A Novel Functional T Cell Hybridoma Recognizes Macrophage Cell Death Induced by Bacteria: A Possible Role for Innate Lymphocytes in Bacterial Infection

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A Novel Functional T Cell Hybridoma Recognizes Macrophage Cell Death Induced by Bacteria: A Possible Role for Innate Lymphocytes in Bacterial Infection

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We have established a novel TCRαβ (TCRVβ6)+CD4−CD8+ T cell hybridoma designated B6HO3. When the B6HO3 cells were cocultured with bacterial-infected J774 macrophage-like cells, IFN-γ production by B6HO3 cells was triggered through direct cell-cell contact with dying J774 cells infected with Listeria monocytogenes (LM), Shigella flexneri, or Salmonella typhimurium that expressed the type III secretion system, but not with intact J774 cells infected with heat-killed LM, nonhemolytic hly− LMG deficient (Hly−) LM, plasmid-cured Shigella, or stationary-phase Salmonella. However, the triggering of B6HO3 cells for IFN-γ production involved neither dying hepatoma cells infected with LM nor dying J774 cells caused by gliotoxin treatment or freeze thawing. Cycloheximide and Abs to H-2Kd, H-2Dd, Iad, CD1d, TCRVβ, and IL-12 did not inhibit the contact-dependent IFN-γ response, indicating that this IFN-γ response did not require de novo protein synthesis in bacterial-infected J774 cells and was TCR and IL-12 independent. Thus, in an as yet undefined way, B6HO3 hybridoma recognizes a specialized form of macrophage cell death resulting from bacterial infection and consequently produces IFN-γ. Moreover, contact-dependent interaction of minor subsets of splenic αβ T cells, including NKT cells with dying LM-infected J774 and bone marrow-derived macrophage (BMM) cells, proved to provide an IFN-γ-productive stimulus for these minor T cell populations, to which the parental T cell of the B6HO3 hybridoma appeared to belong. Unexpectedly, subsets of γδ T and NK cells similarly responded to dying LM-infected macrophage cells. These results propose that innate lymphocytes may possess a recognition system sensing macrophage cell “danger” resulting from bacterial infection. The Journal of Immunology, 2006, 176: 7576–7588.

Macrophages are well known for their scavenger function. They are able to ingest and digest a variety of particulate bodies, such as macromolecular complexes, aged erythrocytes, apoptotic cells, and microbial pathogens (1). Upon infection, in addition to ingesting microbial pathogens, macrophages recognize pathogen-associated molecular patterns of bacteria, such as LPS or bacterial CpG DNA, via pattern recognition receptors that include several members of the TLR family (2), and consequently produce a variety of soluble mediators, such as cytokines and chemokines that recruit immune cells and induce inflammation (1). Among other things, IL-12 produced by macrophages (3), in conjunction with other cytokines like IL-18 (4), induces NK cells (5, 6) and some TCRαβ-bearing memory-type CD8+ T cells (7–9) to produce IFN-γ, which in turn activates macrophages to ultimately kill internalized bacteria via generation of reactive nitrogen and oxygen intermediates (10). The macrophages, therefore, constitute both sentinels and the first line of defense against bacterial pathogens, and IFN-γ is the most important factor of early host resistance against bacterial infection.

Microbes, on the other hand, have evolved a variety of countermeasures to macrophage defense mechanisms (1, 11). Intracellular bacterial pathogens evade the macrophage killing mechanisms that eliminate intracellular pathogens, and thus they can survive and replicate inside macrophages (12–15). In addition, a variety of bacterial pathogens are known to induce cell death in macrophages (15–26). However, the biological significance of the pathogen-induced cell death in macrophages is not fully defined with respect to the host-parasite relationship and it may differ with individual pathogens; viz, the macrophage cell death may be one evasion mechanisms of bacteria from killing by macrophages, or it may constitute a host defense response to the incoming pathogen to halt its survival within macrophages and/or to present Ags to the adaptive immune system through bystander dendritic cells (27, 28), or it may be the result of a bacterial strategy to promote disease through activation of caspase-1 which can cleave the proforms of the inflammatory cytokines IFN-β and IL-18 (18–20).

In previous studies, we developed a novel T cell hybridization system, referred to as a functional T cell hybridoma, which comprised production of growth-arrested hybrids between the B6HO3 T cell lymphoma (29) and normal T cells and subsequent spontaneous cell transformation of the hybrids (30, 31). Compared with the ordinary T cell hybridomas using the BW5147 T cell lymphoma (32), our hybridoma system is unique in that the hybridomas dictate most of the terminally differentiated phenotypes of normal parental T cells, thus preserving the effector functions of T lymphocytes, such as T cell contact helper activity (30) and cytotoxic activity (31). Thus, we were interested in the feasibility of constructing functional T cell hybridomas with hitherto unknown phenotypes by using this hybridization system (32), as T lymphocytes were becoming recognized as consisting of functionally more heterogeneous populations than previously anticipated (33–36). In a series of attempts to produce functional T cell hybridomas derived from minor T cell populations, we obtained a novel T cell hybridoma designated B6HO3. We focused in this study on the characterization of this hybridoma and found that the B6HO3 T cell hybridoma recognizes and responds to a specialized form of macrophage cell death resulting from bacterial infection.
cell death in bacterial-infected J774 macrophage cells with the production of IFN-γ, a crucial cytokine for microbicidal functions of macrophages. This finding predicted that a minor subset of T cells should have the ability to recognize cell death in macrophages resulting from bacterial infection. In fact, we have shown in this study that not only minor subsets of spleen αβ T cells but also subsets of γδ T and NK cells produce IFN-γ in response to direct cell-cell contact with dying Listeria monocytogenes (LM)2-infected macrophage cells. Thus, this study suggests that the innate immunity may involve a lymphocyte recognition system sensing macrophage cell "danger" resulting from bacterial infection.

**Materials and Methods**

**Mice and cell lines**

We derived TCRαβ-deficient T cell line YC11 from the hypoxanthine-guanine phosphoribosyltransferase-negative YACUT T cell line (29). YC11 cells and J774 macrophage-like cells were maintained in DMEM medium containing 10% FCS. Mouse hepatoma cell line I-6-37 and mouse fibroblast cell line L929 were obtained from Riken Cell Bank and maintained in DMEM supplemented with 10% FCS and 4.5 g/ml glucose. Bone marrow-derived macrophages (BMM) were grown from marrow cells harvested from C57BL/6 mice according to the method described elsewhere (38). C3H/He, DBA/2, and C57BL/6 mice were purchased from Japan SLC and Oriental Yeast. Mice were used in accordance with the institutional guidelines.

**Bacteria**

Virulent LM (EGD strain; Hly+) and its nonhemolytic strain (ATCC15316; Hly-) were obtained from Dr. T. Fujimura (Department of Dermatology, Kitasato University School of Medicine, Sagamihara, Japan). A mutant strain of LM, DP-L4048 (39), which produces a mutant listeriolysin O (LLO) protein that is not subjected to phosphorylation, was obtained from Dr. D. A. Portnoy (Department of Molecular and Cell Biology, University of California, Berkeley, CA). LM strains were grown in tryptic soy broth overnight. Escherichia coli (ATCC25922), Salmonella typhimurium (ST; a stock strain of our laboratory), and Staphylococcus aureus (SA; a stock strain of our laboratory) were grown in brain-heart infusion broth overnight. The bacteria were washed twice with PBS by centrifugation and stored at −80°C until use. Heat-killed LM (HKLKM) was prepared by incubating suspension of bacteria at 70°C for 1 h. S. typhimurium expressing type III secretion system (STSS) was prepared according to the method described elsewhere (21). Briefly, overnight cultures of S. typhimurium were diluted to an OD measured at 600 nm of 0.1 in L-broth containing 0.3 M sodium chloride, incubated at 37°C for 3 h with shaking, and used immediately after washing twice with PBS by centrifugation. B. subtilis (B. subtilis 168) was grown inbrain-heart infusion broth overnight. The bacteria were washed twice with PBS by centrifugation and stored at −80°C until use. Heat-killed MLKM (HKLKM) was prepared by incubating suspension of bacteria at 70°C for 1 h. S. typhimurium expressing type III secretion system (STSS) was prepared according to the method described elsewhere (21). Briefly, overnight cultures of S. typhimurium were diluted to an OD measured at 600 nm of 0.1 in L-broth containing 0.3 M sodium chloride, incubated at 37°C for 3 h with shaking, and used immediately after washing twice with PBS by centrifugation. B. subtilis (B. subtilis 168) was grown inbrain-heart infusion broth overnight. The bacteria were washed twice with PBS by centrifugation and stored at −80°C until use.

