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Macrophages Express Multiple Ligands for $\gamma\delta$ TCRs

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Abstract

As only a handful of ligands have been identified, the general nature of the ligands recognized by $\gamma\delta$ T cells remains unresolved. In this study, soluble multimerized $\gamma\delta$ T cell receptors (smTCRs) representing the TCRs of two $\gamma\delta$ T cell subsets common in the mouse were used to detect and track their own ligands. Ligands for both subsets were found on resident peritoneal macrophages taken from untreated mice, and the expression of both was further induced by *Listeria monocytogenes* infection. Nevertheless, the two types of ligand differ from one another in abundance, in the kinetics of their induction following *Listeria* infection, and in their ability to be induced by in vitro culture with LPS. Surprisingly, because both are detectable on normal macrophages, these host-derived ligands are likely expressed constitutively, but are induced to higher levels of expression by stress or inflammation. In contrast to T22 and other known cell-surface ligands for $\gamma\delta$ T cells in mice and humans, expression of these smTCR-defined ligands does not depend on β_2 -microglobulin, suggesting that they are not MHC class I or class I-like molecules.

Keywords

Rodent; T cells; Cell Surface Molecules; T Cell Receptors

Introduction

$\gamma\delta$ T cells have attracted much interest because of their protective potential in malignancies and their ability to regulate immune responses and tissue repair, but little is known about how their functions are triggered. Presumably, these functions are induced via the T cell receptor (TCR), but the nature of ligands for the $\gamma\delta$ TCR and a coherent theory of ligand recognition remain elusive.

Despite this, some ideas about ligand recognition by $\gamma\delta$ T cells and the nature of $\gamma\delta$ TCR ligands have gained broad acceptance. Because of structural similarities between the putative ligand-binding-sites of the $\gamma\delta$ TCRs and the BCR (Rock et al., 1994), and because several cell-surface-expressed ligands for $\gamma\delta$ T cells are apparently recognized as intact molecules without the need for antigen processing or presentation (Crowley et al., 2000; Groh et al., 1998; Sciammas et al., 1994; Spada et al., 2000), ligand recognition by $\gamma\delta$ TCRs has been postulated to have

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features in common with that of immunoglobulin molecules. Conversely, this concept is difficult to reconcile with the observed stimulation of certain human $\gamma\delta$ T cells by small pyrophosphate-containing compounds, such as isopentenyl pyrophosphate (IPP), because they are too small by themselves to provide TCR crosslinking. IPP has been found to accumulate in cells when the mevalonate biosynthetic pathway has been blocked by drugs, infection, or mutation, providing an explanation as to why these particular $\gamma\delta$ T cells can be stimulated in many different ways (Bonneville and Scotet, 2006). However, recognition of IPP may more resemble antigen recognition by the $\alpha\beta$ TCR than by immunoglobulin, because IPP is thought to be “presented” in some fashion by a larger cell surface molecule, including as a possible candidate an F1-ATPase-apolipoprotein complex which has been found on certain tumor cells and has also been implicated as a ligand (Scotet et al., 2005).

The idea that ligands for $\gamma\delta$ TCRs are stress-inducible is also supported by much of the available evidence. Most of the currently acknowledged ligands are induced to higher levels of expression under conditions of cellular stress, including in mice the non-classical MHC class I molecules T22 and T10 (Crowley et al., 2000), and in humans the class I-like molecules MIC-A, MIC-B (Groh et al., 1998), and CD1c (Spada et al., 2000), as well as IPP (Bonneville and Scotet, 2006).

Progress has also been made in defining the $\gamma\delta$ TCR components needed for ligand recognition. At least one ligand, the murine T22 molecule, turned out to be recognized almost exclusively by the CDR3 component of the TCR- δ chain (Adams et al., 2005; Shin et al., 2005). In fact, nearly all of the required motif could be supplied by the D δ 2 element when expressed in one of 3 possible reading frames. In contrast, no contributions from the γ chain were seen, and only one amino acid was supplied by V δ , a residue that could alternatively be encoded by D δ 1, or by appropriately-placed N or P nucleotides in the rearranged δ gene. The interaction of this $\gamma\delta$ TCR with its ligand contrasts markedly to that typical of $\alpha\beta$ TCRs with MHC-peptide ligands, both in the involvement of only the CDR3 of one TCR chain, rather than a combination of variable region and CDR3 contributions from both chains, and in the tilted angle of binding that was seen (Adams et al., 2005), as opposed to the nearly parallel alignment generally observed for the $\alpha\beta$ TCR bound to peptide/MHC complex. Further examples are needed in order to determine whether or not this is typical of $\gamma\delta$ TCR ligand recognition. The problem remains that no method has been available that would allow for the detection and characterization of ligands for any given $\gamma\delta$ TCR.

