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Fas-Fas Ligand Interactions Are Essential for the Binding to and Killing of Activated Macrophages by γδ T Cells

Jane E. Dalton,* Gareth Howell,* Jayne Pearson,* Phillip Scott,‡ and Simon R. Carding†

γδ T cells have a direct role in resolving the host immune response to infection by eliminating populations of activated macrophages. Macrophage reactivity resides within the Vγ1/Vδ6.3 subset of γδ T cells, which have the ability to kill activated macrophages following infection with Listeria monocytogenes (Lm). However, it is not known how γδ T cell macrophage cytoidal activity is regulated, or what effector mechanisms γδ T cells use to kill activated macrophages. Using a macrophage-T cell coculture system in which peritoneal macrophages from naïve or Lm-infected TCRγ−/− mice were incubated with splenocytes from wild-type and Fas ligand (FasL)-deficient mice (gld), the ability of Vγ1 T cells to bind macrophages was shown to be dependent upon Fas-FasL interactions. Combinations of anti-TCR and FasL Abs completely abolished binding to and killing of activated macrophages by Vγ1 T cells. In addition, confocal microscopy showed that Fas and the TCR colocalized on Vγ1 T cells at points of contact with macrophages. Collectively, these studies identify an accessory or coreceptor-like function for Fas-FasL that is essential for the interaction of Vγ1 T cells with activated macrophages and their elimination during the resolution stage of pathogen-induced immune responses. The Journal of Immunology, 2004, 173: 3660–3667.

Immunoregulation is an emerging paradigm of γδ T cell function (reviewed in Refs. 1–4). Experiments using mice genetically engineered to be deficient in γδ T cells as animal models of infectious or autoimmune-driven immune responses have provided compelling evidence for the direct involvement of γδ T cells in influencing the activities of a variety of immune cells (5, 6). In particular, chronic inflammation and exaggerated tissue necrosis seen in γδ T cell-deficient mice infected with different microbial and parasitic pathogens has identified a requirement for γδ T cells in resolving pathogen-mediated immune responses and preventing immune-mediated tissue injury (5–10). Our own studies have identified a mechanism by which γδ T cells down-modulate pathogen-induced immune responses. In response to infection by the Gram-positive intracellular bacterium, Listeria monocytogenes (Lm), γδ T cells recognize and kill populations of activated proinflammatory macrophages after pathogen clearance (5). Macrophage cytoidal activity is restricted to a subset of γδ T cells reactive with the 2.11 mAb that recognizes GVS11 (Vγ1, TCR Vγ nomenclature used is that of Heilig and Tonegawa (11)-encoded TCRs (12). The ability to bind to and kill macrophages by Vγ1 T cells is an inherent property of these cells and does not require any prior activation or response to infection (13). An important question raised by these findings is how is the inherent cytotoxic activity of Vγ1 T cells controlled and targeted appropriately to pathogen-elicted activated macrophages, but not other populations of macrophages?

Studies of other populations of cytotoxic γδ T cells, particularly the epithelia-associated γδ T cells in the skin (dendritic-epidermal T cells (DETC)) and small intestine (intestinal intraepithelial lymphocytes (IELs)), have shown that in addition to TCR-ligand interactions, γδ T cell recognition of stressed or damaged epithelial cells requires the involvement of additional ligand-receptor interactions. Murine DETCs and human peripheral blood (Vγ2Vδ2+) γδ T cells express NK cell receptors, such as NKG2D, which upon ligation by Rae-1 and H60 or MHC class I polypeptide-related sequence (MIC) A and B expressed by murine and human target cells, respectively, delivers an activating stimulus, resulting in an enhancement of TCR-dependent responses (14–16). By binding to CD80α, the MHC class I-related thymus leukemia (TL) Ag can also serve as an accessory-like molecule for CD80α− γδ IELs (17). Because expression of all of these coreceptor or accessory molecules are up-regulated in response to stress, infection, or transformation, this provides a mechanism by which the cytotoxic activity of epithelia-associated γδ T cells can be appropriately targeted and healthy cells that do not express these molecules are protected.

