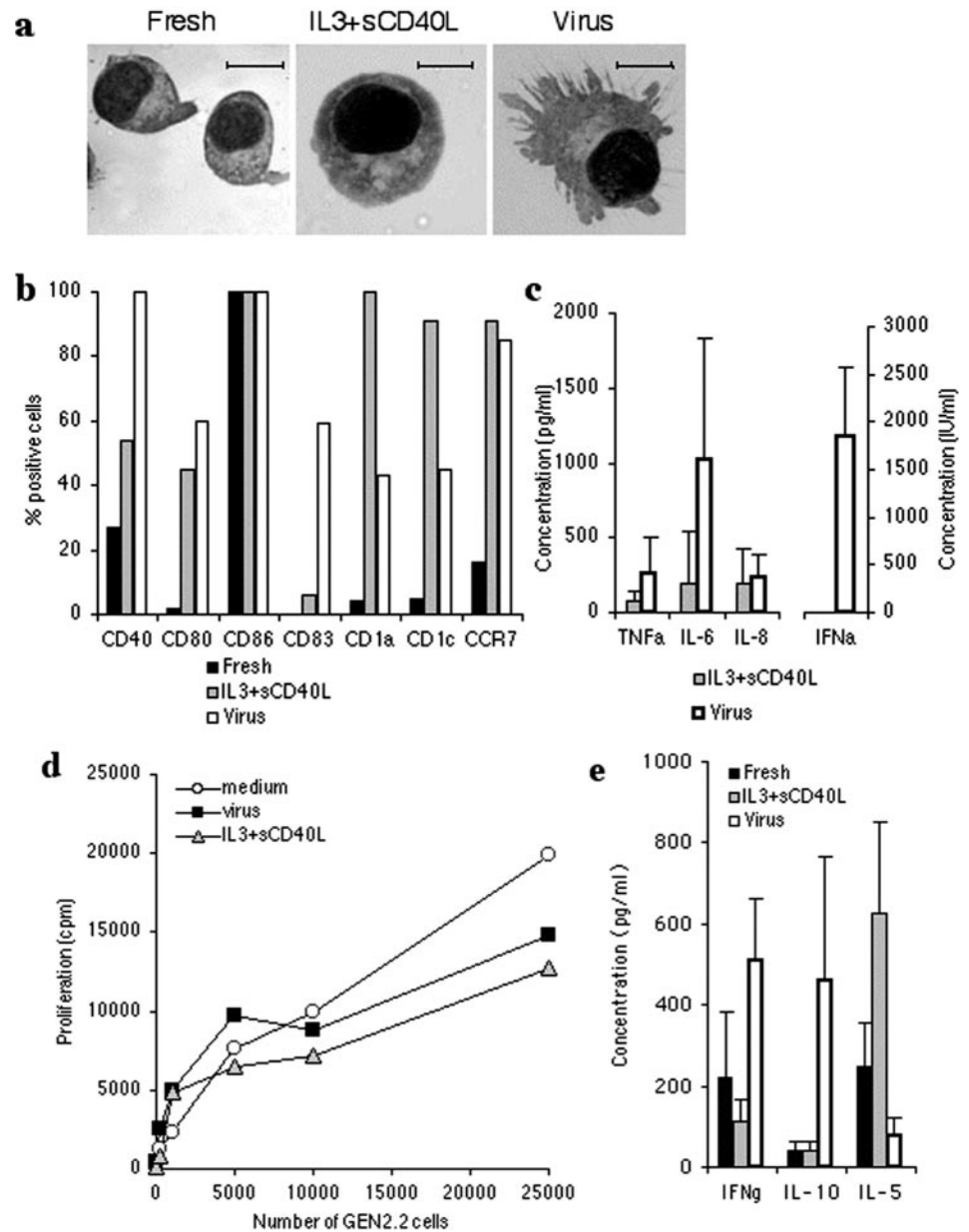


FIGURE 2. Activation and stimulatory functions of GEN2.2 cells. *a*, Morphology of fresh and 2-day activated GEN2.2 cells (scale bar: 10 μ m). *b*, Expression of molecules associated with DC maturation on control and activated GEN2.2 cells. After 2 days in the presence of inactivated influenza virus or IL-3+sCD40L, the phenotype of GEN2.2 cells was analyzed by flow cytometry. These values are representative of at least two independent experiments. *c*, Inflammatory cytokines and type I IFN levels in the supernatants of activated GEN2.2 (mean of six independent experiments). *d*, Proliferation of naive T cells after 6-day coculture with fresh, virus-, and IL-3+sCD40L-activated GEN2.2 (representative of three experiments). *e*, IFN- γ , IL-10, and IL-5 levels in the supernatants of naive CD4⁺ T cells primed by GEN2.2 cells after a 6-day mixed lymphocyte culture (mean of three independent experiments).

characterized by their expression of CD4, HLA ABC, HLA DR, CD45RA, and CD123 (Table I and Fig. 1*a*).

Thirty-five percent of the cells highly expressed CD56, whereas other T, B, and NK cell-associated surface markers (CD3, CD8, CD19, CD20, CD16, and CD57) were negative, as we described for fresh tumor cells (31). Besides CD33, they are negative for myeloid markers (CD13, CD11b, CD11c, CD14, and CD64). They expressed BDCA-2 and BDCA-4, high levels of CD86, and moderate levels of CD40, whereas CD80 was undetectable. CD1a, CD1c, and CD83 were negative at the surface of GEN2.2 cells. Chemokine and homing receptors such as CCR5, CCR6, CCR7, and CXCR2 were weakly expressed, and