Towards this end, we have taken the approach of generating soluble multimeric $\gamma\delta$ TCRs (smTCRs), which can be used as reagents to detect their own specific ligands (Aydintug et al., 2004). We demonstrated that an smTCR derived from the T22-specific $\gamma\delta$ T cell hybridoma KN6 readily detects the cell-surface-expressed transfected T22 molecule, and showed, by staining with several other smTCRs representative of common subsets of $\gamma\delta$ T cells in mice, that many immortalized cell lines express unknown ligands for $\gamma\delta$ TCRs. Whether this would also be true for normal non-stressed, non-transformed cells was not clear.

We now show that normal resident peritoneal macrophages also stain with two smTCRs representative of common subsets of $\gamma\delta$ T cells in mice, suggesting that they express at least two different ligands for $\gamma\delta$ T cells, even in untreated mice. Because of changes in the smTCR staining patterns noted during an infection of mice with the bacterium *L. monocytogenes*, or following *in vitro* stimulation with lipopolysaccharide (LPS), our data further suggest that these ligands are induced to higher levels of expression under conditions of cellular stress. In contrast to T22 and other previously described cell-surface-expressed ligands in mice, these newly detected ligands for $\gamma\delta$ T cells do not require expression of β_2 -microglobulin, which is typically associated with class I and class I-like MHC molecules.

Materials and Methods

Mice

Fully backcrossed C57BL/6 background mice homozygous for a genetically inactive TCR- δ constant region gene [B6.TCR $\delta^{-/-}$ mice (Mombaerts et al., 1993)] were used for most of the experiments and were bred in our facility from commercially available stock (Jackson Labs, Bar Harbor, ME). Additional strains examined include B6.FcR $\gamma^{-/-}$, B6. β 2-microglobulin $^{-/-}$, and C57BL/6 wildtype mice, also bred in-house from commercially available stock (Jackson Labs, Bar Harbor, ME). C57BL/6 mice for some experiments were obtained from Harlan Sprague Dawley, Inc., (Indianapolis, IN). These studies were reviewed and approved by the National Jewish Institutional Animal Care and Use Committee.

Unstimulated peritoneal macrophages

Cells from naive or *Listeria*-infected mice (see below) were isolated from the peritoneal cavity of untreated mice by peritoneal lavage. Briefly, ~7 ml of Hank's BSS containing 5% heat-inactivated FBS (Atlanta Biologicals) were injected into the peritoneal cavity, and the abdomen massaged before withdrawing the BSS. The cell preparation was treated with Gey's solution to remove any RBCs, and the cells enumerated using a Coulter counter. The cells were then placed in culture medium (O'Brien et al., 1992) containing 10% heat-inactivated FBS. To purify/enrich macrophages, the cells were next cultured at 37°C for 1–2 hours, to allow the macrophages to adhere to tissue culture flasks or plates. Any non-adherent cells were then poured off. Next, the bound macrophage-enriched population was removed from the plate by culturing them for 30 minutes at 37°C in calcium/magnesium-free PBS containing 10 mM EDTA, followed by vigorous pipetting. The cells were then placed in round-bottom 96-well tissue culture plates, stained and analyzed by flow cytometry, taking care to keep the cells on ice or refrigerated at all times during the staining to reduce adherence to the culture plate.

In vivo stimulation of macrophages

Mice were infected with *Listeria monocytogenes*, strain 10403S (Portnoy et al., 1988) (kind gift of Dr. Laurel Lenz, National Jewish Medical and Research Center). Fresh overnight cultures in tryptose phosphate broth were grown from frozen stock, then diluted in pyrogen-free PBS on ice, just prior to injection. An approximate dose of 2×10^4 colony-forming units was given per mouse by i.p. injection; the number of *Listeria* injected was verified by plating a small aliquot of the inoculum on Tryptic Soy agar plates.