However, it is not known how comprehensive this requirement for additional molecular interaction is for cytotoxic γδ T cells and whether they are required for other populations of cytotoxic γδ T cells, not normally associated with epithelial cells. The studies we present here provide the first report for the requirement of an additional cell surface receptor for the recognition of target cells by systemic murine γδ T cells. In the course of investigating the mechanism of Vγ1 T cell cytotoxicity, we have demonstrated that the recognition and subsequent killing of activated macrophages by Vγ1 T cells is dependent upon T cell expression of Fas ligand (FasL) and its interaction with Fas expressed by target cells.

Materials and Methods

Mice and infections

C57BL/6 wild-type were purchased from Harlan Laboratories (Bicester, U.K.). C57BL/6 TCRγ−/−, C57BL/6 TCRβ−/−, B6Smn.C3-Tnfsf6gldl1 (gld), and C57BL/6 CD45.1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific pathogen-free conditions at University of Leeds and used between 6 and 8 wk of age.

*School of Biochemistry and Microbiology, University of Leeds, Leeds, United Kingdom; and †Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA 19104

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1 Address correspondence and reprint requests to Dr. Simon R. Carding, School of Biochemistry and Microbiology, University of Leeds, Leeds, LS2 9JT, U.K. E-mail address: S.R.Carding@Leeds.ac.uk

2 Abbreviations used in this paper: Lm, Listeria monocytogenes; CMA, concanamycin A; DETC, dendritic-epidermal T cell; DIOc, 3,3′-dioctadecyloxycarbocyanine; FasL, Fas ligand; IEL, intestinal intraepithelial lymphocyte; IS, immunological synapse; PEC, peritoneal exudate cell; TL, thymus leukemia; MIC, MHC class I polypeptide-related sequence.
Mice were infected i.p. with 1.5 × 10⁴ CFU Lm (strain 10403S) in PBS, and cells were harvested 8 days later. In some cases, previously infected C57BL/6 mice were reinfected with 1.5 × 10⁸ CFU, and 3 days later, lymph node mononuclear cells were harvested for CTL assays.

### Abs and flow cytometry
F(ab)² of Abs specific for TCR Vγ1 (clone 2.11), TCR Vβ6.3 (17C), and TCRβ (GL3) were obtained as described previously (13). The 2.11 hybridoma cell line (18) was kindly provided by Dr. P. Pereira (Institut Pasteur, Paris, France). Commercial Abs used included anti-mouse TCRγ6 (GL3), CD3 (145 2C11), CD95, Fas (Jo2), CD178, (FasL, MFL3), or CD11b (M1/70.15), which were used as biotin or fluorochrome conjugates and were purchased from Caltag-Medsystems (Towncester, U.K.) or BD Pharmingen (Oxford, U.K.). Anti-CD178, (Fasl, 3C82) was purchased from Alexis Biochemicals (Nottingham, U.K.). Streptavidin-PE, FITC (Caltag-Medsetsyms), or Alexa Fluor 633 (Molecular Probes, Eugene, OR) were used as secondary reagents. To block nonspecific Ab binding, cells were incubated with anti-FcR Abs (10 μg/ml) (Caltag-Medsetsyms). Isotype-matched Abs of irrelevant specificity were used to determine the level of nonspecific staining. Stained cells were analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences, Oxford, U.K.).

### Cell isolation
Peritoneal exudate cells (PECs) were obtained by two rounds of peritoneal lavage with 5 ml of HBSS containing 10 U/ml heparin. Cells were collected by centrifugation, washed twice, and then resuspended in RPMI 1640 medium supplemented with 10% FCS. To obtain macrophages, PECs were plastic adhered (<5 × 10⁶ cells/ml) for 1 h at 37°C in 5% CO₂. Splenocytes, lymph node mononuclear cells, and thymocytes were prepared by homogenizing tissue using a glass homogenizer, and passing the cell suspension through a 0.7-μm nylon filter. Erythrocytes were lysed with 0.8% w/v ammonium chloride solution.

