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

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

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

We previously identified a subpopulation of monocyte/macrophage lineage cells expressing both CD4 and CD8. 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/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 an NK-like phenotype and function has been previously described (2). This subpopulation, referred to as rat splenic 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 National Institute of Genetics (Ashigarakami, 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 1 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 (H1/109; BD Pharmingen) was also used. Rabbit and goat polyclonal Abs used were anti-FLAG (Sigma-Aldrich) and anti-granzyme B (N-19; Santa Cruz Biotechnology), respectively. Mouse IgG1 and IgG2 (C2L600P and C2L601P, 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 n/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, syngenic 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 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 cultures 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 EGFp-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 RNaseasy 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 BC-5. The primer sequences for PCR were 5' - ATCGCTTTTGCTGTCTTTGG-3' (with a HindIII site at its 5' end) and 5' - CTGGTGGATCTTTGAGTTTGT-3' (with a HindIII 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 FuGENE6 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' - CGAATCCGAGTTTGTGGATTTGT-3' (with a HindIII site at its 5' end) and 5' - CTGCTACTAAGCGAGACTT-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). Accuracy of the construct was verified by DNA sequencing. This chimera was amplified by PCR using the primers 5' - GGAATTCATGTTAGCCATGGGG-3' (sense) and 5' - CTGCTACTAAGCGAGACTT-3' (anti-sense). After digestion with BglII and NheI, the PCR products were ligated to the pUSE-hIgG-Fc2 expression vector devoid of the IgG1-Fc sequence. Accuracy of the construct was verified by DNA sequencing. This construct, designated as NKR-P2-hIgG-pUSE, enabled the expression of rat NKR-P2 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>CD48</td>
<td>5'-ATCGCTTTTGCTGTCTTTGG-3'</td>
<td>5'-AACTGGTTGCTCTCAGAGTGTT-3'</td>
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<td>CD112</td>
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<td>5'-TTGACATCTGGACTCACAGA-3'</td>
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<td>CD155</td>
<td>5'-CATTGCTTCCCTTCTTTTTTG-3'</td>
<td>5'-CCAGGTCTTCTTCTTCAGTT-3'</td>
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<td>5'-CTGGAGAACCACAAAGTGAGGA-3'</td>
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<td>RAET1</td>
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<td>5'-GAAGATCTGTGGAGTGCCCACCTTGAAA-3'</td>
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<td>NKR-P1A</td>
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<td>5'-AACTGGTTGCTCTCAGAGTGTT-3'</td>
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<td>5'-CACTGGTTGCTCTCAGAGTGTT-3'</td>
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<td>TLR2</td>
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<tr>
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<td>TLR10</td>
<td>5'-CTGCTACTAAGCGAGACTT-3'</td>
<td>5'-AACTGGTTGCTCTCAGAGTGTT-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 mononuclear 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 TLR10 are expressed at high levels in DCs. When we examined TLR expression profiles, TLR3 and TLR10 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 ± 10.4 to 5.9 ± 3.0; 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, B and C). In contrast, adhesion to NBT-II was significantly less extensive (83.3 ± 9.0/field) than that to MT cells. Because MT and NBT-II cells were killed by CD4+CD8+ macrophages equivalently (see Fig. 4B), 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.

FIGURE 4. Cell-cell contact is required for killing by CD4⁺CD8⁺ macrophages. A, Splenic CD4⁺CD8⁺ macrophages were added to NBT-II cells with an E:T ratio of 30 (1.2 × 10⁶ effector cells and 4 × 10⁴ target cells/well in 24-well plates). For blocking the cell-cell contact, a cell culture insert (pore size, 0.4 μm; BD Biosciences was used. After incubation for 18 h, cytotoxicity was measured. Data are represented as mean ± SD of experiments done in triplicate.

B, Tumor cells were incubated in 8-well chamber slides to form a monolayer at 37°C. Then, splenic CD4⁺CD8⁺ macrophages isolated from EGFP-transgenic rats were added to the culture. After incubation for 2 h, the chamber slides were gently washed with PBS and then GFP⁺ cells that adhered to the tumor cell sheet were observed using a fluorescence microscope. Total magnification, ×200. C, GFP⁺ cells that adhered to the tumor cell sheet in three high-power fields (×400) were counted. Data are represented as mean ± SD. *, p < 0.05.