In vitro stimulation of macrophages

Fresh peritoneal resident macrophages were isolated and maintained at 37°C in a 10% CO₂ atmosphere in culture medium (O'Brien et al., 1992) containing 10% heat-inactivated FBS, as described above, along with LPS from *Escherichia coli* O55:B5 (Sigma-Aldrich Co., St. Louis, MO) at a concentration of 3 μ g/ml. Tripalmitoyl-S-glycerylcysteine (Pam3Cys), a TLR2 agonist, was the kind gift of Dr. Ross Kedl, Univ. of Colorado at Denver and Health Sciences Center, and was used at a concentration of 200 ng/ml. Cells were cultured for 2–4 days in T-75 culture flasks, and removed from the flasks for staining as described above.

Preparation of spleen macrophages

Spleens were dispersed in cold BSS + 5% heat-inactivated FBS by passing the cells through a mesh screen. After letting the debris settle, this single cell suspension was spun down at 1000 rpm for 8 minutes in the cold, and the RBCs lysed with Gey's solution. The cells were then resuspended in culture medium and transferred to T-75 culture flasks (Corning Corp., Corning, NY) using 2 flasks per spleen, and incubated for 3 hours at 37°C in a 10% CO₂ atmosphere. The nonadherent cells were at this time poured off, fresh medium was added, and the cells

were then again cultured overnight. Adherent cells were removed from the flasks for staining as described above.

Flow Cytometry

Cells were stained in round-bottom 96-well plates, containing 5×10^5 or fewer cells/well. Cells were pre-blocked to prevent Fc-receptor background binding by treatment with 40 $\mu\text{g/ml}$ of a purified rat monoclonal antibody against mouse CD16/CD32 [clone 2.4G2 (Unkeless, 1979)] prepared in our laboratory, plus 20–40 $\mu\text{g/ml}$ mouse IgG (Jackson ImmunoResearch, West Grove, PA), for 45 minutes at 4°C. Affinity-purified $\gamma\delta$ smTCRs were generated in our laboratory after the method of Crawford et al. (Crawford et al., 2004), and used to stain cells as previously described (Aydintug et al., 2004), using a TCR concentration of 30 $\mu\text{g/ml}$. Unless otherwise indicated in the figure legends, a low-level biotinylated anti-C δ “core” monoclonal antibody [clone 403A.10 (Itoharu et al., 1989)], first tetramerized using Alexa fluor-647 streptavidin (Invitrogen Corp., Carlsbad, CA), was used to generate individual smTCRs; anti-histidine-tag monoclonal antibody (anti-HisTag, clone AD1.1.10, Serotec, Oxford, UK), and an anti-FLAG-tag monoclonal antibody (clone M2, Sigma-Aldrich Co., St. Louis, MO) were also used as core monoclonal antibodies in particular experiments as noted in the figure legends. In all experiments, core monoclonal antibodies used to generate the smTCRs were matched, except in one experiment in which the use of different core monoclonal antibodies was necessary to avoid cross reactions (shown in Fig. 5D). In experiments in which monomeric soluble TCRs were used, the preparations were spun at 16,000 g for 15–20 minutes before use, to remove any aggregated material. In most experiments, dead cells were excluded from the analyses based on staining with 7-AAD or propidium iodide. In some experiments, cells were also co-stained with rat anti-mouse F4/80-FITC [a pan-macrophage marker (Austyn and Gordon, 1981; Schaller et al., 2002); clone BM8, eBioscience, San Diego, CA], and hamster anti-mouse C δ FITC [prepared in our own laboratory, clone GL3 (Goodman and Lefrancois, 1989)]. Unlabeled antibodies used to inhibit staining, or as a negative control, include a rat anti-mouse V δ 6.3 [17C (Belles et al., 1996); prepared in our laboratory], and polyclonal rat IgG (Sigma-Aldrich Co., St. Louis, MO). All flow cytometry experiments shown were carried out at least twice to ensure that the results were reproducible, allowing for some differences in the intensity of staining when using different smTCR preparations. Nomenclature used for expressed V γ genes is as in (Heilig and Tonegawa, 1986) and is in accordance with the IMGT numbering system; however, V δ 1 is termed TRDV4 in the IMGT system and V δ 6.3 is termed DV6D-1.