**mAbs and reagents**

The following mAbs were used: FITC-conjugated, PE-conjugated, or biotinylated mAbs specific for TCRαβ (H-57-59), CD4 (GK1.5), CD8 (53-6.7), CD11c (HL-3), CD69 (1H2F3), CD45RB/B220 (RA3-6B2), DX5, TCRVβ6 (44-22-1), CD25 (3C7), CD3 (145-2C11), NK1.1 (PK136), FcRII/III (2.4G2), and mouse IFN-γ (XMG1.2) (all purchased from BD Pharmingen). Anti-CD1d (1B1) mAb and PE-Cy5-conjugated streptavidin were purchased from BD Pharmingen. Anti-CD1d mAb and PE-Cy5-conjugated streptavidin were purchased from eBioscience. PE-conjugated anti-TCRγδ (GL3), PE-conjugated hamster IgG and PE-conjugated anti-mouse IL-4 (BDV6-24G2) were purchased from Caltag Laboratories. Anti-CD244 mAb (C9.1) was produced and fluorescein conjugated in our laboratory (41). Biotinylated anti-CD121i mAb (TM-β1) was obtained from Dr. T. Tanaka (Osaka University, Suita, Japan). Anti-mouse IL-12 polyclonal Abs and its control mAb (TM-11001) were diluted to an OD measured at 600 nm of 0.1 in L-broth containing 0.3 M sodium chloride, incubated at 37°C for 3 h with shaking, and used immediately after washing twice with PBS by centrifugation. B. subtilis (B. subtilis 168) was grown inbrain-heart infusion broth overnight. The bacteria were washed twice with PBS by centrifugation and stored at −80°C until use.

2 Abbreviations used in this paper: LM, Listeria monocytogenes; BMM, bone marrow-derived macrophage; CHX, cycloheximide; EtA-1, early T lymphocyte activation protein 1; LDH, lactate dehydrogenase; LLO, listeriolysin O; MOI, multiplicity of infection; NWNNA, nylon wool nonadherent; TSS, type III secretion system; SA, Staphylococcus aureus; ST, Salmonella typhimurium.

**Cell fusion and hybrid selection**

YC11 cells (10 × 10^6) were hybridized with 10 × 10^6 secondary C3H/He anti-DBA/2 MLC cells as described previously with slight modifications (31). Briefly, after fusion, cells were suspended in culture medium (DMEM supplemented with 10% FCS, 10 mM HEPES, 5 × 10^-3 M 2-ME, and 1 mM nonessential amino acids) containing hypoxanthine/aminopterin/thymidine, distributed into the wells of a 24-well microculture plate and cultured for 18 days to eliminate unhybridized YC11 cells. Eighteen days after fusion, unhybridized MLC cells were eliminated by the panning method as described previously (30). The adherent cells on the panning plate were recovered by vigorous pipetting with culture medium, mixed with 6 × 10^6 irradiated DBA/2 mouse spleen cells and then pelleted by centrifugation. The collected cells were suspended in culture medium and distributed into the wells of a 24-well microculture plate and then incubated at 37°C in a CO2 incubator. On the next day, human rIL-2 (25 U/ml) was added to the culture. After 5 days, growing cells were collected and distributed at one cell per well into 96-well microculture plates containing irradiated DBA/2 spleen cells, and IL-2 was added at day 2. After 5 days, growing cells in each well were passaged into a 24-well microculture plate containing irradiated DBA/2 spleen cells and IL-2, then diluted to 1/50. After this passaging, the hybrid cells were repeated five times with intervals of 2 wk. Autonomously proliferating cells appeared in one of the wells. This hybridoma cell line was designated B6H03 and used in the present study.

**Flow cytometric analysis of surface Ags**

This method was described previously (30).

**Ab-mediated cross-linking of the TCR-CD3 complex**

Anti-CD3 mAb (25 μg/ml) diluted in Tris buffer (0.05 M Tris-HCl, pH 9.5) was incubated in a 96-well microculture plate at 4°C overnight. After unbound mAb was removed, 50 × 10^5 of B6H03 cells or YACUT cells were added to each well and cultured for 16 h.

**RNA extraction and RT-PCR**

Total RNA was extracted from B6H03 hybridoma and YACUT lymphoma by using the Pure RNA isolation kit (Boehringer Mannheim). RT-PCR was performed by using the Access RT-PCR system (Promega) with two primer sets, β2-microglobulin-specific primers as an endogenous standard (42) and cytokine-specific primers, in the same tube. Primers used for PCR are shown in Table 1. They were synthesized by SIGM Genosys and designed with Primer3 software (Broad Institute, Cambridge, MA).

**Cytokine assay**

Mouse IFN-γ, TNF-α, and GM-CSF levels in culture supernatants were determined by ELISA using commercial ELISA kits (BioSource International). For early T lymphocyte activation protein 1 (Eta-1), a mouse osteopontin determination kit (ImmunoBiologicals) was used. For intracellular cytokine staining, cells stimulated with the blocking anti-FCyR mAb (2A4G2) were stained with mAbs against surface markers, and then the cells were permeabilized using the Cytofix/Cytoperm Plus kit (BD Pharmingen) and stained using either FITC-conjugated anti-IFN-γ or PE-conjugated anti-IL-4 mAb. The data were acquired using FACSscan and analyzed using CellQuest software (BD Bioscience). For measuring intracellular IFN-γ production by B6H03 cells, B6H03 cells were separated from J774 cells using magnetic cell sorting. Briefly, cells in coculture of B6H03 with LM-infected or noninfected J774 cells were collected in microcentrifuge tubes and incubated with biotinylated anti-TCRγδ followed by addition of streptavidin-conjugated microbeads (Miltenyi Biotec), since this mAb non-specifically bound to J774 cells, but not to B6H03 cells. Magnectically labeled J774 cells were removed by placing the tube in the magnetic field of a MACS separator and then the rest of the cells were assessed for intracellular IFN-γ. For measuring intracellular IFN-γ production by NWNA spleen cells, dyeing LM-infected J774 cells were excluded on the basis of forward and side light scatter.
Table 1. Primer sequences used for RT-PCR and expected sizes of the PCR products

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer Sequences (5’–3’)**</th>
<th>Product Sizes (bp)*</th>
</tr>
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<tbody>
<tr>
<td>β2-microglobulin</td>
<td>GGTCCGCTCGGTTGGATCGCAGTCGGTCTTTT</td>
<td>301</td>
</tr>
<tr>
<td>IL-2</td>
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<tr>
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<tr>
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<td>IFN-γ</td>
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<td>247</td>
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<tr>
<td>GM-CSF</td>
<td>GTACAGTTTCTGAGCTGATTAGG</td>
<td>261</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>MCP-1</td>
<td>GACTAGCTGTTCGGGCTGGCTTGAAC</td>
<td>243</td>
</tr>
</tbody>
</table>

*The sense sequence is shown above the antisense sequence.

