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Rat CD4⁺CD8⁺ Macrophages Kill Tumor Cells through an NKG2D- and Granzyme/Perforin-Dependent Mechanism

Tomohisa Baba,²* Sari Iwasaki,* Takako Maruoka,* Akira Suzuki,* Utano Tomaru,* Hitoshi Ikeda,* Takashi Yoshiki,* Masanori Kasahara,* and Akihiro Ishizu³†

We previously identified a subpopulation of monocyte/macrophage lineage cells expressing both CD4 and CD8. This subpopulation was expanded in rat peripheral blood and spleen after immunization with adjuvants containing killed tuberculosis germs. CD4⁺CD8⁺ monocytes/macrophages obtained from preimmunized rats exhibited a Th1-type cytokine/chemokine profile, expressed high levels of Fas ligand, perforin, granzyme B, and NKR-P2 (rat ortholog of human NKG2D), and killed certain tumor cells. In the present study, we confirmed that CD4⁺CD8⁺ monocytes/macrophages are distinct from splenic dendritic cells (DCs) or IFN-producing killer DCs. In vitro cytotoxic assays revealed that CD4⁺CD8⁺ macrophages killed tumor cells in a cell-cell contact-dependent manner and that expression of the retinoic acid early transcript 1 (a ligand for NKG2D) made tumor cells susceptible to killing by CD4⁺CD8⁺ macrophages. Furthermore, inhibitors of granzyme and perforin significantly decreased cytotoxic activities of CD4⁺CD8⁺ macrophages. Consistent with these in vitro findings, preimmunization with adjuvants containing killed tuberculosis germs elevated the expression of granzyme B in tumor-infiltrating CD4⁺CD8⁺ macrophages and significantly inhibited the growth of inoculated tumor cells. Our current work demonstrates that CD4⁺CD8⁺ macrophages are a unique subpopulation of monocyte/macrophage lineage cells that kill tumor cells in an NKG2D- and granzyme/perforin-dependent mechanism. The Journal of Immunology, 2008, 180: 2999–3006.

Monocyte/macrophage lineage cells and dendritic cells (DCs) play critical roles in innate immunity. In our previous study, we identified a novel subpopulation of monocyte/macrophage lineage cells expressing both CD4 and CD8 (1). When rats were immunized with adjuvants containing killed tuberculosis germs, CD4⁺CD8⁺ monocytes and macrophages were expanded in peripheral blood and spleen, respectively. CD4⁺CD8⁺ macrophages in immunized rats expressed cytotoxic factors such as granzyme B and perforin at high levels and exhibited antitumor cytotoxicity in vitro. In rats, a unique DC subpopulation with a NK-like phenotype and function has been previously described (2). This subpopulation, referred to as rat spleen DCs, expresses the activating NK receptor NKR-P1A and is cytotoxic against NK-sensitive YAC-1 cells. Recently, a new DC subpopulation named IFN-producing killer DCs (IKDCs) was discovered (3, 4). Unlike conventional DCs, IKDCs express CD49b and NK receptors, including NKG2A/C/E, NKG2D, and Ly49. Furthermore, IKDCs express NK-related cytotoxic factors, such as granzyme B and perforin, and kill typical NK target tumor cells. These findings indicate that CD4⁺CD8⁺ macrophages, rat splenic DCs, and IKDCs all play important roles in antitumor immunity.

In the present study, we first show that CD4⁺CD8⁺ monocytes/macrophages have characteristics, including surface markers, different from those of rat splenic DCs or IKDCs and hence constitute a distinct subpopulation. We then show that they kill tumor cells expressing the retinoic acid early transcript 1 (RAET1, a ligand for NKG2D) through a granzyme/perforin-dependent mechanism. To our knowledge, this is the first report demonstrating the existence of a unique subpopulation of monocytes/macrophages that kill tumor cells through an NKG2D- and granzyme/perforin-dependent pathway.

Materials and Methods

Rats

Closed-colony Wistar rats were purchased from Charles River. The EGFP-transgenic rats ubiquitously expressing GFP (5) were obtained from the YS Institute (Usamonya, Japan) and Tohoku University (Sendai, Japan). All experiments using rats were done according to the Guidelines for the Care and Use of Laboratory Animals in the Hokkaido University Graduate School of Medicine.