Proliferation Assays

V γ 6/V δ 1 [line 134, (Sim et al., 1995)] transgenic spleen cells were prepared and passed over nylon wool to enrich for T cells, then were treated with a biotinylated anti-C β monoclonal antibody (Kubo et al., 1989), washed, incubated with streptavidin-conjugated magnetic beads, and then passed over a MACS-streptavidin bead column (Miltenyi Biotec, Gladbach, Germany) to remove most of the $\alpha\beta$ TCR-bearing cells. The nonadherent cells were collected and placed on a 96-well plate at 2×10^4 T cells/well. Irradiated XB-2 cells were added at various effector:target (E:T) ratios, using triplicate wells for each condition, and the plate incubated for 3 days, after which 1 $\mu\text{Ci/well}$ of ^3H -thymidine was added to each well. After an additional 18-hour incubation, wells were harvested and counts per well determined. The stimulation index was calculated by dividing the cpm obtained per well by the average obtained from triplicate wells containing the T cells only. As a control, the same experiment was carried out using $\alpha\beta$ T cells together with XB-2 cells. The $\alpha\beta$ T cells were enriched from the spleen of a B6.TCR $\delta^{-/-}$ mouse, using nylon wool purification.

Competitive soluble TCR binding

XB-2 keratinocytes, 3×10^5 , were first pre-incubated for 45 minutes at 4°C with unlabeled V γ 6/V δ 1 monomeric soluble TCR at a concentration of 1.2 mg/ml. Following a single wash in staining buffer, the cells were then stained with labeled V γ 1/V δ 6.3 smTCR having an anti-C γ 4 monoclonal antibody core [clone 2.11 (Pereira and Boucontet, 2004; Pereira et al., 1995)] at a concentration of 16 μ g/ml for the TCR. Staining with smTCR only was carried out as previously described (Aydintug et al., 2004). To verify binding of the unlabeled V γ 6/V δ 1 monomer, XB-2 cells were incubated with unlabeled V γ 6/V δ 1 soluble TCR monomer at 200 μ g/ml, which was subsequently detected by using labeled tetrameric anti-C δ monoclonal antibody [clone 403A.10 (Ito et al., 1989)] as a secondary reagent. As a control experiment to demonstrate that preincubation with V γ 6V δ 1 monomer can block itself, pretreatment of XB-2 cells with unlabeled V γ 6V δ 1 soluble TCR monomer was carried for 45 minutes at 4°C using a concentration of 800 μ g/ml, followed by a single wash, and then by staining with soluble V γ 6V δ 1-HisTag monomer at a concentration of 125 μ g/ml. Binding of V γ 6V δ 1-HisTag was detected with labeled tetrameric anti-HisTag monoclonal antibody as a secondary reagent.

Results

Normal peritoneal macrophages express ligands for at least two common $\gamma\delta$ TCRs

Previously, we reported that two unrelated $\gamma\delta$ TCRs of mice, a V γ 1V δ 6.3 TCR and the invariant V γ 6V δ 1 TCR, stained many of the same mouse cell lines, most of which were derived from epithelial cells, and that multimeric versions of these TCRs (smTCRs) gave superior staining compared to monomeric soluble TCRs (Aydintug et al., 2004). We have found that staining of these cell lines with the V γ 1V δ 6.3 smTCR is consistently brighter than that obtained with the V γ 6V δ 1 smTCR, although in a previous study (Aydintug et al., 2004), we underestimated the degree of staining with the V γ 1V δ 6.3 TCR due to use of either monomeric TCR, which stains poorly for this TCR, or of a different anti-C δ core monoclonal antibody to generate the smTCR, which gave higher backgrounds than the anti-C δ core monoclonal antibody used in this study. We speculated that the expression of multiple $\gamma\delta$ TCR ligands might be a consequence of the transformed nature of immortal cell lines, and we thus went on to see whether ligands for the same TCRs could be detected on normal mouse cells as well. Although no substantial staining was detected on suspensions of spleen or liver cells (data not shown), a large portion of normal resident peritoneal cells was found to stain well with both the V γ 1V δ 6.3 and V γ 6V δ 1 smTCRs. Resident peritoneal cells include both macrophages, which stain with the monoclonal antibody F4/80 that detects a pan-macrophage marker (Austyn and Gordon, 1981), and B and T lymphocytes, which do not, and the two populations are also largely distinguishable by differences in their forward/side scatter properties (Fig. 1A). The F4/80 positive population, much like the epithelial cell lines we previously studied, stained brightly with the V γ 1V δ 6.3 smTCR, and weakly but clearly with the V γ 6V δ 1 smTCR. In contrast, a $\gamma\delta$ smTCR derived from the hybridoma KN6 (Ito et al., 1990), which recognizes the nonclassical MHC class I molecule T22 (Crowley et al., 2000), showed no detectable staining for the F4/80 positive population, and with the invariant V γ 5V δ 1 smTCR, which is closely related to the V γ 6V δ 1 TCR and carries an identical δ chain, it was also essentially negative (Fig. 1A). In contrast, the F4/80 negative population showed no detectable staining with the V γ 6V δ 1, the V γ 5V δ 1, or the KN6-derived smTCRs, although these cells showed a small but reproducible shift when stained with the V γ 1V δ 6.3 smTCR (Fig. 1A), suggesting low level expression of a ligand for this TCR on lymphocytes.