**Predicted size of the PCR product.

In vitro stimulation of B6HO3 hybridoma and NWNA spleen cells by bacterial-infected J774 cells

J774 cells (1 × 10^6 cells/well) or BMM cells (5 × 10^4 cells/well) suspended in culture medium without antibiotics were seeded into a 96-well microculture plate and cultured at 37°C overnight in a CO2 incubator. After each well was washed with DMEM without antibiotics, bacteria were inoculated into each well (100 μl/well) of the microculture plate at various multiplicity of infection (MOI) and the plate was centrifuged at 800 × g for 1 min and then incubated at 37°C in a CO2 incubator. After a 1-h infection period, 50 × 10^3 B6HO3 cells or 8 × 10^5 NWNA spleen cells per 0.2 ml of culture medium containing gentamicin (200 μg/ml) were added to each well. The microculture plate was incubated at 37°C for 24 h in a CO2 incubator. For B6HO3 cells, culture supernatants were harvested and assessed for IFN-γ levels by ELISA. For NWNA cells, intracellular IFN-γ staining was conducted and analyzed on FACSscan.

Macrophage cytotoxicity assay

Cytotoxicity was assessed by measuring the release of cytosolic lactate dehydrogenase (LDH) into the supernatants. J774 cells were incubated with bacteria for a 1-h infection period and then cultured in medium containing 200 μg/ml gentamicin. At various time points, the culture supernatants were harvested and their LDH levels were measured using the colorimetric Cytotox 96 kit (Promega). The relative LDH release was calculated as 100 × (experimental release – spontaneous release)/total release – spontaneous release, where spontaneous release is the amount of LDH activity in the supernatant of uninfected J774 cells and total release is the activity in cell lysates.

Results

Surface membrane phenotype of B6HO3 hybridoma

The B6HO3 hybridoma and its parental YC11 lymphoma were characterized by immunofluorescence for surface staining of Abs specific for a variety of T cell surface Abs. Fig. 1 mainly illustrates FACS profiles of the Abs that were specifically expressed by the B6HO3 cells. The B6HO3 cells expressed the H-2Dβ of the C3H/He parental lymphocytes and H-2Dβ of the YC11 lymphoma (Fig. 1, A–D), which, along with the fact that the mean chromosome numbers of B6HO3 were 80 (data not shown), indicates that the B6HO3 cells were hybrids. B6HO3 cells expressed 2B4 (CD24), CD11c, IL-2Rα (CD25), IL-2Rβ (CD122) and TCRαβ utilizing the VB6 gene segment (Fig. 1, I–T), but did not express CD4 and CD8 (Fig. 1, E–H), whereas none of these Abs were expressed on the parental YC11 cells. CD244 and CD11c, which are primarily expressed by NK cells (41) and dendritic cells, respectively, are known to be expressed on activated/memory-type T cells (43, 44). Other activation markers for T cells, such as CD69, CD45RBhigh, and CD44high (45), were also detected on the B6HO3 cells as well as the parental YACUT cells (data not shown). These results demonstrate that the B6HO3 is the CD4+ CD8– TCRαβ+ T cell hybridoma exhibiting the activated/memory phenotype. Since some double-negative T cells have been reported to express surface markers such as B220, CD16/CD32 (46), or DX5 (35), we also examined the expression of these Abs on B6HO3 cells and found that B6HO3 cells were negative for the Abs (data not shown).

Cytokine production by B6HO3 cells upon Ab-mediated cross-linking of the TCR-CD3 complex

B6HO3 cells were next studied for their ability to produce cytokines following activation of the cells by cross-linking of the TCR-CD3 complex with solid-phase anti-CD3 mAb. Fig. 2, A and B, show the analysis of RT-PCR products from GM-CSF, IFN-γ, IL-2, IL-4, and TNF-α mRNAs extracted from the B6HO3 hybridoma and the YACUT lymphoma before and after cross-linking of the CD3-TCR complex. The CD3-TCR ligation induced B6HO3 cells to express GM-CSF and IFN-γ mRNA, but not IL-2 and IL-4 mRNA, while parental YACUT lymphoma cells transcribed only IL-4 mRNA in response to CD3-TCR ligation, whose responsiveness was thus suppressed in the B6HO3 hybridoma. TNF-α mRNA was constitutively expressed in both YACUT and B6HO3 cells, and CD3-TCR ligation did not enhance the levels of its expression in both of the cells. Since some double-negative T cells have been reported to produce MCP-1 (CCL2), Eta-1 (47), or IL-10 (34), investigation of their mRNAs expression was included in this experiment. As shown in Fig. 2C, although YACUT cells did not express Eta-1 mRNA before and after CD3-TCR ligation, B6HO3 cells constitutively expressed Eta-1 mRNA, and the expression level was enhanced by CD3-TCR ligation. MCP-1 and IL-10 mRNAs were not detectable in both B6HO3 and YACUT cells before and after CD3-TCR ligation (data not shown).

To see production of cytokines at a translational level, secretion of GM-CSF, IFN-γ, TNF-α, and Eta-1 into culture supernatants following activation of B6HO3 and YACUT cells by Ab-mediated cross-linking of the TCR-CD3 complex was assessed by ELISA.
intracellular cytokine staining (Fig. 3B). Approximately 56% of cells with each one of the following bacteria: LM, HKLM, LLO-deficient LM, ST, and SA, and then supernatants harvested at 24 h of culture were assessed for IFN-γ levels by ELISA as illustrated in Fig. 3A. High amounts of IFN-γ were detectable only in cocultures of B6HO3 cells with LM-infected J774 cells. J774 cells cultured with HKLM and J774 cells infected with Hly−LM, Escherichia, Salmonella, or Staphylococcus displayed no significant ability of stimulating B6HO3 cells to produce IFN-γ. Interestingly, among the bacterial-infected J774 cells examined, wild type LM-infected J774 cells were the only cells that were microscopically observed to undergo cell death.

To verify that B6HO3 cells themselves produce IFN-γ, flow cytometric analysis of IFN-γ production was performed by using intracellular cytokine staining (Fig. 3B). Approximately 56% of

B6HO3 hybridoma produces IFN-γ when cocultured with dying LM-infected J774 macrophage cells

In the course of the cell fusion experiments, we noticed that the IL-2-dependent hybrid cells, precursor cells of the B6HO3 hybridoma before its transformation, were gathering around the macrophages that had been used for feeder cells. Thus, we hypothesized that the hybridoma cells might functionally interact with macrophages. To test this possibility, we cocultured B6HO3 cells with the J774 macrophage-like cell line alone or J774 cells infected with each one of the following bacteria: LM, HKLM, LLO-deficient (Hly−) LM, E. coli, ST, and SA, and then supernatants harvested at 24 h of culture were assessed for IFN-γ levels by ELISA as illustrated in Fig. 3A. High amounts of IFN-γ were detectable only in cocultures of B6HO3 cells with LM-infected J774 cells. J774 cells cultured with HKLM and J774 cells infected with Hly−LM, Escherichia, Salmonella, or Staphylococcus displayed no significant ability of stimulating B6HO3 cells to produce IFN-γ. Interestingly, among the bacterial-infected J774 cells examined, wild type LM-infected J774 cells were the only cells that were microscopically observed to undergo cell death.