Cell lines

The cloned malignant epithelial thymoma cell line MT was established from the transgenic rats carrying the human T cell leukemia virus type I pX gene in our laboratory (6). The rat mammary cancer cell line SST-2 was provided from Dr. J. Hamada (Hokkaido University, Sapporo, Japan). The COS-7 cell line, rat colon cancer cell line RCN-9, rat bladder cancer cell line NBT-II, and murine mastocytoma cell line P-815, which expresses FcγRs, were purchased from RIKEN Cell Bank (Tsukuba, Japan). MT, SST-2, and RCN-9 were derived from F344 rats and NBT-II from Wistar rats.

Antibodies

Murine mAbs used were anti-rat CD4 (OX-35; BD Pharmingen), anti-rat CD8 (OX-8; BD Pharmingen), anti-rat CD11c (8A2; Serotec), anti-rat CD25 (OX-39; BD Pharmingen), anti-rat CD68 (ED-1; Serotec), anti-rat CD80 (3H5; BD Pharmingen), anti-rat CD103 (OX-62; BD Pharmingen), anti-rat CD163 (ED-2; Serotec), anti-rat MHC class II (MRC OX-6; Serotec), anti-rat Fas ligand (MFL4; BD Pharmingen), and anti-rat NKR-P1A (10/78; BD
Pharmingen). The hamster mAb for rat CD49b (Ha129; BD Pharmingen) was also used. Rabbit and goat polyclonal Abs were used anti-FLAG (Sigma-Aldrich) and anti-granzyme B (N-19; Santa Cruz Biotechnology), respectively. Mouse IgG1 and IgG2 (CBL600P and CBL601P, respectively; Chemicon International) and rabbit or goat IgG (Sigma-Aldrich) served as controls.

**Immunization of rats**

Killed tuberculosis germs were added to IFA (Sigma-Aldrich) to reach the concentration of 100 mg/ml, which were then emulsified with an equal volume of PBS. The resultant emulsion containing killed tuberculosis germs was inoculated into bilateral footpads (100 µl/site) of 8-wk-old rats.

**Tumor inoculation into rats**

Eight-week-old Wistar rats (n = 9) were divided into two groups. One group of rats was immunized with adjuvants containing killed tuberculosis germs (n = 5) and the other group was not treated (n = 4). Seven days later, syngeneic NBT-II carcinoma cells were inoculated s.c. into the back of each rat (1 × 10^7/0.5 ml per site). Then, the tumor size was measured three-dimensionally (length, width, and height) every other day and the volume was calculated as (length × width × height)/2.

**Flow cytometry (FCM) and MACS**

Peripheral blood cells were stained by the direct method without removal of serum. After reaction with Abs, erythrocytes were depleted by treatment with ammonium chloride. Splenic mononuclear cells isolated with Histopaque-1083 (Sigma-Aldrich) were resuspended in PBS supplemented with 3% FBS and then stained. Expression of cell surface molecules was monitored by FCM using the anti-FLAG Ab.

**Isolation of CD4^+ CD8^+ macrophages and NK cells**

Eight-week-old rats were immunized with the adjuvant containing killed tuberculosis germs. One week later, mononuclear cells were isolated from the spleen using Histopaque-1083 and incubated in plastic dishes for 20 min at 37°C. Resultant adherent cells were collected and then divided into CD8^+ or CD8^- cells using the MACS system. We previously showed that the majority of CD8^- cells were CD4^+ CD8^- macrophages (1). NKR-P1A^- cells were collected from nonadherent mononuclear cells using the MACS system and used as splenic NK cells.

**Cytotoxic assay**

Effector cells were added to the culture of tumor cells. After incubation for 6 or 18 h, cytotoxicity was measured using a CytoTox 96 test kit (Promega).

**Adhesion assay**

Tumor cells were incubated in 8-well chamber slides (Nalge Nunc) to form a monolayer at 37°C. CD4^+ CD8^- macrophages isolated from EGF-Fc transgenic rats were added to the culture. After incubation for 2 h, the chamber slides were gently washed with PBS. Then, GFP^+ cells that adhered to the tumor cell sheet were observed using a fluorescence microscope (ECLIPSE E600; Nikon).

**RT-PCR**

Total RNAs were extracted from cells using a RNeasy Mini Kit (Qiagen) and then reverse-transcribed with M-MLV reverse transcriptase (Invitrogen Life Technologies). PCR was done using the cDNAs, 2 mM dNTP mix (GeneAmp dNTPMix; Applied Biosystems), TaqDNA polymerase (AmpliTaq Gold; Applied Biosystems), and primer sets for 30 cycles of 95°C for 40 s, 56°C for 40 s, and 72°C for 40 s. The primer sequences for PCR are listed in Table I.