### Macrophage-T cell coculture
Splenocytes from C57BL/6 or C57BL/6 TCRβ⁻/⁻ were incubated with plastic-adherent macrophages from C57BL/6 TCRβ⁻/⁻ mice or the IC-21 peritoneal macrophage cell line (American Type Culture Collection, Manassas, VA) in complete medium at 37°C for 1 h. In some experiments, a range of concentrations of F(ab)² of anti-VCβ6.3 recombinant FasL, Abs control, Abs, or CMA were included. Nonadherent effector cells were removed by repeated washing with warm PBS (37°C) before adding propidium iodide. Dead target cells were identified by coincident green-membrane and red-nuclear staining. Slides were observed under UV illumination using a Zeiss Axiosvert 200 M microscope (Zeiss, Welwyn, Garden City, U.K.) using Axiosview image analysis software (Imaging Associates, Bicester, U.K.). Counting at least 100 live and/or dead cells in four separate fields of view.

### Immunohistochemistry
PECs (5 × 10⁶ cells) recovered from C57BL/6 mice 8 days after infection with Lm were allowed to adhere to eight-well chamber slides (Valeant Pharmaceuticals, Basingstoke, U.K.) in complete medium at 37°C for 1 h. Nonadherent cells were removed by repeated washing with warm PBS and the remaining adherent cells were fixed (4% paraformaldehyde PBS, pH 7.3) for 15 min and incubated with 0.5% (w/v) BSA, 5% rabbit serum, and 20 μg/ml mouse IgG at 4°C for 30 min, before staining with biotinylated anti-Vγ1 (2.11) and F/480-FTIC or biotinylated CD8α (CT-CD8α; Caltag-Medsetsyms) for 1 h. Cells were washed and then incubated with streptavidin-Texas Red (Caltag-Medsetsyms) at 4°C for 30 min. For detection of cytoplasmic perforin, splenocytes from C57BL/6 were cyt centrifuged onto microscope slides, permeabilized with 0.2% (w/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 30 min before incubation with monoclinal anti-perforin Ab (CB5.4; Alexis Biochemicals) at 4°C for 60 min. Bound Ab was detected by incubating with FITC-goat anti-rat Ab at 20°C for 30 min. Samples were then incubated for 1 min with the nuclear counterstain.

### FIGURE 1. FasL is essential for the binding of Vγ1 T cells to activated macrophages. Splenocytes from C57BL/6 (A) or Fasl-deficient, B6Smn.C3-Tafsfligd/U (B) mice were incubated with plastic-adherent peritoneal macrophages from Lm-infected C57BL/6-TCRβ⁻/⁻ mice for 1 h, after which nonadherent and adherent cells were analyzed for γδ and Vγ1 T cells by Ab staining and flow cytometry. Vγ1 T cells were identified and quantitated by electronic gating of CD3⁺, TCRγδ⁺ cells (upper and lower left-hand side plots of A and B), which were then analyzed for Vγ1 expression (upper and lower right-hand side plots of A and B). The percentage values shown in the individual dot plots represent the proportion of CD3⁺, TCRγδ⁺, or CD3⁻, TCRγδ⁺, Vγ1⁺ cells. The bar graph (C) summarizes the fold enrichment of Vγ1 T cells after incubation with activated macrophages, which was determined by comparing the proportion of CD3⁺, TCRγδ⁺, Vγ1⁺ cells in the macrophage-adherent (MØ Ad) or nonadherent (MØ Non-Ad) fraction with that in the starting population. The results shown were obtained from four independent experiments. The error bars represent SEM.
Vγ1 T CELL-MACROPHAGE INTERACTIONS