To verify that B6HO3 cells themselves produce IFN-γ, flow cytometric analysis of IFN-γ production was performed by using intracellular cytokine staining (Fig. 3B). Approximately 56% of the B6HO3 cells cocultured with LM-infected J774 cells were positive for intracytoplasmic IFN-γ (Fig. 3B, upper panel), whereas no IFN-γ was detectable in B6HO3 cells cocultured with noninfected J774 cells (Fig. 3B, lower panel). These results indicate that the B6HO3 hybridoma produces IFN-γ in response to dying LM-infected J774 cells.

Cell-cell contact is necessary for activation of B6HO3 cells by dying LM-infected J774 cells, but the TCR is not involved in its recognition mechanism

We next addressed a question of whether soluble mediators secreted by LM-infected J774 cells were responsible for the IFN-γ production by B6HO3 cells. To this end, we compared the ability of LM-infected J774 cells to induce IFN-γ production by B6HO3 cells with that of LM-infected J774 supernatants collected at 24 h postinfection (Fig. 4A). Since IL-12 plays a pivotal role in the production of IFN-γ by T and NK cells (3), we also examined whether IL-12 played a role in this response by coculturing B6HO3 cells with LM-infected J774 cells in the presence of neutralizing Ab specific for IL-12. Fig. 4A shows IFN-γ levels in supernatants collected from those 24-h cocultures. A culture supernatant from LM-infected J774 cells was incapable of inducing IFN-γ by B6HO3 and addition of anti-IL-12 Ab to the coculture did not inhibit the production of IFN-γ. These results indicate that the IFN-γ response is IL-12 independent and not triggered by soluble mediators.

To determine whether cell-cell contact between B6HO3 cells and dying LM-infected J774 cells is required for the production of IFN-γ, B6HO3 cells were cocultured with LM-infected J774 cells
in a Transwell to separate both cells with a permeable membrane, which inhibited cell-cell contact (Fig. 4B). Separation of B6HO3 cells from dying LM-infected J774 cells failed to induce IFN-γ, indicating that cell-cell contact between B6HO3 cells and LM-infected J774 cells was required for the activation of B6HO3 cells.

To evaluate the contribution of the TCR expressed on B6HO3 cells in the cognitive interaction between B6HO3 cells and dying LM-infected J774 cells, we examined the effect of Fab of anti-TCRVβ6 mAb and mAbs to H-2Kb, H-2Dd, Iaβ, and CD1d, potential ligands for the TCR, on the IFN-γ response by adding each one of these mAbs to cocultures of B6HO3 cells with LM-infected J774 cells (Fig. 4C). Fab of anti-TCRVβ6 mAb as well as anti-H-2Dd, anti-Iaβ, and anti-CD1d mAbs did not inhibit IFN-γ production by B6HO3 cells. Anti-H-2Kb mAb and a mixture of anti-H-2Kd and anti-H-2Dd mAbs slightly enhanced the IFN-γ production for an unknown reason. These results indicate that the TCR expressed on B6HO3 cells is not involved in the mechanism underlying the cognitive interaction between B6HO3 cells and dying LM-infected J774 cells, and that the IFN-γ response is TCR independent.

De novo protein synthesis is not required for B6HO3 hybridoma cell recognition of dying LM-infected J774 cells

Since a variety of proteins, such as cytokines and heat shock proteins, are induced to synthesize in bacterial-infected macrophages, we next asked whether de novo protein synthesis in bacterial-infected macrophages is necessary for B6HO3 cell recognition of dying LM-infected J774 cells. To this end, J774 cells that had been treated with CHX, an inhibitor of protein synthesis, were infected with LM, and at 6 h postinfection the infected cells were cocultured with B6HO3 cells without removing CHX. After 4 h of coculture, total RNA was extracted from the cultures and assessed for the expression of IFN-γ mRNA by RT-PCR (Fig. 4D). Although no IFN-γ mRNA was detectable in cultures of J774 cells, LM-infected J774 cells, and B6HO3 cells alone, IFN-γ mRNA expression was detected in cocultures of B6HO3 cells with LM-infected J774 cells, and this IFN-γ mRNA expression was not inhibited by the treatment of J774 cells with CHX. The inhibitory effect of CHX on protein synthesis was confirmed by the parallel experiment showing that TNF-α production by LM-infected J774 cells was strongly inhibited in the presence of CHX (Fig. 4E). These results indicate that the B6HO3 cell recognition of dying LM-infected J774 cells does not require de novo protein synthesis in LM-infected J774 cells.

IFN-γ production by B6HO3 hybridoma is also induced through direct cell-cell contact with dying J774 cells infected with S. flexneri or ST that expressed TTSS

Since S. flexneri and ST had been reported to induce cell death in macrophages (16, 19–26), we next determined whether cell death in J774 cells caused by Shigella or Salmonella infection could also induce B6HO3 cells to produce IFN-γ. Fig. 5A shows the time course of cell death following infection of J774 cells with LM or Shigella as assessed by LDH release into culture supernatants. Infection of J774 cells with LM resulted in cell destruction beginning at 8 h postinfection, reaching a maximum of 60% death within 20 h. Infection of J774 cells with wild-type Shigella YSH6000 resulted in almost complete destruction of the cells within 16 h, while plasmid-cured Shigella-infected J774 cells did not undergo cell death. Fig. 5B shows IFN-γ levels in supernatants from 24-h cocultures of B6HO3 cells with wild-type Shigella- or plasmid-cured Shigella-infected J774 cells. Like dying LM-infected J774 cells, dying wild-type Shigella-infected J774 cells induced IFN-γ production by B6HO3 cells, whereas intact J774 cells infected with plasmid-cured Shigella YSH6200 did not significantly induce B6HO3 cells to produce IFN-γ. Fig. 5C shows the result of an experiment using a Transwell to separate B6HO3 cells from dying wild-type Shigella-infected J774 cells, and the result of an experiment in which the ability of a supernatant collected from a 24-h culture of wild-type Shigella-infected J774 cells to induce IFN-γ production by B6HO3 cells was examined. Separation of wild-type Shigella-infected J774 cells and B6HO3 cells by a permeable membrane completely abrogated the IFN-γ production by B6HO3 cells, and a culture supernatant from wild-type Shigella-infected J774 cells had no ability to stimulate B6HO3 cells to produce IFN-γ. These results indicate that direct cell-cell contact between B6HO3 cells and dying Shigella-infected J774 cells is necessary for the IFN-γ production by B6HO3 cells.

FIGURE 3. IFN-γ production by B6HO3 hybridoma cocultured with LM-infected J774 cells. A, Supernatants were collected from 24-h cultures of the following combinations: J774 cells alone, B6HO3 cells alone, B6HO3 and J774 cells, B6HO3 and LM, LM-infected J774 cells, B6HO3 and LM-infected J774 cells, Hly-deficient (Hly + ) LM-infected J774 cells, B6HO3 and Hly + LM-infected J774 cells, HKLM and J774 cells, B6HO3 and HKLM-inoculated J774 cells, E. coli-infected J774 cells, B6HO3 and E. coli-infected J774 cells, ST-infected J774 cells, B6HO3 and ST-infected J774 cells, SA-infected J774 cells, or B6HO3 and SA-infected J774 cells. Infection was performed at MOI of 50:1. IFN-γ levels of supernatants were measured by ELISA. B, IFN-γ production by B6HO3 cells was assessed by intracellular cytokine staining. B6HO3 cells were cultured alone (open curve) or cocultured with J774 cells (lower panel, shaded curve) or LM-infected J774 cells (upper panel, shaded curve) for 24 h. Monensin was added for the last 10 h of culture. B6HO3 cells were separated from J774 cells by magnetic cell sorting, stained for intracellular IFN-γ, and analyzed using FACScan. Depletion of J774 cells was confirmed by the fact that no cells were detected in the forward and light scatter area gated for B6HO3 after magnetic cell sorting of control LM-infected J774 cells. Representative data from one of two experiments are shown.
The same results were obtained with ST as shown in Fig. 5, D–F. J774 cells infected with Salmonella grown under conditions for TTSS expression underwent rapid cell death (Fig. 5D) and simultaneously stimulated B6HO3 cells to produce IFN-γ (Fig. 5E). However, J774 cells infected with Salmonella in stationary phase neither underwent cell death (Fig. 5D) nor induced IFN-γ production by B6HO3 cells (Fig. 5E). In the same way as Shigella, the necessity of cell-cell contact between B6HO3 cells and dying Salmonella-infected J774 cells for the induction of IFN-γ was verified by both the cell separation experiment and the experiment using a supernatant collected from 24-h cultures of J774 cells infected with Salmonella grown under conditions for TTSS expression (Fig. 5F).