**Construction of the rat RAET1 expression plasmid**

The entire coding region of rat RAET1, excluding the leader sequence, was obtained by PCR using cDNA derived from the bladder cancer cell line NBT-II. The primer sequences for PCR were 5' -CAAGCTTACACTCTCTCTAGTTGCAA-3' (with a HindIII site at its 5' end) and 5' -GGGATACAGGCCGTCAAGAC-3' (with a SalI site at its 5' end) and 5' -AGGAAGTTTCTGTTCTTACGCTGAGACCT-3' (with a HindIII site at its 5' end) and 5' -GGGTGAGCTTGTGGATTTGT-3' (with a SalI site at its 5' end). After digestion with HindIII and BamHI, the PCR products were ligated to the HindIII/BamHI-digested pFLAG-CMV-3 expression vector (Sigma-Aldrich). Accuracy of the construct was verified by DNA sequencing. This construct, designated as RAET1-pFLAG-CMV-3, enabled the expression of rat RAET1 with an N-terminal FLAG tag when transfected into mammalian cells. DNA for transfection was isolated with a plasmid purification kit purchased from Qiagen.

**Transfection**

To obtain transient transfectants expressing rat RAET1, COS-7 cells were transfected with the RAET1-pFLAG-CMV-3 plasmid using the FuGENE 6 Transfection Reagent (Promega). The efficiency of transfection was monitored by FCM using the anti-FLAG Ab.

**Production of rat NKR-P2 and human Ig (hIg) fusion proteins**

The entire extracellular region of rat NKR-P2 was obtained by PCR using cDNA derived from rat CD8^- lymphocytes as templates. The coding sequence for human IgG1-Fc was obtained by PCR using the pFUSe-hIgG-Fc2 expression vector (Invitrogen Life Technologies) as templates. The primer sequences for NKR-P2 were 5' -CTCGTTACAGGGGCTTACAGGAA-3' (with a KpnI site at its 5' end) and 5' -CTGGGAACCAAAGTGAAGGA-3' (with a BamHI site at its 5' end). After digestion with HindIII and BamHI, the PCR products were ligated to the HindIII/BamHI-digested pFLAG-CMV-3 expression vector (Sigma-Aldrich). The accuracy of the construct was verified by DNA sequencing. This construct was designated as NKR-P2-pFLAG-HC, and the expression of NKR-P2-HC dimers, each with an N-terminal human IgG1-Fc tag. The plasmid DNA

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**Table I. Primer sets used for RT-PCR and real-time quantitative RT-PCR**

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<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>CD48</td>
<td>5'-ATCGCTTTTGTGCTCTTTGG-3'</td>
<td>5'-AAGTTGGTGGTTCTCAGAAGTG-3'</td>
</tr>
<tr>
<td>CD112</td>
<td>5'-GAAGAAACCCAGGACCT ACA-3'</td>
<td>5'-TTGACATTGGACCCAGCACA-3'</td>
</tr>
<tr>
<td>CD155</td>
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<td>5'-CGACGTTCCTCCTTCTTCA-3'</td>
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<tr>
<td>Fas</td>
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<td>5'-ACAGTTGAGAGAAGTAAGTTGT-3'</td>
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<tr>
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<td>5'-CTGGGACACAAAGTGAGAA-3'</td>
</tr>
<tr>
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<td>5'-CCTCTTCCTGTTGTTGAGAC-3'</td>
<td>5'-GGGATACAGGCCGTCAAGAC-3'</td>
</tr>
<tr>
<td>NKR-P1A</td>
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<td>5'-CAGGACAGAAGAAATGTTGT-3'</td>
</tr>
<tr>
<td>TLR1</td>
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<td>5'-GGGATACAGGCCGTCAAGAC-3'</td>
<td>5'-CAGGACACACACTCCAAGAAAC-3'</td>
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was transfected into COS-7 cells using the FuGENE 6 Transfection Reagent. Soluble NKR-P2-hIg was purified from the culture supernatant of the transfectants using the HiTrap Protein G HP column (Amersham Biosciences).

**Immunocytochemistry**

Mononuclear cells separated from rat spleen were cultured in chamber slides (Nalge Nunc International) for 1 h. Resultant adherent cells were fixed using cold acetone for 5 min or 4% paraformaldehyde for 15 min at room temperature and then stained. After washing with PBS, the slides were mounted in Fluorescent Mounting Medium (DakoCytomation). Immunofluorescence was detected using the fluorescence microscope.