CXCR3, CXCR4, and CD62L were positive, whereas CCR1, CCR2, CCR4, and CXCR1 were negative. The presence of mRNA coding for spiB, pre-T α , and λ like chain was detected in the GEN2.2 cell line, as with leukemic cells from GEN patient, but not in MS-5 (Fig. 1*b*). The presence of mRNA coding for TLR6, 7, 9, and 10 was also detected in the GEN2.2 cell line such as in leukemic cells from GEN patient but neither in MoDC nor in MS-5, except for TLR6 in

MoDC, whereas mRNA coding for TLR8 was only detected in MoDC (Fig. 1*c*). When GEN2.2 cells were cultured without MS-5 cells for 24 h in the presence of IL-3 and CD40L, or of the influenza virus, they rapidly formed large clusters while they remained isolated without stimulation (data not shown). MGG staining of cytopins showed enlargement of the cells and the acquisition of a dendritic morphology in the presence of the virus (Fig. 2*a*). GEN2.2 cells cultured with the virus or with IL-3+CD40L up-regulated their expression of surface molecules associated with APC functions, HLA ABC, HLA DR (data not shown), and CD40, CD80, and CD86, and acquired a mature DC phenotype (Fig. 2*b*) characterized by the expression of CD83 and CCR7. Whereas GEN2.2 cells activated by IL-3+sCD40L secrete only TNF- α , IL-6, and IL-1, GEN2.2 cultured with an influenza virus produced high levels of IFN- α (Fig. 2*c*). Resting and mature GEN2.2 cells activated the proliferation of naive CD4⁺ T lymphocytes (Fig. 2*d*). T cells primed with virus-activated GEN2.2 cells secrete IFN- γ and IL-10, whereas T cells primed with IL-3+sCD40L-activated GEN2.2 cells secreted IL-5 (Fig. 2*e*).