These results indicate that cell death in bacterial-infected J774 cells is closely associated with the ability of the infected cells to stimulate B6HO3 cells to produce IFN-γ in a direct cell-cell contact-dependent manner.

Cell death in J774 cells induced by gliotoxin or freeze thawing and cell death in hepatoma cells caused by LM infection do not trigger B6HO3 hybridoma to produce IFN-γ

To determine whether B6HO3 cells recognize macrophage cell death per se, we examined whether cell death in J774 cells induced by the treatment of gliotoxin (48) or freeze thawing, which induce apoptosis and necrosis, respectively, in macrophages, could also induce B6HO3 to produce IFN-γ. Fig. 6A shows the time course of cell destruction following a 3-h treatment of J774 cells with gliotoxin as assessed by LDH release into culture supernatants. The gliotoxin treatment of J774 cells resulted in cell destruction with similar kinetics to cell death in LM-infected J774 cells. However, the dying gliotoxin-treated J774 cells, when cocultured with B6HO3 cells, did not induce IFN-γ production by B6HO3 cells as assessed by IFN-γ levels in culture supernatants (Fig. 6B). Fig. 6B also shows that dead J774 cells subjected to freeze thaw did not stimulate B6HO3 cells to produce IFN-γ. These results indicate that the B6HO3 hybridoma discriminates between typical apoptotic and necrotic cell death in macrophages and macrophage cell death caused by bacterial infection.

We next addressed a question of whether B6HO3 cells could produce IFN-γ in response to cell death in LM-infected cells other than macrophages. To this end, we infected Hepa 1-6 hepatoma cells with mutant LM strain DP-L4048, because infection of Hepa 1-6 cells with wild-type LM did not result in cell death in Hepa 1-6 cells (data not shown). The mutant strain DP-L4048 expresses mutant LLO protein, whose potential phosphate acceptor residues in the PEST-like sequence were all changed to a residue that cannot accept phosphate, and thus the LLO proteins are not subjected to degradation in the cytosol, being capable of inducing rapid membrane damage (39). Fig. 6, C–F, show phase-contrast microscopic observations of normal Hepa 1-6 cells and J774 cells (Fig. 6, C and E) and those infected with the mutant LM strain DP-L4048 (Fig. 6, D and F) in the absence of gentamicin at 6 h postinfection. As has been reported (39), J774 cells infected with the mutant strain underwent rapid cell death within 6 h (Fig. 6F). Similarly,
J774 and Hepa 1-6 cells were cocultured with B6HO3 cells for marker DX5. A representative result is shown in Fig. 7, A–K. Hepa 1-6 cells did not. These results indicate that the IFN-γ response of B6HO3 is a bacterial-infected macrophage death-specific phenomenon.

A minor subset of αβ T cells and subsets of γδ T and NK cells respond to dying LM-infected J774 cells with the production of IFN-γ in a cell-cell contact-dependent manner

The above-mentioned functional phenotype of the T cell hybridoma B6HO3 predicted that a minor subset of T cells should recognize dying LM-infected J774 cells. To address this question, wild-type LM- or Hly− LM-infected J774 cells were cocultured with C3H/He mouse nylon wool nonadherent (NWNA) spleen cells in ordinary wells or Transwells in the presence of either neutralizing Ab specific for IL-12 or control Ab, and 24 h after coculture the NWNA spleen cells were assessed for IFN-γ production by intracellular cytokine staining in combination with surface staining of TCRαβ and NK cell marker DX5. A representative result is shown in Fig. 7, A–K. Although coculture of NWNA spleen cells with Hly− LM-infected J774 cells, which had not undergone cell death, did not result in the production of IFN-γ by NWNA spleen cells (Fig. 7B), dying wild-type LM-infected J774 cells indeed induced 2.3 ± 0.5% of αβ T cells to produce IFN-γ (Fig. 7D), of which the proportion was decreased to 0.7 ± 0.1% by addition of anti-IL-12 Ab (Fig. 7G). It is noteworthy that the IFN-γ production of αβ T cells expressed intermediate levels of the TCRαβ. Unexpectedly, dying LM-infected J774 cells also induced a small percentage of non-αβ T cells (6.1 ± 0.6% of total NWNA spleen cells) to produce IFN-γ (Fig. 7D, lower right quadrant), and those cells were decreased to 3.0 ± 0.4% in the presence of anti-IL-12 Ab (Fig. 7G, lower right quadrant). When the dot plots were gated on IFN-γ-producing non-αβ T cells and assessed for DX5 expression, 89.2 ± 4.2% and 92.1 ± 2.7% of the IFN-γ-producing non-αβ T cells were DX5+ in the absence and presence of anti-IL-12 Ab, respectively (Fig. 7, E and H), indicating that most of the IFN-γ-producing non-αβ T cells were NK cells. Of total NK cells (αβTCR−DX5+ cells), 41.4 ± 5.0% were IFN-γ+ cells, which were reduced to 26.3 ± 2.6% in the presence of anti-IL-12 Ab (data not shown). Since ~10% of IFN-γ-producing non-αβ T cells were not NK cells, we next determined whether γδT cells also produced IFN-γ. As shown in Fig. 7, I–K, when NWNA spleen cells cocultured with dying LM-infected J774 cells in the presence of anti-IL-12 Ab (Fig. 7I) or control Ab (Fig. 7J) were stained with PE-conjugated mAb specific for TCRγδ (Fig. 7, I and J) or control PE-conjugated hamster IgG (Fig. 7K) followed by intracellular IFN-γ staining, 21 ± 0.2% of γδ T cells, which comprised 2.4 ± 0.3% of the NWNA spleen cell population, produced IFN-γ in the absence of anti-IL-12 Ab and 6.4 ± 1.6% of γδ T cells produced IFN-γ in the presence of anti-IL-12 Ab. Fig. 7, C and F, show that separation of NWNA spleen cells from dying LM-infected J774 through permeable membrane failed to induce NWNA spleen cells to produce IFN-γ, indicating that IFN-γ production by a minor subset of αβ T cells and subsets of γδ T and NK cells is completely dependent on direct cell-cell contact with dying LM-infected J774 cells. To ensure that LM-infected J774 culture supernatants do not include soluble mediators that
can induce T and NK cells to produce IFN-γ, NWNA spleen cells were cultured for 24 h with (Fig. 7M) or without (Fig. 7L) a supernatant collected from 24-h cultures of LM-infected J774 cells, and the production of IFN-γ by NWNA spleen cells was assessed by intracellular cytokine staining. As shown in Fig. 7M, a culture supernatant from LM-infected J774 cells was incapable of inducing IFN-γ by NWNA spleen cells.