Specificity of the soluble TCR staining

We previously found that staining of cell lines with soluble $\gamma\delta$ TCRs could be inhibited by pre-treatment of the soluble TCRs with relevant, but not irrelevant, anti-V region specific monoclonal antibodies, a result that is consistent with binding of the soluble TCR via specific

recognition of a ligand through the variable portion of the molecule. We therefore attempted to verify the specificity of binding of the V γ 1V δ 6.3 smTCR to peritoneal macrophages in a similar way. As shown in Fig. 1B, pretreatment of the V γ 1V δ 6.3 smTCR with an anti-V δ 6.3 monoclonal antibody greatly reduced its ability to bind macrophages, whereas pretreatment with nonspecific rat IgG had no effect. This result implies that the V γ 1V δ 6.3 smTCR binds specifically to peritoneal macrophages. Since peritoneal macrophages stain only weakly with the V γ 6V δ 1 smTCR, and because the one available monoclonal antibody which specifically recognizes the V γ 6V δ 1 TCR is a low affinity IgM antibody (Roark et al., 2004), we were not able to carry out a similar experiment for the V γ 6V δ 1 smTCR. Because these $\gamma\delta$ smTCRs contain monoclonal antibody “cores,” there was still a possibility that the V γ 1V δ 6.3 and V γ 6V δ 1 smTCRs might be binding nonspecifically via Fc receptors on the macrophages, even though the KN6-derived smTCR and V γ 5V δ 1 smTCR which contain the same core failed to bind. Although we attempted to block any potential Fc receptor binding by pretreatment with unlabeled monoclonal antibody 2.4G2 and mouse IgG in all staining experiments shown in this study, potential background from Fc γ RI, which is not specifically recognized by monoclonal antibody 2.4G2, was still possible. We therefore attempted to stain, using the same $\gamma\delta$ smTCRs, peritoneal macrophages from B6.FcR $\gamma^{-/-}$ mice, which lack all activating Fc γ Rs, including Fc γ RI (Takai et al., 1994). Although these mice can potentially express the inhibitory receptor Fc γ RII, monoclonal antibody 2.4G2 was again used in this experiment to preblock the cells, which specifically recognizes Fc γ RII as well as activating receptor Fc γ RIII (Araujo-Jorge et al., 1993; Takai et al., 1994). As shown in Fig. 1C, the staining profile of FcR $\gamma^{-/-}$ peritoneal macrophages was similar to that of B6.TCR $\delta^{-/-}$ peritoneal macrophages, indicating that the binding of the V γ 1V δ 6.3 and V γ 6V δ 1 smTCRs to peritoneal macrophages is not the result of nonspecific binding via FcRs.

In addition to other lymphocytes, the F4/80 negative population potentially contains $\gamma\delta$ T cells, which might be able to bind soluble $\gamma\delta$ TCR multimers via the core anti-C δ monoclonal antibody used to multimerize them. Because they are a rare T cell type (about 3% of the lymphoid T cells in C57BL/6 mice) compared to the large peritoneal macrophage populations that stained with both V γ 6V δ 1 and V γ 1V δ 6.3 smTCRs, the presence of $\gamma\delta$ T cells cannot explain the staining seen among F4/80 positive cells. Many experiments shown in this study (including Fig. 1A and B) were carried out using cells from B6.TCR $\delta^{-/-}$ mice, to prevent this possibility. However, peritoneal macrophages derived from wildtype C57BL/6 mice showed a very similar staining pattern to those from B6.TCR $\delta^{-/-}$ mice (right panel, Fig. 1C).