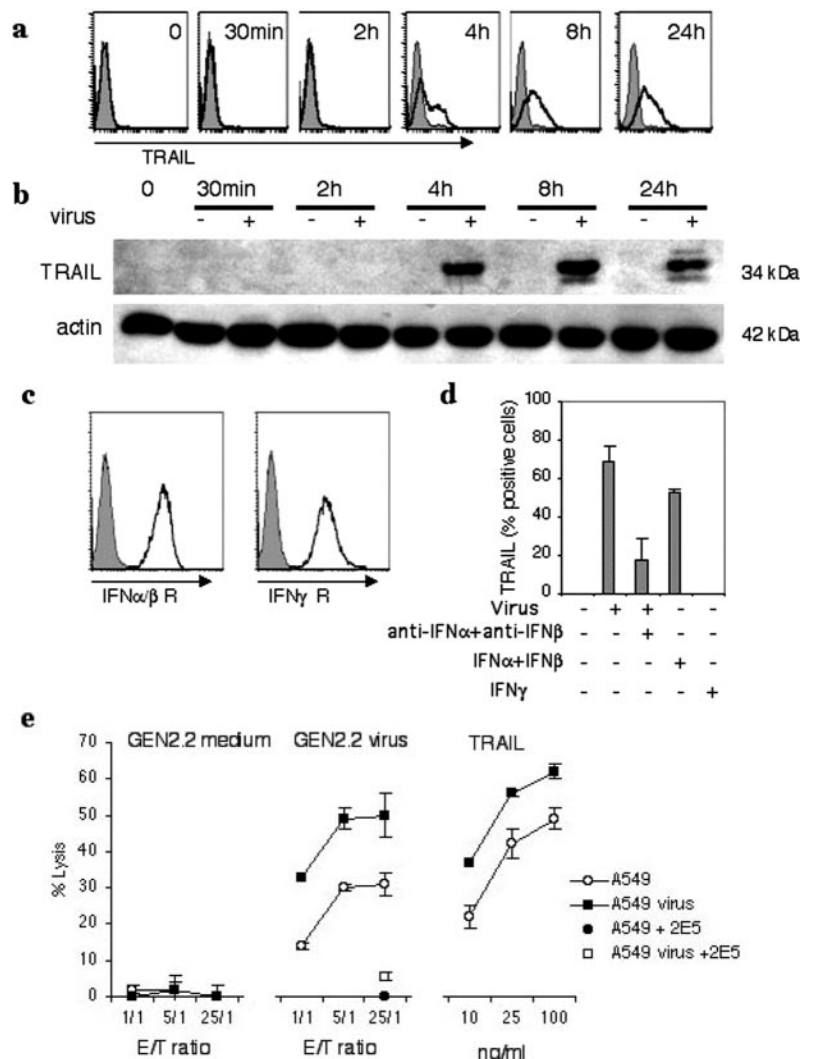


FIGURE 3. Virus-activated GEN2.2 cells up-regulate TRAIL and become killer cells. *a*, Flow cytometric detection of TRAIL expression on GEN2.2 cells activated by inactivated influenza virus. *b*, Western blot detection of TRAIL on GEN2.2 cells with or without activation with inactivated influenza virus. *c*, Expression of IFN- $\alpha\beta$ and IFN- γ receptors by GEN2.2 cells. *d*, Expression of TRAIL on GEN2.2 cells with or without activation with influenza virus, IFN- α , and IFN- β or IFN- γ , in the presence or absence of neutralizing anti-IFN- α and anti-IFN- β Abs (mean of three independent experiments). *e*, Cytotoxicity of GEN2.2 preactivated or not 24 h with influenza virus against A549 lung epithelial carcinoma cells. Killing A549-infected cells was also measured. As a control, cytotoxicity induced by soluble TRAIL was determined in the same experiment. Cytotoxicity was measured by a 20-h ^{51}Cr release assay, in the presence or absence of anti-TRAIL 2E5 mAb. Data show mean \pm SD of one experiment performed in triplicates and are representative of three independent experiments.