To evaluate the role of IL-12, various amounts of anti-IL-12 Ab were added to the cocultures and their effects on the IFN-γ production by NWNA spleen cells were examined (Fig. 7, N and O). Addition of a range of concentrations between 0.01 and 0.3 μg/ml anti-IL-12 Ab resulted in a decrease in the numbers of IFN-γ-producing αβ T cells and non-αβ T cells by ∼25 and ∼50%, respectively, but higher concentrations than 1 μg/ml anti-IL-12 Ab no longer reduced the number of IFN-γ-producing cells. These results indicate that 1 μg/ml anti-IL-12 Ab was sufficient to neutralize IL-12 and that ∼25% of the IFN-γ-producing αβ T cells and ∼50% of IFN-γ-producing non-αβ T cells did not require IL-12 for the production of IFN-γ.

Overall, these results indicate that a small percentage of αβ T cells recognize and respond to dying LM-infected J774 cells by producing IFN-γ in a similar way as the B6H3O hybridoma. Furthermore, subsets of γδ T and NK cells proved to have the same ability of responding to dying LM-infected J774 cells as this minor subset of αβ T cells.

Surface membrane phenotype of IFN-γ-producing αβ T cells
We further examined CD4 and CD8 expression on the IFN-γ-producing αβ T cells responding to dying LM-infected J774 cells by using three-color flow cytometric analysis. C3H/He mouse NWNA spleen cells that had been cocultured with LM-infected J774 cells in the presence of anti-IL-12 Ab or control Ab were stained for TCRαβ, intracellular IFN-γ, and CD4 or CD8, subjected to flow cytometry, and then dot plots were gated on IFN-γ-producing αβ T cells and analyzed for the expression of CD4 and CD8. A representative result is shown in Fig. 8A. Of the IFN-γ-producing αβ T cells, 5.2 ± 2.3% were CD8+ and 55.8 ± 9.2% were CD4+ in the absence of anti-IL-12 Ab, and 7.3 ± 2.4% were CD8+ and 43.9 ± 9.4% were CD4+ in the presence of anti-IL-12 Ab. These results indicate that the IFN-γ-producing αβ T cell population mainly consists of CD4+ (~50%) and CD4−CD8− (~44%) αβ T cells. It was noteworthy that the level of CD4 expression on the surface of the IFN-γ-producing αβ T cells (Fig. 8, middle and bottom panels) was lower than that of ordinary T cells (Fig. 8, top panel).

IFN-γ-producing αβ T cells involve NKT cells
Because the surface membrane phenotype of the IFN-γ-producing αβ T cells responding to dying LM-infected J774 cells was similar to that of NKT cells (35), we next determined whether the IFN-γ-producing αβ T cells were NKT cells or not. To this end, NWNA spleen cells from a C57BL/6 mouse instead of a C3H/He mouse were cocultured with dying LM-infected J774 cells in the presence of anti-IL-12 Ab or control Ab, stained with mAbs specific for TCRαβ and NK1.1 followed by intracellular IFN-γ and IL-4 staining, and then three-color flow cytometric analysis was performed (Fig. 8B). Dying LM-infected J774 cells did not induce NWNA spleen cells to produce IL-4, but induced 2.9 ± 2.3% CD8+ T cells and 7.3 ± 3.2% CD4+ T cells. It was noteworthy that the level of CD4 expression on the surface of the IFN-γ-producing αβ T cells (Fig. 8, middle and bottom panels) was lower than that of ordinary T cells (Fig. 8, top panel).

IFN-γ-producing αβ T cells were further examined by using three-color flow cytometric analysis. C3H/He mouse NWNA spleen cells that had been cocultured with LM-infected J774 cells in the presence of anti-IL-12 Ab or control Ab were stained for TCRαβ, intracellular IFN-γ, and CD4 or CD8, subjected to flow cytometry, and then dot plots were gated on IFN-γ-producing αβ T cells and analyzed for the expression of CD4 and CD8. A representative result is shown in Fig. 8A. Of the IFN-γ-producing αβ T cells, 5.2 ± 2.3% were CD8+ and 55.8 ± 9.2% were CD4+ in the absence of anti-IL-12 Ab, and 7.3 ± 2.4% were CD8+ and 43.9 ± 9.4% were CD4+ in the presence of anti-IL-12 Ab. These results indicate that the IFN-γ-producing αβ T cell population mainly consists of CD4+ (~50%) and CD4−CD8− (~44%) αβ T cells. It was noteworthy that the level of CD4 expression on the surface of the IFN-γ-producing αβ T cells (Fig. 8, middle and bottom panels) was lower than that of ordinary T cells (Fig. 8, top panel).

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**FIGURE 7.** Subsets of αβ T cells, γδ T cells, and NK cells produce IFN-γ in response to dying LM-infected J774 cells. C3H/He mouse NWNA spleen cells were cocultured for 24 h with J774 cells alone (A), Hly− LM-infected J774 cells (B), or LM-infected J774 cells in an ordinary 24-well plate (D and G) or a Transwell (C and F) in the presence (2 μg/ml) of anti-IL-12 Ab (F and G) or control Ab (C and D). Monensin was added for the last 6 h of culture. NWNA spleen cells were assessed for TCRαβ and DX5 expression by double-labeled surface staining and for IFN-γ production by intracellular cytokine staining. The histograms represent TCRαβ IFN-γ+ gated cells stained for DX5. A–K, C3H/He NWNA spleen cells cocultured with dying LM-infected J774 cells in the presence of anti-IL-12 Ab (I) or control Igs (J) were assessed for TCRγδ expression and intracellular IFN-γ production. Control staining was conducted by using PE-conjugated hamster IgG (K). L and M, C3H/He mouse spleen cells were cocultured for 24 h with (M) or without (L) a 24-h culture supernatant collected from dying LM-infected J774 cells and were assessed for TCRαβ expression and intracellular IFN-γ production. N and O. Effect of various concentrations of anti-IL-12 Ab on the IFN-γ production by αβ T and non-αβT cells was examined. IFN-γ+ cells were expressed as the percentage of IFN-γ+ cells in the presence of anti-IL-12 Ab vs control Ab. The numbers shown in the upper right quadrants represent mean percentages of IFN-γ+ cells in αβ T cells or γδ T cells, and those in the lower right quadrants represent mean percentages of IFN-γ− non-αβ T cells in total spleen NWNA cells from four experiments.

αβ T cells to produce IFN-γ, of which the proportion was decreased by 0.6 ± 0.2% in the presence of anti-IL-12 Ab. Of the IFN-γ-producing αβ T cells, 27.2 ± 2.3% were NK1.1+ in the absence of anti-IL-12 Ab, and 24.8 ± 1.8% were NK1.1+ in the presence of anti-IL-12 Ab. The NK1.1+ T cells comprised 1.5 ± 0.2% of the NWNA spleen cell population and this proportion remained unchanged before and after coculture with LM-infected J774 cells (data not shown). These results indicate that IFN-γ-producing αβ T cells consist of NK1.1+ NKT cells (~25%) and non-NKT cells (~75%). When the opposite analysis was done by gating on NK1.1+ T cells and evaluating the percentage of IFN-γ-producing cells in this NKT cell population, we found that 60 ± 5.5% and 10 ± 2.7% of the NK1.1+ NKT cells were IFN-γ+ in the absence and presence of anti-IL-12 Ab, respectively (Fig. 8C).

Because most of NK1.1+ NKT cells carry an invariant TCR restricted to interactions with the class I-like molecule CD1d, we determined whether CD1d was involved in the NKT cell recognition of dying LM-infected J774 cells. To this end, blocking anti-CD1d mAb was added to cocultures of C57BL/6 mouse NWNA spleen cells with LM-infected J774 cells in the presence of anti-IL-12 Ab or control Ab, and the numbers of IFN-γ+ NK1.1+ NKT cells per 107 NWNA spleen cells were determined by three-color flow cytometric analysis (Fig. 8D). Addition of anti-CD1d mAb did not reduce the numbers of both the IL-12-dependent and -independent IFN-γ+ NK1.1+ NKT cells that responded to dying LM-infected J774 cells. This result indicates that the Vα14 invariant TCR expressed on NKT cells is not involved in the mechanism by which NKT cells recognize dying LM-infected J774 cells. Also this result is consistent with the conclusion that the contact-dependent interaction between B6HO3 T cell hybridoma and dying LM-infected J774 cells is not mediated by the TCR of B6HO3 cells (Fig. 4C).