FIGURE 5. Interaction of NKR-P2 and RAET1 is critically involved in the killing mechanism of CD4⁺CD8⁺ macrophages. A. The expression profiles of genes for Fas, RAET1, CD48, CD112, and CD155, known to be involved in antitumor immunosurveillance, were compared among the four tumor cell lines by RT-PCR. B. The RAET1 expression plasmid was transfected into COS-7 cells, the transfectants were stained with rabbit anti-FLAG Ab followed by FITC-conjugated goat anti-rabbit IgG Ab, and then analyzed on FCM. Gray and black unfilled histograms represent the expression of FLAG-tagged RAET1 when transfection was performed with 1 and 2 μg of DNA, respectively. The gray-filled histogram represents the negative control transfected with the empty vector DNA. The numbers in the panel represent the percentage of FLAG⁺ cells. C. Splenic CD4⁺CD8⁺ macrophages were added to the culture of COS-7 cells transfected with the empty vector DNA or 1 or 2 μg of RAET1 cDNA with an E:T ratio of 30 (2.4 × 10⁵ effector cells and 8 × 10³ target cells/well in 96-well plates). After incubation for 6 h, cytotoxicity was measured. Data are represented as mean ± SD of experiments done in triplicate. *, p < 0.05. D. COS-7 cells transfected with 2 μg of DNA of the rat RAET1 expression plasmid (unfilled histogram) or with the empty vector (gray-filled histogram) were incubated with soluble rat NKR-P2-hIg fusion protein followed by staining with FITC-conjugated anti-human IgG Ab and then analyzed on FCM. The numbers in the panel represent the percentage of hIg⁺ cells. E, To block the interaction of NKR-P2 and RAET1, soluble NKR-P2-hIg (sNKR-P2) was added to the coculture of the effector and target cells. For control experiments, the same volume of PBS was added. After incubation for 6 h, cytotoxicity was measured using a CytoTox 96 test kit. Data are represented as mean ± SD of experiments done in triplicate. *, p < 0.05.
examine the involvement of the Fas/Fas ligand system, we pre-treated 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 we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence we isolated RAET1 cDNA from NBT-II cells. This cDNA had a sequence completely identical to the predicted RAET1 sequence.

We confirmed that the rat RAET1 molecule we identified has the ability to bind to soluble NKR-P2-hlg fusion proteins and thus functions as a ligand for NKG2D (Fig. 6D). We then examined whether soluble NKR-P2-hlg can inhibit the cytotoxicity mediated by CD4+CD8+ macrophages. Addition of soluble NKR-P2-hlg significantly inhibited the cytotoxicity of CD4+CD8+ macrophages against the RAET1 transfectants (percent specific lysis was decreased from 17.8 ± 4.4 to 2.7 ± 6.5; Fig. 5E). Because CD4+CD8+ macrophages express NKR-P2 (rat ortholog of human NKG2D) (1), collective evidence indicates that CD4+CD8+ monocytes/macrophages kill target cells via NKG2D ligand recognition.

**NKR-P1A is involved in the killing mechanism of CD4+CD8+ macrophages**

CD4+CD8+ monocytes in rat peripheral blood express the activating NK receptor NKR-P1A (1). We examined whether rat splenic CD4+CD8+ macrophages also express NKR-P1A (Fig. 6, A and B). By RT-PCR and FCM, CD4+CD8+ macrophages were found to express NKR-P1A at higher levels than CD8+ macrophages. Furthermore, cross-linking of NKR-P1A with a specific mAb significantly augmented directed killing of P-815 cells by CD4+CD8+ macrophages (percent specific lysis were 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 (DC1; a broad serine protease inhibitor) and EGTA (a chelator of Ca2+), respectively (14). Both DC1 and EGTA significantly inhibited cytotoxic activities of CD4+CD8+ macrophages against the NBT-II cell line (percent

**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 × 10⁶ effector cells and 8 × 10⁴ 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.