Activated PDC become killer cells

PDC, by their ability to secrete high levels of type I IFN, are involved in the early response to viral infection. Because TRAIL is a death-inducing molecule implicated in antiviral responses, and often regulated by type I IFN, we asked whether PDC could express TRAIL and become killer cells upon viral infection. We exposed GEN2.2 cells to inactivated influenza virus (30 min to 24 h) and looked for TRAIL expression. A clear expression of TRAIL was detected by flow cytometry and Western blot analysis from 4 h of coculture with virus but remained undetectable in medium alone (Fig. 3, *a* and *b*). Because both IFN- $\alpha\beta$ and IFN- γ can induce TRAIL expression on various cells, we wondered whether they could control TRAIL expression on GEN2.2. GEN2.2 cells highly expressed both IFN- $\alpha\beta$ and IFN- γ receptors (Fig. 3*c*). We evaluated whether IFN produced by GEN2.2 after activation could up-regulate TRAIL expression in an autocrine manner. Virus-induced TRAIL expression was partially inhibited by either anti-IFN- α or anti-IFN- β mAb (data not shown) and dramatically reduced by neutralizing IFN- α and IFN- β together (Fig. 3*d*). Moreover, type I IFN, but not IFN- γ , induced TRAIL expression on GEN2.2 as did the viral infection (Fig. 3*d*).

Thereafter, we wondered whether GEN2.2 activated by the influenza virus could exert TRAIL-mediated cytotoxicity. Because the virus enters the organism following endocytosis by epithelial cells from the respiratory system, we used the TRAIL-sensitive

A549 lung epithelial carcinoma cells as a target cell model. A549 cells were lysed by virus-activated GEN2.2 (30% lysis at E:T ratio = 25:1) (Fig. 3*e*), and this cytotoxic activity was induced by the virus because resting GEN2.2 did not lyse A549. Moreover, as described with respiratory syncytial virus (40), we found that the influenza virus enhanced A549 cell sensitivity to TRAIL (Fig. 3*e*), without modifying their level of expression of DR4 or DR5 (data not shown). Furthermore, A549 became more sensitive to GEN2.2-mediated lysis after contact with the virus (50% at E:T ratio = 25:1). Treatment of virus-activated GEN2.2 cells with an inhibitory anti-TRAIL mAb completely abolished this cytotoxicity, confirming that PDC-killer activity was mediated by TRAIL (Fig. 3*e*).

We asked whether infection could also turn normal PDC into killer cells. We purified normal PDC from healthy human blood and showed that normal PDC also expressed TRAIL after an 18-h exposure to the influenza virus but not after activation with IL-3 (Fig. 4*a*). Accordingly, they became able to lyse A549 cells (50% lysis at 15:1 E:T ratio) via TRAIL because blocking TRAIL inhibited this cytotoxicity (Fig. 4*b*). This cytotoxicity was not found with nonactivated PDC or IL-3-activated PDC.

TRAIL-mediated antitumor cytotoxicity of GEN2.2 cells

Thanks to the expression of TLR9 and TLR7, PDC can be activated by CpG ODN and resiquimod (R848), respectively. These immune modifiers are under development to treat cancers, and we

wondered whether PDC could also become killer cells following activation with such TLR agonists and hence play a role in the elimination of tumors treated with TLR ligands. Both CpG 2336 and R848 are able to activate GEN2.2 cell line, as evidenced by the up-regulation of CD80 and CD40 (Fig. 5*a*). GEN2.2 cells activated with CpG 2336 produced type I IFN in the same range as with the influenza virus, whereas they did not after activation with R848 (data not shown). TRAIL was detected on GEN2.2 cells after activation with CpG ODN 2336, or R848 (Fig. 5*b*). Thereafter, we wondered whether virus- or TLR-activated GEN2.2 could exert TRAIL-mediated antitumor cytotoxicity. In melanoma, tumor regressions have been described after treatment with the TLR7 agonist imiquimod, both in man and mice, and in the latter PDC were found recruited. We used a TRAIL-sensitive melanoma cell line (Mel) as a target cell model. Resting Mel cells were slightly sensitive to TRAIL and slightly lysed by GEN2.2 either activated by CpG2336, R848, or influenza virus (10–20% lysis at E:T ratio = 25:1) (Fig. 5*c*). However, when Mel cells were preincubated with IFN- β , their sensitivity to soluble TRAIL increased, and hence, they were lysed by activated GEN2.2 cells (40–50% lysis at E:T ratio = 25:1) (Fig. 5*c*). Treatment of activated GEN2.2 cells with an inhibitory anti-TRAIL mAb diminished this cytotoxicity, confirming that their killer activity was partly mediated by TRAIL (Fig. 5*c*).