We have previously shown that the ability of γδ T cells to bind to activated macrophages is restricted to the Vγ1+ subset (13). By binding to Lm-elicited activated macrophages, Vγ1 T cells are enriched >4-fold compared with the starting population of spleen cells (Fig. 1A). In subsequent experiments investigating the molecular basis of this γδ T cell-macrophage interaction and the mechanism of macrophage killing, we found that the ability of Vγ1 T cells to bind to activated macrophages was dependent upon Fas-FasL interactions. Using splenocytes from gld mice that lack functional Fasl, there was no evidence of any Vγ1 T cell enrichment (Fig. 1B). In comparing the distribution of Vγ1+ T cells in the starting population with that in the macrophage-adherent and nonadherent fractions, the vast majority (>80%) of the Vγ1+ T cells were present in the nonadherent fraction. The proportion of (FasL−) Vγ1+ T cells in the macrophage-adherent fraction was less than that in the starting population (0.6% vs 1%), consistent with there being minimal binding to macrophages and no enrichment of Vγ1+ T cells.

To confirm this observation and investigate further the possible involvement of Fas-FasL interactions in Vγ1+ T cell-macrophage binding, Abs to the TCR-Vδ6.3 and FasL were used to attempt to block binding of Vγ1+ T cells with activated macrophages from Lm-infected TCRδ−/− mice. Anti-Vδ6.3 Abs reduced the binding of Vγ1+ T cells to activated macrophages by ~60% (Fig. 2). A similar level of inhibition was seen using anti FasL Abs. Combining both Abs virtually abolished all binding (≥98% inhibition) of Vγ1+ T cells to activated macrophages (Fig. 2). By contrast, isotype-matched control Abs or an Ab to CD45 that is expressed at high levels by hemopoietic cells, including γδ T cells, had no significant effect on the binding of Vγ1+ T cells to macrophages (Fig. 2A). Together with the results of the experiments using FasL−/− γδ T cells, these findings demonstrate a requirement for Fas-FasL interactions in the binding of Vγ1+ T cells to activated macrophages. A similar requirement for Fas-FasL interactions in the binding of Vγ1+ T cells to the peritoneal macrophage cell line, IC-21, was also demonstrated (Fig. 2B). Incubation of IC-21 macrophages with C57BL/6 splenocytes resulted in a >5-fold enrichment of Vγ1+ T cells (data not shown). Whereas, anti-TCR and FasL Abs alone only partially inhibited (50–60%) the binding of Vγ1+ T cells to IC-21 macrophages, together they virtually abolished (≥98% inhibition) the binding of Vγ1+ T cell.

Factors influencing Fas and FasL expression by Vγ1 T cells and macrophages

To establish the requirements for, and conditions under which, Fas-FasL expression is acquired by Vγ1 T cells, the distribution of Fas and FasL by Vγ1 cells before and after exposure to microbial infection was evaluated. Because Vγ1 cells are first produced in
the newborn and neonatal thymus (19), we also determined whether these cells acquire Fas and FasL expression during their intrathymic development. A large proportion of V$\gamma$$\delta^+$ T cells in the thymus of newborn mice already expressed both Fas (80%) and FasL (40%) (Fig. 3A), the levels of which were not significantly different from that in naive adult (Fig. 3, B and C) animals (6–8 wk old). The distribution of Fas and FasL among splenic and peritoneal V$\gamma$$\delta^+$ T cells of adult animals was similar, although the proportion of splenic V$\gamma$$\delta^+$ T cells positive for both Fas (≤30%) and FasL (≥75%) was somewhat lower than that of peritoneal V$\gamma$$\delta^+$ cells (Fig. 3C, day 0). In response to infection, the proportion of Fas$^+$ V$\gamma$$\delta^+$ T cells increased within 48 h in the spleen and remained high throughout the course of infection (Fig. 3C). By contrast, the change in FasL expression by peritoneal V$\gamma$$\delta^+$ T cells and in Fas expression by both splenic and peritoneal V$\gamma$$\delta^+$ T cells was less striking with levels of positive cells increasing only slightly. These findings demonstrate that Fas and FasL expression is acquired by a large proportion of V$\gamma$1 cells during their intrathymic development, the levels of which are modulated further in response to microbial challenge.