Levels of ligand for both the V γ 6V δ 1 and V γ 1V δ 6.3 TCRs are higher on macrophages from *Listeria*-infected mice

Responses by both V γ 1V δ 6.3+ and V γ 6V δ 1+ $\gamma\delta$ T cells have been previously reported in mice infected with *Listeria monocytogenes* (Belles et al., 1996; Dalton et al., 2003; O'Brien et al., 2000; Roark et al., 1996). Therefore, we tested whether the ligands on macrophages for either of these two $\gamma\delta$ TCRs were affected by infection of the mice with *Listeria*. We found that staining with the V γ 1V δ 6.3 smTCR was stronger overall on macrophages of mice infected intraperitoneally with *Listeria*. As well, a small population that stained brightly with the V γ 6V δ 1 smTCR was induced on *Listeria*-infected macrophages. We next examined the expression of these ligands for the $\gamma\delta$ TCRs over a timecourse during *Listeria* infection (Fig. 2). Staining of macrophages with the V γ 1V δ 6.3 smTCR rose gradually over time, increasing from about 1.4 times above the uninfected level at day 4 to more than 2.5 times above the uninfected level by day 8 of the infection, based on mean fluorescence values. The level of V γ 1V δ 6.3 smTCR staining was still comparably high on days 8 and 10 (not shown) following infection, although we have not followed the expression further than this. In contrast, staining with the V γ 6V δ 1 smTCR decreased over time, such that the mean fluorescence of the peak of bright cells was clearly reduced by day 8. By day 10, staining of a V γ 6V δ 1-bright peak was

even lower, nearly down to the background level in uninfected macrophages (not shown). In contrast, the KN6-derived smTCR, which generally does not stain uninfected macrophages, remained essentially unchanged in macrophages from *Listeria*-infected mice, as was staining with the V γ 5V δ 1 smTCR (not shown). Thus, it appears that ligands for both V γ 1V δ 6.3+ and V γ 6V δ 1+ $\gamma\delta$ T cells are indeed induced on macrophages following *Listeria* infection, but to different degrees and with different kinetics, when compared with one another. In contrast, *Listeria* infection under the conditions used does not appear to induce ligands for the KN6-derived or the V γ 5V δ 1 canonical TCR.

Stimulation of macrophages in vitro can also induce $\gamma\delta$ TCR ligands

During the course of a peritoneal *Listeria* infection, neutrophils and macrophages are recruited to the site of infection, whereas many of the resident macrophages disappear (Barth et al., 1995; Skeen et al., 2004). Therefore, the changes we saw in expression of ligands for the V γ 6V δ 1 and V γ 1V δ 6.3 TCRs during *Listeria* infection could reflect differential expression of ligands on newly recruited macrophages, rather than a change in ligand expression following activation of resident macrophages present before infection. We therefore attempted to stimulate resident peritoneal macrophages in vitro, by culture in medium containing LPS, to see whether levels of ligands for either of these two TCRs increase. As shown in Fig. 3, after 4 days of culture in LPS, staining of the cells with the V γ 6V δ 1 smTCR was greatly enhanced, such that over 50% of the F4/80-positive cells now stained with this reagent. This change was not yet evident after 2 days of culture with LPS (data not shown). In contrast, staining with the V γ 1V δ 6.3 smTCR was not induced by this treatment at either timepoint. Culture of peritoneal macrophages with the synthetic lipopeptide TLR2 agonist Pam3Cys also induced a ligand for the V γ 6V δ 1 but not the V γ 1V δ 6.3 smTCR, although fewer cells were induced (data not shown), implying that stimulation through particular Toll-like receptors, including TLR4 and TLR2, is sufficient for induction of this ligand. Macrophages cultured alone, without LPS, showed a decrease in the expression of ligands for all smTCRs tested, compared to freshly isolated peritoneal macrophages (except for the KN6-derived smTCR, which stained neither fresh nor cultured macrophages), and perhaps expression of other cell surface proteins as well, since nonspecific background staining with the core monoclonal antibody was also evidently reduced.