Discussion

DC, described as professional APC, are capable of initiating immune responses. MoDC can be easily generated *in vitro*, so their functions can be described extensively. PDC, which represent a distinct DC subset, are able to secrete high levels of type I IFN in response to viral infections and to recruit immune effectors (41, 42), so they might play a central role in innate immunity. We focused here on the functions of PDC during the early phases of their activation and see whether they could behave as effector cells in the innate phases of immune responses. The methodology for differentiating PDC from CD34⁺ hemopoietic progenitor cells (43, 44), or their purification from blood, is rather expensive and time-consuming, so poor availability of cells usually limits functional studies of PDC. For this reason, we tried to derive a cell line from leukemic PDC (31). In the present study, we describe the generation of a cell line called GEN2.2. This cell line has been grown in coculture with the murine marrow-derived stromal MS-5 cells. As we demonstrated for LPDC (31, 34), such as normal PDC, GEN2.2 cells mature upon activation with IL-3+sCD40L or influenza virus and then induce naive CD4⁺ T cell proliferation and polarization toward Th2 or Th1 pathways. In the presence of the influenza virus, GEN2.2 cells secrete IFN- α ; they are also activated by R-848 and CpG ODN, which is not surprising, because mRNA coding for TLR7 and TLR9 were detected in the cell line, as described for normal PDC (15, 16). GEN2.2 cells were also found to express low levels of mRNA coding for TLR6 and 10, but mRNA coding for TLR8 was not observed. Altogether, these initial results demonstrate the validity of the GEN2.2 cell line to study PDC functions.

Besides its direct antiviral effects, type I IFN can activate cytotoxic functions for various kinds of effector cells, mainly by inducing TRAIL expression (29, 30, 39, 45, 46). So, because PDC are the natural IFN-producing cells, they may induce cytotoxic functions of other locally recruited effector cells. Indeed, in favor of this hypothesis, it has been shown that after stimulation by CpG ODN, PDC via their secretion of IFN- α , induced TRAIL on monocytes (47). In the present study, we examined whether PDC could share such innate cytotoxic functions. We explored the initial phases of PDC activation upon encounter with the influenza virus, and up-regulation of TRAIL expression on both GEN2.2 cells and normal PDC was observed. The activation triggered by the influ-

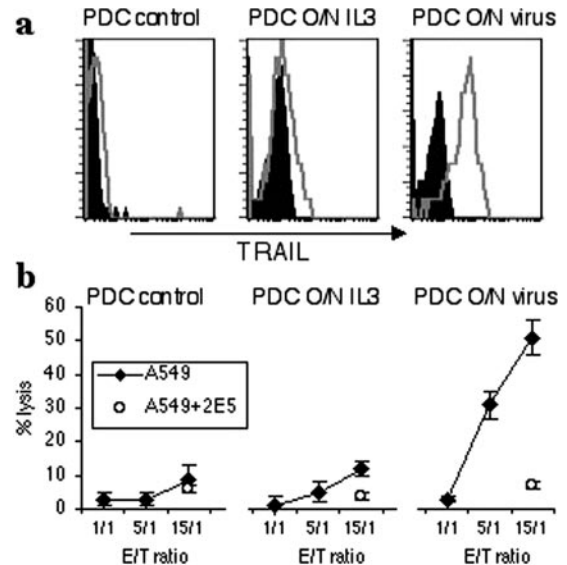


FIGURE 4. Virus-activated normal PDC up-regulate TRAIL and become killer cells. *a*, The expression of TRAIL was measured by flow cytometry after an overnight incubation of purified normal PDC with medium alone, influenza virus, or IL-3. *b*, Cytotoxicity of virus-activated normal PDC. Normal PDC were preactivated 16 h with influenza virus, and their cytotoxicity was measured by a 20-h ⁵¹Cr release assay, in the presence or absence of anti TRAIL 2E5 mAb. Data show mean \pm SD of one experiment performed in triplicates and are representative of two independent experiments.