To determine whether expression of Fas by macrophages changed in response to infection, macrophages before infection and at 8 days postinfection with Lm, at which the highest levels of macrophage cytotoxic V$\gamma$1 T cells are seen, were examined for Fas expression. Approximately 20% of peritoneal macrophages from naive adult C57BL/6 mice expressed Fas (Fig. 3D). As a consequence of infection, Fas expression by peritoneal macrophages was much higher with ≥75% of macrophages recovered from mice 8 days postinfection being Fas$^+$ (Fig. 3D). These results indicate that a small but significant population of peritoneal macrophages constitutively express Fas, and that following infection, expression of Fas is significantly up-regulated.

**Killing of macrophages by V$\gamma$1 T cells is dependent on Fas-FasL interactions and not perforin**

The functional significance of Fas-FasL interactions between V$\gamma$1 cells and macrophages, and the probability that V$\gamma$1 cell-macrophage cytoidal activity is mediated via the Fas-FasL pathway was investigated using the Live/Dead Cytotoxicity Cell assay. As seen in Fig. 4, preincubation of effector splenocytes with Abs to the TCR and FasL resulted in similar levels of partial inhibition (≤50%) of macrophage killing. When combined, however, anti-V66.3 and anti-FasL Abs almost completely inhibited (≥95% inhibition) macrophage killing by V$\gamma$1 cells. By contrast, Abs to an
unrelated Ag, CD45, which is expressed at high levels by γδ T cells and binds independently of the TCR, had no significant effect on macrophage killing (Fig. 4).

In view of previous studies suggesting use of the perforin-granzyme pathway by cytotoxic γδ T cells (20–22), we determined whether this pathway made any contribution to Vγ1 T cell killing of macrophages. Vγ1 T cells from C57BL/6 mice infected 8 days previously with Lm, a time during infection at which the highest levels of γδ T cell macrophage cytotoxic activity was seen (13), were examined for perforin expression by immunohistochemical analysis. Intracellular staining of Vγ1 T cells revealed no reactivity of anti-perforin Abs (Fig. 5B), whereas CD8+ cells from Lm-infected mice were reactive with the anti-perforin Abs (Fig. 5A). These findings were corroborated in macrophage killing assays in which the inhibitor of perforin-dependent killing, CMA, was included. Whereas CMA effectively inhibited the killing of macrophages from Lm-infected TCRΔ−/− mice by CD8+ T cells (Fig. 5D), it had no effect on Vγ1 T cell macrophage cytotoxic activity (Fig. 5C) even at concentrations as high as 100 nM.