Evidence that the molecule detected by staining with an smTCR is actually a ligand

In a previous study (Aydintug et al., 2004), we showed that as for peritoneal macrophages, XB-2 cells, a keratinocyte-derived cell line, also stain with both the V γ 6V δ 1 and V γ 1V δ 6.3 smTCRs. This was somewhat surprising, because V γ 5V δ 1+ $\gamma\delta$ T cells, but not V γ 6V δ 1+ or V γ 1V δ 6.3+ $\gamma\delta$ T cells, are normally found in the skin in close association with keratinocytes (Jameson et al., 2004). If the molecules these smTCR detect on XB-2 are actually ligands, they should be capable of stimulating the relevant $\gamma\delta$ T cells. To test whether XB-2 cells can stimulate V γ 6V δ 1+ T cells, we cultured XB-2 together with V γ 6V δ 1-purified cells from a TCR-transgenic mouse strain. As shown in Fig. 4, a response to XB-2 by V γ 6V δ 1 $\gamma\delta$ T cells was evident, whereas $\alpha\beta$ T cells did not respond. This supports the interpretation that the molecule detected on XB-2 cells by the V γ 6V δ 1 soluble TCR is in fact a ligand. We were unable to similarly verify the presence of a ligand for the V γ 1V δ 6.3 soluble TCR due to a lack of appropriate V γ 1V δ 6.3 transgenic mice to provide a clonally identical T cell population.

Evidence that the ligands detected by different soluble $\gamma\delta$ TCRs are distinct molecules

Because they are affected differentially following a *Listeria* infection or after culture with LPS, the molecules that macrophages express which bind to the V γ 6V δ 1 and the V γ 1V δ 6.3 smTCR appear to be distinct moieties. To test whether the molecules detected on cells by these two soluble TCRs are in fact different from one another, we carried out experiments with the XB-2

cell line rather than macrophages, due to the variable background staining characteristics of macrophages, which fluctuate considerably among different preparations; the XB-2 line in contrast has reproducibly low background staining. We previously found that the V γ 6V δ 1 and V γ 1V δ 6.3 smTCRs stain XB-2 cells differentially, and as shown in Fig. 5A, although variations are seen among different smTCR preparations, the V γ 1V δ 6.3 smTCR consistently stains them more brightly even when the smTCRs are generated with a different core monoclonal antibody, suggesting that the ligands for these TCRs are indeed distinct. To more rigorously assess whether these ligands are different molecules, we tested whether it was possible to block the staining of a soluble TCR of one type by pre-treating the cells to be stained with unlabeled soluble TCR of another type. Specifically, we pretreated XB-2 cells with an excess of unlabeled V γ 6V δ 1 monomer, and then compared them with untreated cells for their ability to bind a labeled V γ 1V δ 6.3 smTCR. Importantly, in this experiment shown in Fig. 5B, the monoclonal antibody core used to generate the V γ 1V δ 6.3 smTCR is specific for V γ 1J γ 4C γ 4, and is thought to recognize C γ 4, the C γ uniquely associated with V γ 1 but not other V γ s (Pereira and Boucontet, 2004). Thus, the core monoclonal antibody cannot cross-react with pre-bound V γ 6V δ 1 monomer (although the need to use a different core monoclonal antibody for the smTCR in this experiment resulted in somewhat weaker staining) The staining of the XB-2 cells with the labeled V γ 1V δ 6.3 smTCR was undiminished by pretreatment with “cold” V γ 6V δ 1 monomer, even when using as much as a 75-fold excess of the monomer. The ability of the V γ 6V δ 1 monomer to bind to XB-2 cells was confirmed by the staining monomer-treated cells subsequently with a labeled secondary reagent. This result implies that these two $\gamma\delta$ TCRs are binding to distinct ligands on XB-2. We were unable to carry out a reciprocal experiment using monomeric V γ 1V δ 6.3 soluble TCR to block staining of the V γ 6V δ 1 smTCR, because the V γ 1V δ 6.3 TCR stains very poorly in monomeric form. However, to validate the experimental method used here, we verified that pretreatment of XB-2 cells with unlabeled V γ 6V δ 1 soluble TCR monomer does effectively block subsequent staining of the cells by a histidine-tagged version of the same TCR (Fig. 5C).

As an additional test, we also compared the binding of these two different smTCRs at the same time. As shown in Fig. 5D, this was accomplished by double-staining macrophages and XB-2 cells with differentially labeled smTCRs prepared with core monoclonal antibodies that cannot cross-react with the other TCR. The two left panels show single staining of XB-2 cells with either the V γ 6V δ 1 smTCR or V γ 1V δ 6.3 smTCR. For the peritoneal macrophages (top row), the single staining is shown next to a specificity control verifying that the core monoclonal antibody used to generate each multimer does not detect the irrelevant TCR. XB-2 cells double-stained with both smTCRs, added at the same time, are shown in the right panel. Clearly, as shown by the mean fluorescence values, each smTCR stains with equivalent brightness whether used to double-stain or single-stain the cells. If the smTCRs recognized the same molecule, when added together for the double staining, they would be expected to stain with about half of the intensity obtained when each was used alone, but no decrease was seen. Therefore, several lines of evidence indicate that the ligands detected by the V γ 1V δ 6.3 TCR and the V γ 6V δ 1 TCR are distinct molecules.