enza virus might involve the recognition of viral ssRNA by TLR-7 (48, 49), which does not depend on the replication of the virus, and hence, it may occur very early upon infection of the cells. Regarding MoDC, monocytes, or epithelial cells, it has been described that TRAIL expression can be induced by viral infection (50, 51), but the mechanism of inducing TRAIL in these experiments performed with replicating virus remained to be explored. Our results also showed that IFN- $\alpha\beta$ produced by GEN2.2, upon viral challenge, by an autocrine loop, was mostly responsible for this up-regulation of TRAIL. Because blockade of type I IFN receptor only partially blocked TRAIL up-regulation, there could also be a direct type I IFN independent up-regulation of TRAIL further enhanced by the IFN autocrine loop. This also suggested that PDC may express TRAIL very rapidly upon infection, independently of other cell subsets.

We then confirmed that expression of TRAIL by PDC endowed them with a cytotoxic activity. We found that both GEN2.2 cells and normal PDC, after contact with the influenza virus, were able to lyse sensitive target cells via TRAIL. This PDC killer activity may be important for early elimination of infected cells. Indeed, up-regulation of DR4 or DR5 death receptors for TRAIL has been reported after certain viral infections (on lung epithelial cells by respiratory syncytial virus (40), on fibroblasts by human CMV (52), and on hepatocytes during hepatitis C virus infection (53)) and can also be induced by IFN- α (54), sensitizing cells to TRAIL-mediated lysis. Because normal cells are thought to be resistant to TRAIL, they should not be concerned by this apoptosis. The acquisition of TRAIL happened as early as 4 h after contact with the virus, suggesting that PDC may constitute the first weapon against infected cells, and this newly described function of PDC reinforces their position as a crucial component of innate immunity. TRAIL was up-regulated earlier than CD40 or CD80 (data not shown), suggesting that first PDC act as killer cells. In a second step, they might capture the cellular debris generated by apoptosis, and migrate to lymph nodes to further activate T cells.