**FIGURE 7.** CD4+CD8+ macrophages kill tumor cells via the granzyme/perforin-dependent pathway. A. Splenic CD4+CD8+ macrophages were stained for CD68 (ED-1, green) and granzyme B (N-19, red) (total magnification, ×4000). B. Splenic CD4+CD8+ macrophages were added to the culture of NBT-II cells with an E:T ratio of 30 (2.4 × 10⁶ effector cells and 8 × 10⁴ target cells/well in 96-well plates). After incubation for 18 h in the presence of 50 μM DC1 or 5 mM EGTA, cytotoxicity was measured. Data are represented as mean ± SD of experiments done in triplicate. *, p < 0.05.
Immunized rats, whereas CD68 tissues. Granzyme B was hardly detectable in CD68 phages infiltrating into degenerative cystic spaces in the tumor tissues (Fig. 8). Specific lysis was decreased from 89.8 to 25.6 with DCI treatment and to 1.1 with EGTA treatment (Fig. 7B). These results indicate that CD4+CD8+ macrophages kill tumor cells via the granzyme/perforin-dependent pathway.

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

To investigate how CD4+CD8+ 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+CD8+ 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 ■, Preimmunized and nontreated rats, respectively. **, p < 0.01.

Discussion

We recently described a novel subpopulation of rat monocytes/macrophages expressing both CD4 and CD8 and investigated its differentiation and functional features (1). The number of CD4+CD8+ monocytes/macrophages was dramatically increased in peripheral blood and spleen after immunization with adjuvants containing killed tuberculosis germs. Interestingly, unlike conventional macrophages, these cells express high levels of granzyme B, perforin, and Fas ligand and exhibit cytotoxicity against tumor cells in vitro. Monocyte/macrophage lineage cells and DCs can have a similar phenotype. Therefore, our first aim was to confirm that CD4+CD8+ monocytes/macrophages represent a cell population distinct from DCs exhibiting a cytotoxic function, namely, rat splenic DCs (2) and the recently described mouse DC subpopulation named IKDC (3, 4). CD4+CD8+ monocytes/macrophages do not express CD25, CD103 (which are characteristically expressed on rat splenic DCs (2, 7)), or CD49b (a marker for IKDC) (3, 4) (Fig. 1). In addition, the morphological features and TLR expression profiles of CD4+CD8+ macrophages were different from those of rat splenic DCs, while they were similar to those of CD8+ conventional macrophages (Fig. 2). These observations indicate that the CD4+CD8+ cells characterized here are monocyte/macrophage lineage cells rather than DC lineage cells.

The second aim of the present study was to understand the molecular mechanisms by which CD4+CD8+ macrophages kill tumor cells. In our previous report, we showed that CD4+CD8+ macrophages can kill allogenic target cells (also shown in Fig. 3A), thus indicating that killing is triggered by a MHC-unrestricted mechanism (1). In the present study, we demonstrated that CD4+CD8+ macrophages killed tumor cell lines selectively (Fig. 3B). Killing required direct cell-cell contact between effector and target cells (Fig. 4A). However, cell-cell contact alone was not sufficient for CD4+CD8+ macrophages to kill target cells (Fig. 4B). These observations suggested that killing by CD4+CD8+ macrophages was critically dependent on putative factors expressed by target cells.

At least four lines of evidence suggested that one of the factors involved was RAET1, a ligand for the C-type lectin-like activating receptor NKG2D (15–18). First, RAET1 was expressed at higher levels in the CD4+CD8+ macrophages-sensitive cancer cell lines MT, NBT-II, and SST-2 than in the resistant line RCN-9 (Fig. 5A). Second, the expression of RAET1 on the cell surface rendered COS-7 cells susceptible to killing by CD4+CD8+ macrophages (Fig. 5, B and C). Third, soluble NKR-P2-hlg, which was shown to bind to RAET1, was able to inhibit the killing of RAET1 transfectants by CD4+CD8+ macrophages (Fig. 5, D and E). Fourth, CD4+CD8+ monocytes/macrophages constitutively express NKR-P2, the rat ortholog of human NKG2D (1). Thus, combined evidence indicates that the interaction of NKR-P2 and RAET1 is critically involved in the killing mechanism of CD4+CD8+ macrophages. At the moment, we know very little about additional factors involved in the killing mechanism of CD4+CD8+ monocytes/macrophages. However, the evidence presented in Fig. 6 indicates that the interaction of NKR-P1A and its undetermined ligands on target
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

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References