**Immunohistochemistry**

After fixation by 10% phosphate-buffered formaldehyde, tissues were embedded in paraffin blocks and cut into 4-μm sections. For immunohistochemistry, the sections were reacted with the anti-rat CD68 Ab (ED-1) at a 1/250 dilution at 4°C overnight. After removing the unbound Ab, the sections were reacted with Alexa 488-labeled anti-mouse IgG and Alexa 568-labeled anti-goat IgG Abs (Invitrogen Life Technologies) at room temperature for 60 min. Fluorescence was detected using Eclipse E600 (Nikon).

**Image processing**

Microscopic photographs were taken using the objective lens (×400/0.75 numeric aperture) in the DP70 system (Olympus). DP Controller software (Olympus) was used for image processing.

**Results**

**CD4<sup>+</sup>CD8<sup>-</sup> monocytes/macrophages are a cell population distinct from DCs with cytotoxic phenotypes**

CD4<sup>+</sup>CD8<sup>-</sup> monocytes/macrophages, rat splenic DCs, and IKDCs all exhibit killer activities against tumor cells (2–4). To confirm that CD4<sup>+</sup>CD8<sup>-</sup> monocytes/macrophages are a cell population distinct from DCs with cytotoxic phenotypes, we examined the expression of surface markers characteristic of DCs on CD4<sup>+</sup>CD8<sup>-</sup> monocytes/macrophages. Rat monocyte/macrophage lineage cells were identified in the CD4<sup>medium</sup> population (R2 gate) in nonmononuclear cells (R1 gate) (Fig. 1A) (1). CD4<sup>+</sup>CD8<sup>-</sup> monocytes/macrophages, which were CD8<sup>-</sup> and CD4<sup>medium</sup>, were abundantly seen in the peripheral blood (52.9% of monocytes) and spleen (26.7% of macrophages) in adjuvant-immunized rats. Histograms were obtained by setting a gate for the CD4<sup>medium</sup>CD8<sup>-</sup> cells (Fig. 1B). CD4<sup>+</sup>CD8<sup>-</sup> monocytes in the peripheral blood and CD4<sup>+</sup>CD8<sup>-</sup> macrophages in the spleen expressed CD11c, CD80,
MHC class II, CD68, and CD163. However, they were negative for CD25, CD103 (both are characteristically expressed on rat splenic DCs (2, 7)) or the IKDC marker CD49b (3, 4). Thus, CD4+CD8+ monocytes/macrophages differ from rat splenic DCs and IKDCs in expression of surface markers.

Next, we examined the morphological features of CD4+CD8+ macrophages and the expression profiles of TLRs in these cells. CD4+CD8+ macrophages were morphologically similar to CD8+ conventional macrophages, but different from rat splenic DCs displaying characteristic dendrites (Fig. 2A). Recent work has shown that the expression profiles of TLRs differ between DCs and monocyte/macrophage lineage cells (8). For example, TLR3 and TRL10 are expressed at high levels in DCs. When we examined TLR expression profiles, TLR3 and TRL10 were abundantly expressed in rat splenic DCs, but only at low levels in CD4+CD8+ and CD8+ macrophages (Fig. 2B). These observations support our conclusion that the CD4+CD8+ cells characterized in this study are macrophage/monocyte lineage cells rather than DC lineage cells.

CD4+CD8+ macrophages kill tumor cell lines selectively

In our previous study, we demonstrated that CD4+CD8+ macrophages were able to kill the malignant thymoma cell line MT in vitro (1). Killing was dose dependent and reached the peak when the E:T ratio was 30. Because the effector/target combination was allogenic (CD4+CD8+ macrophages were derived from Wistar rats and MT from F344 rats), killing was induced by a MHC-unrestricted mechanism. After coculture with CD4+CD8+ macrophages for 18 h, almost all MT cells were destroyed, whereas control CD8+ macrophages barely killed the target cells (Fig. 3A).

To examine the killing specificities of CD4+CD8+ macrophages, four tumor cell lines were used as targets (Fig. 3B). CD4+CD8+ macrophages killed MT and NBT-II cells efficiently (percent specific lysis was 73.8 ± 6.8 and 77.4 ± 20.4, respectively) and SST-2 cells to some extent (percent specific lysis was 11.8 ± 1.8). However, RCN-9 cells were resistant to killing (percent specific lysis was −1.8 ± 0.5). These findings indicate that not all tumor cell lines are sensitive to lysis by CD4+CD8+ macrophages and that killing is critically dependent on putative factors expressed by target cells.