**Cell death in LM-infected BMM cells is also associated with the IFN-γ production by subsets of αβ T cells, γδ T cells, and NK cells**

Since LM had been reported to induce nonapoptotic cell death in BMM (17), we reiterated the same experiments using BMM instead of the J774 macrophage cell line to corroborate that dying LM-infected macrophages have the ability to stimulate subsets of lymphocytes to produce IFN-γ in a cell-cell contact-dependent manner. C57BL/6 mouse BMM cells were infected with LM and cocultured with syngeneic C57BL/6 mouse NWNA spleen cells in ordinary wells or Transwells in the presence of either neutralizing anti-IL-12 Ab or control Ab, and 20 h after coculture the NWNA spleen cells were assessed for intracellular IFN-γ production by three-color flow cytometric analysis (Fig. 9). LM induced cell death in BMM (Fig. 9B), as has been reported, and coculture of dying LM-infected BMM cells with NWNA spleen cells induced 1.6 ± 0.6% of αβ T cells, part of non-αβ T cells (2.0 ± 0.3% of total NWNA spleen cells; Fig. 9D), and 15 ± 2% of γδ T cells (Fig. 9I) to produce IFN-γ. When anti-IL-12 Ab was added to the coculture, the proportion of IFN-γ-producing αβ T cells, non-αβ T cells, and γδ T cells decreased to 0.4 ± 0.2%, 1.1 ± 0.2%, and 3.9 ± 0.6%, respectively. Addition of higher concentrations than 2 μg/ml anti-IL-12 no longer reduced the number of IFN-γ-producing cells (data not shown). When the dot plots were gated on TCRαβ NK1.1+ cells (NKT cells), we found that 50.9 ± 7.0% and 16 ± 5.2% were IFN-γ− in the absence or presence of anti-IL-12 Ab, respectively (Fig. 9, G and H). Of TCRαβ NK1.1+ cells (NK cells), 46 ± 9% were IFN-γ−, of which the proportion was decreased to 27 ± 5% in the presence of anti-IL-12 Ab (data not shown). Separation of NWNA spleen cells from dying LM-infected BMM cells in a Transwell failed to induce IFN-γ production (Fig. 9F). These results indicate that in addition to a minor subpopulation of αβ T cells, subsets of γδ T cells, NK cells, and NKT cells produced IFN-γ in response to direct cell-cell contact with dying LM-infected BMM.

Mutant LM strain DP-L4048 infection has been reported to induce cell death in macrophages in a manner that is inversely correlated to the presence of gentamicin in culture medium (39); accordingly, we examined whether the IFN-γ production by NWNA...
spleen cells was also inversely correlated to the presence of gentamicin. As shown in Fig. 10, A and B, when gentamicin was added 45 min after infection, DP-L4048-infected BMM did not undergo cell death, probably because bacteria that escaped from host phagosomes were killed by the antibiotic that was allowed to flow into the cytoplasm through the plasma membrane permeabilized by the mutant LLO (39), whereas in the absence of gentamicin DP-L4048-infected BMM underwent cell death within 10 h. When NWNA spleen cells were cocultured with these BMM cells, dying DP-L4048-infected BMM cells, but not intact DP-L4048-infected BMM cells, induced IFN-γ production by NWNA spleen cells (Fig. 10, C and D). The same results were obtained with B6HO3 hybridoma. When B6HO3 cells were cocultured with the dying or intact DP-L4048-infected BMM cells, only the dying DP-L4048-infected BMM cells induced B6HO3 cells to produce IFN-γ (Fig. 10E).

These results indicate that cell death in BMM caused by LM infection is closely associated with its capability of stimulating subsets of lymphocytes and the B6HO3 hybridoma to produce IFN-γ, and thus this phenomenon is not limited to the J774 macrophage cell line.

Discussion

We have established in the present study a novel double-negative T cell hybridoma line designated B6HO3, which expresses activated/memory markers such as CD25, CD69, CD44high, and CD45RBhigh and produces IFN-γ, TNF-α, GM-CSF, and Eta-1, but not IL-2 and IL-4 upon CD3-TCR ligation. We have also shown that the B6HO3 hybridoma recognizes a specialized form of macrophage cell death caused by bacterial infection in a TCR-independent manner and consequently produces IFN-γ in an IL-12-independent manner.

A variety of bacterial pathogens induce cell death in macrophages (16), but the mechanisms by which cell death is induced seem to be manifold and there exists considerable controversy; i.e., combinations of different types of macrophage cells and bacteria have been reported to induce different modes of cell death in bacterial-infected macrophages, with some exhibiting apoptotic or autophagic cell death and others exhibiting necrosis or oncosis (15–26). The mode of cell death in LM-infected J774 cells has been shown to be nonapoptotic (Ref. 17 and my unpublished data), but
the exact mechanism is yet undefined. Mutant LM DP-4048, which produces mutant LLO protein that is not subjected to intracellular degradation, has been reported to induce cell death in J774 cells by rapid pore formation in the plasma membrane (19). *Salmonella typhimurium* was first shown to induce apoptosis in J774 cells in a caspase-1-dependent manner (16, 18), but recent reports have shown that an effector function of T cells (30, 31), the unique functional phenotype of the B6HO3 hybridoma predicted existence of a hitherto unknown minority population that should respond to pathogen-induced macrophage cell death by producing IFN-γ. In fact, the present study has revealed that in response to direct cell-cell contact with dying LM-infected J774 cells, some CD8+ T cells produce IFN-γ in an IL-12-dependent and -independent manner, respectively. Two IFN-γ-producing CD8+ T cell subpopulations consisted of CD4+ (~50%), CD4-CD8+ (~44%), and CD4-CD8- (6%) T cells expressing intermediate levels of TCRαβ. Among these T cells, the CD4-CD8+ T cells that produce IFN-γ independently from IL-12 signaling appears to be the parental T cell of the B6HO3 hybridoma. As B6HO3 cells expressed CD244 and CD11c, we examined whether IFN-γ-producing CD4+ T cells also expressed these markers. Although we did not observe any significant differences in the expression of CD4+ T cells between B6HO3 and parental B6.6 HO3 cells, the parental T cell of this hybridoma might have happened to be the cell expressing these markers. Like the B6HO3 hybridoma, all of the IFN-γ-producing CD8+ T cells responding to dying LM-infected J774 cells had a CD44hiCD69hi phenotype (data not shown). Since this phenotype is expressed upon T cell

FIGURE 9. IFN-γ production by subsets of αβ T cells, γδ T cells, and NK cells in response to dying LM-infected BMM. C57BL/6 mouse BMM cells were infected with wild-type LM (MOI, 50:1) and cocultured for 24 h with C57BL/6 mouse NWA spleen cells in an ordinary 24-well plate (C–E and G–K) or a Transwell (F) in the presence of (2 μg/ml) of anti-IL-12 Abs (E and H) or control Ab (D). The expression of TCRαβ, NK1.1, and IFN-γ was analyzed using FACS. The histograms in D and E represent TCRαβ, IFN-γ+ gated cells stained for NK1.1, and the histograms in G and H represent TCRαβ, NK1.1+ gated cells (NKT cells) stained for IFN-γ. I–K, The same NWA spleen cells in the presence of anti-IL-12 Ab (J) or control Ab (I) were assessed for TCRγδ expression and intracellular IFN-γ production. Control staining was conducted by using PE-conjugated hamster IgG (K). Data are representative from one of three experiments.