**Colocalization of the TCR and Fas on Vγ1 T cells interacting with macrophages**

Taken together, the above findings show that the binding to and subsequent killing of activated macrophages by Vγ1 T cells is dependent on the Fas/FasL pathway consistent with a coreceptor or accessory molecule-like function for Fas-FasL. To investigate this possibility further, we attempted to demonstrate colocalization of the TCR and FasL among Vγ1 cells bound to macrophages using confocal microscopy. An indirect method in which FasL localization was based upon detection of Fas expression by macrophages (and Vγ1 T cells; Fig. 3) was used because cell-cell contact is dependent upon Fas-FasL interaction (Fig. 1), and there is no evidence to suggest that Fas binds to any ligands other than FasL (23). This approach also enabled us to look at the distribution of Fas on both Vγ1 T cells and macrophages, identify any changes in Fas distribution on the surface of macrophages bound by Vγ1 T cells, and provided good contrast for the imaging of both cell types by confocal microscopy. IC-21 macrophages were plated out onto glass microscope slides and then incubated with splenocytes from C57BL/6 TCRβ−/− mice. After washing off nonadherent cells, bound Vγ1 were visualized by staining with F(ab′)2 FITC-anti-Vγ1 and biotinylated anti-Fas Ab and streptavidin-Texas Red to localize Fas. All of the Vγ1 T cells retained in the macrophage-adherent fraction were found in close association or contact with macrophages; it was not possible to identify any Vγ1 T cells that were in isolation and not in contact with macrophages. A series of 400-nm eight-bit optical sections were captured through the samples with an LSM510 META laser scanning confocal microscope. The merged images in Fig. 6, B and D were obtained from Imaris software and the ImarisColoc module to highlight pixels present in the same location in both the FITC and Texas Red channels. Colocalized data was then added to the original two-color data set as a third channel and pseudocolored yellow for visualization. The images produced provided evidence of colocalization of Fas and the TCR at the Vγ1 T cell-macrophage contact site as indicated by the yellow staining found at points of contact (Fig. 6, A–D). The inability to detect any yellow staining by Vγ1 T cells or macrophages other than at points of contact (Fig. 6, A–D) or among either population of cells in isolation and not in contact with each other (data not shown), indicated that Fas and TCR colocalize in discrete aggregates on Vγ1 T cells at points of contact with activated macrophages. This distribution pattern is consistent with colocalization of TCR and FasL on the γδ T cell bound to macrophages. Furthermore, the three-dimensional volume rendered images of γδ T cell in contact with macrophages in which the Vγ1 T cell is electronically “removed” from the γδ T cell-macrophage complex (Fig. 6, E and F) demonstrates that the TCR and Fas are indeed in close proximity and restricted to the point of contact between the T cell and macrophage.

**Discussion**

We have previously established that the ability of γδ T cells to interact with Lm-elicited activated macrophages is restricted to the Vγ1/Vδ6.3 subset, which are inherently cytotoxic and kill populations of activated macrophages (5). This study now extends these findings and has identified the molecular requirements for γδ (Vγ1) T cell-macrophage interactions and a possible mechanism by which the cytotoxic activity of Vγ1 T cells and killing of target cells is regulated. The results presented demonstrate a requirement for Fas-FasL interactions in addition to TCR engagement for the interaction between Vγ1 T cells and activated macrophages to occur. Disrupting Fas-FasL interactions prevents both the binding to, and subsequent killing of, activated macrophages by Vγ1 T cells. Together with the observation that Fas and the TCR colocalize on the surface of Vγ1 T cells at the point of contact(s) between Vγ1 T cells and macrophages, these findings suggest that Fas-FasL might function as a coreceptor or accessory molecule to enhance TCR/ligand interactions. The requirement for additional molecular interactions for Vγ1 T cells to kill target cells may serve as a means of regulating and restricting the activity of inherently CTLs and protecting nonactivated (Fas−) macrophages.

The importance of Fas in the maintenance of immune cell homeostasis is highlighted by the lymphoproliferative and autoimmune disorder present in both Fas (lpr)- and FasL (gld)-deficient mice (23, 24). The observation that the transfer of Fas-expressing T cells to
FIGURE 5. Vγ1 T cell killing of activated macrophages is perforin-granzyme independent. A, Cytocentrifuge preparations of splenocytes from Lm-infected C57BL/6 mice stained with either (i) anti-perforin (FITC), (ii) isotype control Abs, or (iii) CD8 (Texas Red) and perforin Abs. B, Splenocytes from adult naive C57BL/6 mice were incubated with plastic-adhered peritoneal macrophages from Lm-infected C57BL/6-TCRβ−/− mice for 1 h, washed to remove nonadherent cells, and adherent cells were stained with Abs to perforin (i), or perforin and TCR-Vγ1 (ii) and (iii). Specimens in A and B were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride, mounted, and visualized by UV light microscopy and Axiosvision image analysis software. The results obtained are representative of those obtained from four independent experiments. Scale bar, 10 μm. C. The perforin inhibitor, CMA, does not interfere with the killing of activated macrophages by Vγ1 T cells. Plastic-adherent peritoneal macrophages from Lm-infected C57BL/6-TCRβ−/− mice were incubated with effecter splenocytes from naive C57BL/6 mice in the presence or absence of CMA, and cell death was quantitated after 60 min using the Live/Dead cell assay as described in Fig. 4. D, CMA inhibits CD8 T cell-mediated killing of target cells. CD8 T cells isolated from lymph nodes of Lm-infected C57BL/6 mice were incubated with adherent macrophages from C57BL/6-TCRβ−/− mice infected 3 days previously with Lm, and live vs dead cells were quantitated as described in C. The percentage of inhibition of killing was determined by comparing the level of macrophage death in the absence vs the presence of CMA. The results shown are representative of those obtained from three independent experiments.