By contrast, the four cell lines were susceptible to killing by purified NK cells although the extent of killing showed considerable variation (percent specific lysis was 31.7 ± 4.5 for MT, 39.9 ± 14.3 for SST-2, 16.1 ± 2.7 for RCN-9, and 68.1 ± 4.3 for NBT-II). Interestingly, MT cells were more susceptible to killing by CD4+CD8+ macrophages than by NK cells, whereas the opposite was the case for SST-2 cells. These observations ruled out the possibility that the cytotoxic activities of CD4+CD8+ macrophages were mediated by contaminating NK cells.

Cell-cell contact is required for killing by CD4+CD8+ macrophages

To examine whether cell-cell contact is required for killing by CD4+CD8+ macrophages, adhesion of the effector cells to target cells was blocked using a cell culture insert. Cytotoxicity against NBT-II was completely inhibited by blocking cell-cell contact (percent specific lysis was decreased from 62.5 ± 8.6 to −2.4 ± 15.2; Fig. 4A). This result indicates that adhesion to the target cells is essential for the killing process by CD4+CD8+ macrophages.

Next, to examine whether differential sensitivities to killing by CD4+CD8+ macrophages are dependent on the adhesive efficiency to the target cells, effector cells derived from EGFP-transgenic rats were cultured on the monolayer of tumor cells. After incubation for 2 h, a large number of GFP+ cells (219 ± 24.9/field) adhered to the monolayer of MT cells (Fig. 4, A and C). In contrast, adhesion to NBT-II was significantly less extensive (83.3 ± 9.0/field) than to MT cells. Because MT and NBT-II cells were killed by CD4+CD8+ macrophages equivalently (see Fig. 3B), differences in adhesive efficiency alone cannot account for differential sensitivities of target cells to CD4+CD8+ macrophages. Interestingly, CD4+CD8+ macrophages also adhered to
RCN-9 (56.3 ± 7.0/field) with efficiency comparable to that of NBT-II. However, RCN-9 was resistant to killing by CD4⁺CD8⁺ macrophages (see Fig. 3B). Thus, adhesion to the targets is essential, but not sufficient for killing by CD4⁺CD8⁺ macrophages.

Interaction of NKR-P2 and RAET1 is critically involved in the killing mechanism of CD4⁺CD8⁺ macrophages

To identify the molecules involved in the killing by CD4⁺CD8⁺ macrophages, the expression profiles of genes for Fas, RAET1, CD48, CD112, and CD155, known to be involved in antitumor immunosurveillance (4, 9–11), were compared among the four tumor cell lines (Fig. 5A). RCN-9 cells, which were resistant to lysis by CD4⁺CD8⁺ macrophages, expressed Fas and RAET1 at significantly lower levels than the other cells. Expression of CD48 was not detected in any of the samples. CD112 and CD155 were expressed at similar levels in the four cell lines. Based on these observations, we assumed that Fas and/or RAET1 could be involved in the killing mechanism of CD4⁺CD8⁺ macrophages. To
FIGURE 6. NKR-P1A is involved in the killing mechanism of CD4+CD8+ macrophages. A. Expression of NKR-P1A mRNA was examined by RT-PCR in splenic CD4+CD8+ (DP) macrophages and CD8+ macrophages. B. Expression of NKR-P1A molecules was analyzed by FCM. Unfilled and gray-filled histograms represent the expression of NKR-P1A on the DP and CD8+ macrophages, respectively. Experiments were repeated three times. Representative data are shown. C. For cross-linking experiments, total splenic macrophages were preincubated with the anti-NKR-P1A Ab followed by cocultivation with P-815 cells. For control experiments, macrophages were preincubated with the isotype-matched Ab. After washing with PBS, CD4+CD8+ macrophages were added to the culture of P-815 cells with an E:T ratio of 30 (2.4 \times 10^5 effector cells and 8 \times 10^5 target cells/well in 96-well plates). After incubation for 18 h, cytotoxicity was measured using a CytoTox 96 test kit. Data are represented as mean ± SD of experiments done in triplicate. *, p < 0.05.

We examined the involvement of the Fas/Fas ligand system, we pretreated effector cells with the anti-Fas ligand Ab. This pretreatment did not alter cytotoxic activities of CD4+CD8+ macrophages, arguing against the involvement of the Fas-Fas ligand interaction (data not shown).