The precise mode of the pathogen-induced macrophage cell death that B6HO3 cells can recognize and the recognition mechanism thereof remain to be further explored.

Since our hybridoma system dictates most of the terminally differentiated programs of parental T cells and thus preserves the effector functions of T cells (30, 31), the unique functional phenotype of the B6HO3 hybridoma predicted existence of a hitherto unknown minority T cell population that should respond to pathogen-induced macrophage cell death by producing IFN-γ. In fact, the present study has revealed that in response to direct cell-cell contact with dying LM-infected J774 cells, some CD8+ T cells produce IFN-γ in an IL-12-dependent and -independent manner, respectively. Two IFN-γ-producing CD8+ T cell subpopulations consisted of CD4+ (~50%), CD4-CD8+ (~44%), and CD4-CD8- (6%) T cells expressing intermediate levels of TCRαβ. Among these T cells, the CD4-CD8+ T cells that produce IFN-γ independently from IL-12 signaling appears to be the parental T cell of the B6HO3 hybridoma. As B6HO3 cells expressed CD244 and CD11c, we examined whether IFN-γ-producing CD4+ T cells also expressed these markers. Although we did not observe any significant differences in the expression of CD4+ T cells between B6HO3 and parental B6.6 HO3 cells, the parental T cell of this hybridoma might have happened to be the cell expressing these markers. Like the B6HO3 hybridoma, all of the IFN-γ-producing CD8+ T cells responding to dying LM-infected J774 cells had a CD44hiCD69hi phenotype (data not shown). Since this phenotype is expressed upon T cell
activation, it was not determined whether it was induced to express on the IFN-γ-producing cells as a result of activation through the interaction with dying J774 cells or this particular subset of αβ T cells naturally expressed this activation phenotype in vivo like innate T lymphocytes (36). However, the latter possibility is more likely, because the functional T cell hybridoma B6HO3 expressed this phenotype and the IFN-γ-producing αβ T cells were shown to involve NKT cells (Fig. 8B), which are known to bear this activation phenotype (35).

The experiments using C57BL/6 mice have shown that ~26% of the IFN-γ-producing αβ T cells responding to dying LM-infected J774 cells are NK1.1+ NKT cells (Fig. 8B) and the rest are NK1.1 negative. It was possible that the NK1.1− αβ T cells were derived from NK1.1+ NKT cells as a result of down-regulation of NK1.1 Ag expression upon activation of the cells (49). However, this possibility is unlikely, because the proportion of NK1.1+ NKT cells in the NWNA spleen cell population remained unchanged before and after coculture with dying LM-infected J774 cells (data not shown). Since NKT cells consist of phenotypically and functionally diver cell populations (35, 50), the definition of NKT cells is expanding and it is now known that there exist NK1.1− NKT cells (50). Because the IFN-γ-producing αβ T cells expressed intermediate levels of the TCR (Fig. 7), which is one of the characteristics of NKT cells (35, 50), it is possible that the NK1.1− αβ T cells may represent NK1.1− NKT cells. Further investigation is necessary to define this IFN-γ+ NK1.1− αβ T cell in view of NKT cell biology.

The present study has also shown that in response to contact-dependent interaction with dying LM-infected J774 cells ~50 and ~10% of splenic NK1.1+ NKT cells produce IFN-γ in an IL-12-dependent and -independent manner, respectively (Fig. 8C) and that this response is not mediated by the Vα14 invariant TCR expressed on the NKT cells (Fig. 8D). Upon TCR ligation, NKT cells rapidly produce many cytokines including Th1 and Th2 cytokines such as IFN-γ and IL-4 and thus NKT cells have been implicated in a variety of immune responses, playing a regulatory role (51, 52). However, the question remains as to how a variety of cytokines produced by NKT cells leads to a regulated immune response (52). The production of IFN-γ, but not IL-4, by NKT cells upon interaction with dying LM-infected J774 cells (Fig. 8B) may explain one of the regulation mechanisms by which NKT cells direct the Th1-type response that is beneficial for eradication of invaded bacteria.

Unexpectedly, this study has revealed that in addition to the minor subset of αβ T cells, which obviously involve NKT cells, ~15% of splenic γδ T cells and ~15% of splenic NK cells in an IL-12-dependent manner and ~6% of the γδ T cells and ~26% of the NK cells in an IL-12-independent manner produce IFN-γ in response to direct cell-cell contact with dying LM-infected J774 cells. NK cells, NKT cells, and γδ T cells are grouped as innate lymphocytes, which are characterized as having germline-encoded autoreactive receptors, being poised to unleash their effector function and participating in innate immune responses (36, 53). The present results suggest that subsets of these innate lymphocytes possess a common recognition system sensing macrophage cell death caused by bacterial infection and consequently produce IFN-γ. These IFN-γ-producing NK and γδ T cell subpopulations remain to be further characterized to shed light on the understanding of NK cell and γδ cell biology.

IL-12 is a pivotal cytokine for IFN-γ production by NK cells and T cells, but IL-12-induced efficient IFN-γ production by these cells requires costimulation mediated by other cytokines such as IL-18 or by interaction with other cognate cells (3). The present study revealed that there are IL-12-dependent and -independent subpopulations among the αβ T cells, γδ T cells, and NK cells that produce IFN-γ in response to direct cell-cell contact with dying LM-infected J774 cells (Fig. 7). The direct cell contact with dying LM-infected macrophages, therefore, provides a novel costimulation signal for the production of IFN-γ by the IL-12-dependent populations, while this signal is necessary and sufficient for the IL-12-independent subsets of the αβ T cells, γδ T cells, and NK cells to produce IFN-γ. These two IL-12-dependent and -independent populations may differ in maturation stages, exhibiting different responsiveness to this costimulation signal, and thus it may be a potentiating role that IL-12 plays in this response.

The experiments with BMM in the present study have corroborated the results obtained using the J774 macrophage cell line. Thus, we may conclude that IFN-γ production by subsets of innate lymphocytes in response to direct cell-cell contact with dying bacterial-infected macrophages is a general phenomenon not limited to the J774 macrophage cell line. The macrophages constitute the first line of defense against bacterial pathogen, and activation of macrophages by IFN-γ is cardinaly important in the early defense against bacterial infection. Therefore, macrophage cell death induced by bacteria is thought to be detrimental to the host defense against bacterial infection, although the biological significance of the pathogen-induced cell death in macrophages is not fully defined. The demonstration of IFN-γ production by NKT cells, NK cells, and γδ T cells as well as B6HO3 T cell hybridoma following direct cell-cell contact with dying LM-infected macrophage cells raises the possibility that there is a novel innate IFN-γ production pathway that is mediated by the innate lymphocyte recognition of macrophage cell death caused by bacterial infection. Provided that this innate IFN-γ production does not require de novo protein synthesis in bacterial-infected macrophages as has been shown with the B6HO3 hybridoma (Fig. 4D), the proposed IFN-γ production pathway may particularly contribute to the host defense at the initial phase of bacterial infection where resting macrophages are killed and exposed to danger by an attack of microbial pathogens before de novo protein synthesis that is required for cytokine production can take place.

The establishment of the functional T cell hybridoma line B6HO3, which has led to the identification of the innate lymphocytes that respond to dying LM-infected macrophage cells, will make it feasible to dissect the mechanisms underlying the lymphocyte recognition of macrophage cell death and the precise mode of the specialized cell death in macrophages caused by bacterial infection. It will also open new insights into the innate immune responses of lymphocytes upon bacterial infection.

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Disclosures

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