lpr mice is sufficient to abrogate the autoimmune disorder further demonstrates the importance of Fas in regulating immune cell homeostasis (25). Although several studies have demonstrated the involvement of Fas/FasL pathway in γδ T cell-mediated killing of various cell targets (26–29), the study presented here is to our knowledge the first to demonstrate it being essential for γδ T cell-target cell interactions. Our findings suggest, but do not prove, that Vγ1 T cells kill activated macrophages through the Fas/FasL pathway. Because macrophage killing by Vγ1 T cells is cell contact dependent and requires Fas/FasL interactions, it was not possible to directly demonstrate Fas-FasL-mediated killing using Fas-deficient (gld) effector cells or neutralizing Abs because these interventions also prevented binding of Vγ1 T cells to target macrophages. However, Fas/FasL-mediated killing of activated macrophages by Vγ1 T cells would be consistent with previous studies demonstrating Fas-mediated killing of CD4 T cells by γδ T cells in individuals infected with Borrelia burgdorferi (27), and γδ T cell-mediated killing of intestinal epithelial cells (28). In addition, we found no evidence for the involvement of the perforin-granzyme pathway in Vγ1 T cell-mediated killing of activated macrophages, although certain populations of human γδ T cells have been shown to kill cells via this pathway (20–22). It is possible that other Fas/FasL-independent mechanisms, such as TNF, are involved in the killing of activated macrophages by Vγ1 T cells. In preliminary experiments, we have been unable to demonstrate the production of TNF-α mRNA or protein by Vγ1 T cells from naive or Lm-infected animals (D. Newton, J. E. Dalton, and S. R. Carding, unpublished observations), suggesting that TNF-α is unlikely to be involved in the killing of activated macrophages by Vγ1 T cells. Despite Lm-elicited Vγ1 T cells expressing both Fas and FasL, and published reports of Fas/FasL-mediated killing of γδ T cells during pathogen-induced responses (30–32), we found no evidence of fratricidal killing or suicide of Vγ1 T cells. Levels of apoptotic Vγ1 T cells before and after macrophage interactions were similar and affected only a small proportion (<5%) of Vγ1 T cells (Ref. 13, and J. E. Dalton and S. R. Carding, unpublished observations). Therefore, other mechanisms may be involved in regulating and terminating γδ T cell responses that might include both cell intrinsic (e.g., limited half-life (33)) and extrinsic effects of other cells and their products.