To examine whether RAET1 is involved in the killing process, we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence (data not shown). We then examined whether soluble NKR-P2-hIg can inhibit the cytotoxicity mediated by CD4+CD8+ macrophages, percent specific lysis was 26.7 ± 4.4 for Ab specific for NKR-P1A and 20.4 ± 3.8 for isotype-matched Ab; Fig. 6C). These results indicate that NKR-P1A is partially involved in the killing mechanism of CD4+CD8+ macrophages.

CD4+CD8+ macrophages kill tumor cells via the granzyme/perforin-dependent pathway

CD4+CD8+ monocytes/macrophages characteristically express cytotoxic molecules such as granzyme B, perforin, and Fas ligand, which are normally seen in NK cells and CTLs (1). Synthesized granzyme B is stored within cytolytic granules along with perforin and is released to the targets upon the influx of Ca2+ into the effector cells (12, 13). We observed that granzyme B was granularly distributed in the cytoplasm of CD4+CD8+ macrophages (Fig. 7A).

To examine whether granzyme B and perforin are involved in the killing mechanism of CD4+CD8+ macrophages, we inhibited granzyme activities and degranulation of the cytolytic granules using 3,4-dichloroisocoumarin (DCI; a broad serine protease inhibitor) and EGTA (a chelator of Ca2+), respectively (14). Both DCI and EGTA significantly inhibited cytotoxic activities of CD4+CD8+ macrophages against the NBT-II cell line (percent
immunized rats, whereas CD68 tissues. Granzyme B was hardly detectable in CD68 phages infiltrating into degenerative cystic spaces in the tumor monocytes in PBMC was significantly decreased in tumor-bearing specific lysis was decreased from 89.8 to 25.6 with DCI treatment and to −1.1 ± 12.9 with EGTA treatment; Fig. 7B). These results indicate that CD4<sup>+</sup>CD8<sup>+</sup> macrophages kill tumor cells via the granzyme/perforin-dependent pathway.

CD4<sup>+</sup>CD8<sup>+</sup> monocytes/macrophages are involved in antitumor immunity in vivo

To investigate how CD4<sup>+</sup>CD8<sup>+</sup> monocytes/macrophages change in the number, phenotype, and function in tumor-bearing hosts, we inoculated NBT-II cells into the subcutis of syngenic rats with or without preimmunization with adjuvants containing killed tuberculosis germs. On day 4 after inoculation, the proportion of CD4<sup>+</sup>CD8<sup>+</sup> monocytes in PBMC obtained from nonimmunized rats. FCM was done using blood samples obtained on day 0 (preinoculation) and day 4 postinoculation. ***, p < 0.01. B and C, Rats were killed on day 6 after tumor inoculation for histological examination. Representative photographs are shown (original magnification, ×400). D, Chronological changes in tumor size. ▲ and ■, Pre-immunized and nontreated rats, respectively. ***, p < 0.01.

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cells is also involved in the killing mechanism of CD4+CD8− macrophages.

Interestingly, CD4+CD8+ macrophages killed tumor cells via the granzyme/perforin-dependent pathway (Fig. 7). This was rather unexpected because this pathway is widely believed to be unique to CTLs and NK cells. Both DC1 and EGTA significantly inhibited cytotoxic activities of CD4+CD8+ macrophages. Whereas EGTA treatment completely abrogated cytotoxic activities of CD4+CD8+ macrophages, treatment with DC1 resulted only in partial inhibition of cytotoxicity. These results suggest that cytotoxic factors other than granzyme B and perforin are likely involved in the killing mechanism of CD4+CD8+ macrophages.

Finally, we provided evidence suggesting that CD4+CD8+ monocytes in peripheral blood infiltrated into tumor tissues and then differentiated into CD4+CD8+ macrophages (Fig. 8, A and B). We suggest that the expression levels of granzyme B in CD4+CD8+ monocytes/macrophages are elevated by preimmunization with adjuvants and that these cells exert cytotoxic effects against tumor cells in vivo (Fig. 8, C and D).

In summary, the present study demonstrates that CD4+CD8+ monocytes/macrophages exhibit antitumor cytotoxicity through recognition of NKG2D ligands. Calcium influx induced by ligand recognition appears to activate the granzyme/perforin pathway, leading to the destruction of target tumor cells. Although further investigations are required for understanding the physiological and pathological significance of CD4+CD8+ monocytes/macrophages, the observation that they exhibit cytotoxicity against tumor cells in vitro and in vivo does raise the possibility that their manipulation may lead to the development of new immunotherapies against tumors.

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Disclosures
The authors have no financial conflict of interest.

References