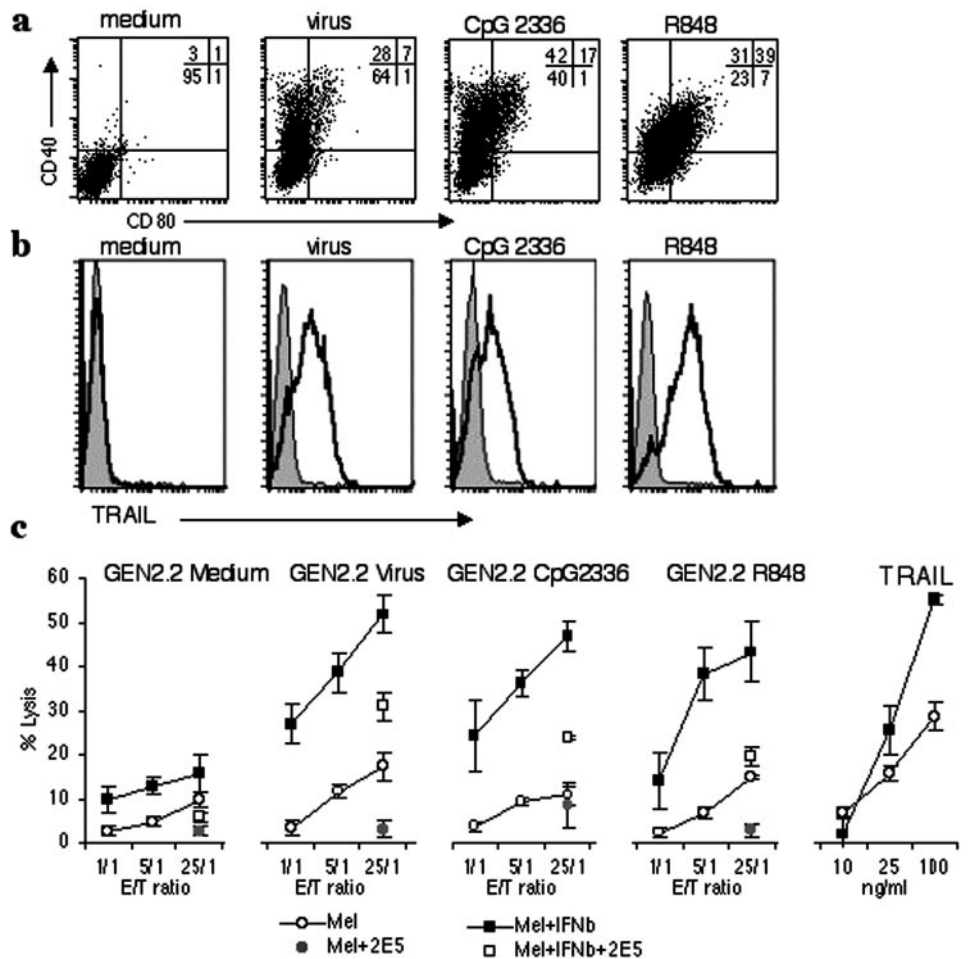


FIGURE 5. GEN2.2 activated by virus, TLR7, or TLR9 agonists acquire TRAIL and antitumor cytotoxicity. *a*, Expression of CD40 and CD80 after a 2-day activation of GEN2.2 cells with or without virus, CpG 2336, or R848. *b*, Expression of TRAIL on GEN2.2 activated 4-h culture with or without virus, TLR9, or TLR7 agonist; closed histograms represent staining with isotype control. *c*, Cytotoxicity of GEN2.2 cells activated or not by influenza virus, CPG2336, or R848. Melanoma cells were presensitized or not by a 24-h incubation with IFN- β . Cytotoxicity was measured by a 20-h ^{51}Cr release assay, in the presence or in the absence of anti-TRAIL blocking 2E5 mAb. Data show mean \pm SD of one experiment performed in triplicates and are representative of at least three independent experiments.

In addition to the effects of virus, we also found up-regulation of TRAIL on GEN2.2 after activation with TLR7 or TLR9 agonists. These molecules represent new ways of modulating the immune response and efforts are being directed toward their use as adjuvants for vaccines or in cancer therapy, with very promising results in the case of basal cell carcinomas treated with imiquimod (25). The local activation of PDC with TLR7 or 9 agonists might favor antitumor immunity by secretion of cytokines, such as IFN- $\alpha\beta$, and by direct killing of tumor cells often sensitive to TRAIL, as our results obtained with a melanoma cell line suggest. In favor of this hypothesis, a recent study showed the association between regression of murine melanoma upon treatment with imiquimod and the recruitment of PDC to the tumor (28). Although rare, PDC can be found in human tumors, but their function may be impaired in the tumor environment (7). However, by the local use of appropriate TLR ligands, their function could be restored, or new fully functional PDC could be recruited to promote antitumor responses.

Altogether, our data reinforce the idea that PDC orchestrate early innate phases of immunity against infectious diseases, and they could also be used to fight against cancer. We showed that a challenge with virus, TLR7 or TLR9 agonists can turn PDC into powerful killer cells against TRAIL-sensitive targets. This phenomenon may be important for the early clearance of TRAIL-sensitive target cells, both in immunity against pathogens and during treatment of cancer or infection by CpG ODN or imidazoquinolines.

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Disclosures

The authors have no financial conflict of interest.

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