The idea that populations of γδ T cells require molecular interactions in addition to TCR-ligand interactions for their effector function to be realized and target cells killed has been previously demonstrated for epithelia-associated γδ T cells in both mice (16) and humans (14, 34). γδ T cell-epithelial cell interactions have been shown to involve molecules including the NK receptor, NKG2D, that interacts with stress-induced ligands such as the human MHC class I-related gene products MICA and MICB, and murine Rae-1 expressed on epithelial cells (14, 16, 34). Analogous to our findings for Fas-FasL-mediated interactions between cytotoxic Vγ1 T cells and activated macrophages, the recognition and killing of epithelial cells by cytotoxic γδ IELs requires additional NKG2D-MICA interactions (14). The nonclassical MHC class I molecule, TL Ag, which binds to CD8αα expressed by γδ as well as αβ IELs, has been found to be a mediator of murine γδ IEL
interaction with epithelial cells (reviewed in Refs. 1–4). The finding that CD8αa/TL interactions potentiate IL-2 release is consistent with a regulatory or accessory-like function for CD8αa/TL interaction (17). CD100 expressed by γδ T cells may also function as an accessory or coreceptor molecule for the DETC recognition of keratinocytes (W. Havran, unpublished observations). Collectively, the analyses of the interaction of cytotoxic γδ T cell interactions with various target cells identify a common mechanism by which γδ T cell cytotoxicity and target cell specificity is controlled; namely through inducible expression of additional accessory or coreceptor molecules. It is interesting to note that in none of these systems has the Ag specificity of cytotoxic γδ T cells yet been determined. Although ligands for these T cells were originally thought to be inducible under specific conditions of stress, transformation, or activation (35), it is possible that they may be constitutively expressed, and that recognition is dependent upon the inducible expression of additional accessory or coreceptor-like molecules such as MICA, Rae-1, and FasL (14–16). The finding of cytotoxic T22/T10 reactive γδ T cells has a higher activation threshold than T22/T10-specific αβ T cells has been interpreted as a requirement for the up-regulation of both γδ TCR ligands and additional molecules by target cells for γδ T cell recognition to occur (36). The recent demonstration that γδ T cells readily form immunological synapses (IS) or supramolecular activation clusters that contain TCR-associated accessory and intracellular signaling molecules (37, 38), but are nonfunctional and require high concentrations of TCR ligand for efficient signaling, is also consistent with this proposal (39). The discrete segregation of molecules on Vγ1 T cells that are essential for their binding to activated macrophages and execution of their effector function is suggestive that FasL and the TCR together form an IS. Additional studies in which cytoskeletal polarization and other likely constituents of IS, such as intracellular signaling molecules, can be shown to colocalize with FasL and the TCR on Vγ1 T cells would confirm that supramolecular activation clusters are indeed formed on these T cells as a result of coming into contact with activated macrophages. Finally, it should also be noted that the activation requirements and relative importance of TCR-ligand vs additional ligand-receptor interactions may be different for the different subsets of

**FIGURE 6.** TCR and Fas colocalize on Vγ1 T cells at points of contact with activated macrophages. IC-21 macrophages were adhered to glass slides and incubated with splenocytes from naive C57BL/6 TCRβ−/− mice. After 60 min, nonadherent cells were washed off and adherent cells were stained with Fas (Texas Red) and Vγ1 (FITC) Abs, and visualized using a LSM510 META laser scanning confocal microscope (A–D). The merged images in B and D were obtained using Imaris software and the ImarisColoc module to highlight pixels present in the same location in both the FITC and Texas Red channels. Colocalized data was then added to the original two-color data set as a third channel and pseudocolored yellow for visualization. E and F, Three-dimensional volume rendered images of the γδ T cell and macrophage, which highlight the position of the Vγ1 T cell in relation to the macrophage (E) and the location of Vγ1 and Fas staining on the surface of the macrophage after electronically removing the Vγ1 T cell (F).
cytotoxic γδ T cells (peripheral vs epithelia-associated) for target cell recognition and killing.

In summary, the results of our study are consistent with Fas-FasL functioning as a coreceptor or accessory molecules for macrophage cytocidal Vγ1 T cells. Because there is no evidence that the Vγ1 TCR reacts with FasL (J. E. Dalton and S. R. Carding, unpublished observations) it is not clear that Fas-FasL satisfies the classical definition of a coreceptor because typical coreceptor molecules such as CD8 and CD4 bind to the same ligand (MHC) as the TCR (40). Therefore, it may be more appropriate to consider Fas as an accessory molecule because it clearly serves to promote and regulate the interaction of Vγ1 TCR with populations of activated macrophages.

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