The V γ 1V δ 6.3 and V γ 6V δ 1 ligands do not appear to be MHC class I or class I-like molecules

In both mice and humans, among the few molecules that have been identified thus far as ligands for known $\gamma\delta$ TCRs, several are MHC class I or class I-like molecules associated with β 2-microglobulin (Crowley et al., 2000; Groh et al., 1998; Spada et al., 2000). Therefore, the possibility that the molecules being detected by the V γ 1V δ 6.3 and V γ 6V δ 1 smTCRs are class I or class I-like was examined by testing whether macrophages from β 2-microglobulin $^{-/-}$ mice also stained with these reagents. As can be seen in Fig. 6A, peritoneal macrophages from these mice gave staining patterns very similar to those from B6.TCR $\delta^{-/-}$ mice, which are wildtype for β 2-microglobulin. Consistently, in other experiments, we found that macrophages from

B6.TAP1^{-/-} mice produce staining patterns with these two smTCRs that are very similar to those of macrophages from B6.TCR δ ^{-/-} or C57BL/6 wildtype mice (data not shown). Thus, the ligands recognized by the V γ 1V δ 6.3 and V γ 6V δ 1 soluble TCRs are not likely to represent class I or class I-like molecules.

Macrophages in other sites also stain with the V γ 1V δ 6.3 and V γ 6V δ 1 smTCRs

To test whether the expression of ligands for the V γ 1V δ 6.3 and V γ 6V δ 1 TCRs is an unusual property of peritoneal macrophages, or instead is common among macrophages in general, we tested both blood monocytes and macrophages derived from spleen for their ability to stain with these smTCRs. To enrich the splenic macrophages enough for analysis, we first cultured splenic plastic-adherent cells overnight before staining, because fresh ex vivo splenic macrophages express low levels of F4/80 and also require enrichment. We found that the V γ 1V δ 6.3 smTCR also stained both blood monocytes and cultured splenic macrophages. Staining with the V γ 6V δ 1 smTCR was weak but also detectable on cultured splenic macrophages and blood monocytes. With the KN6-derived smTCR, staining varied, and was as strong as that of the V γ 6V δ 1 smTCR on blood monocytes, but lower than V γ 6V δ 1 on cultured splenic macrophages (Fig. 5B). It therefore appears that strong expression of a V γ 1V δ 6.3 ligand, and weak expression of a V γ 6V δ 1 ligand, are common properties of macrophages in general, even before they exit the blood for the tissues.

Discussion

In a previous study, we showed that soluble forms of $\gamma\delta$ TCRs can be used as reagents to detect their own ligands, using as a control the $\gamma\delta$ TCR from the hybridoma KN6, whose ligand, T22, had already been identified. Although the monomeric form of this soluble TCR was sufficient for ligand-detection, a multimerized form provided brighter staining with less reagent. Using soluble multimerized forms of other $\gamma\delta$ TCRs (smTCRs) representative of several of the major $\gamma\delta$ T cell subsets in mice, we then went on to show that a variety of transformed cell lines express ligands for $\gamma\delta$ T cells as defined by smTCR-staining, and often several different ones simultaneously. Because these ligands were protease-sensitive, they appear to be protein or protein-associated molecules expressed on the cell surface (Aydintug et al., 2004). These first experiments established cell-staining with smTCRs as a viable approach for the systematic detection and discovery of new $\gamma\delta$ T cell-ligands. They also raised the question of whether such endogenous ligands can be expressed in unstimulated normal cells or are only induced as a consequence of transformation or stress.

In this study, we have addressed this question, and have extended our findings to show that normal cells, in this case peritoneal macrophages identified by their scatter-profile and expression of F4/80, also express ligands for at least two common $\gamma\delta$ TCRs in mice: the invariant V γ 6V δ 1 TCR and a representative V γ 1V δ 6.3 TCR. This staining revealed co-expression of these two smTCR-detected ligands, indicating that the expression of multiple ligands for certain $\gamma\delta$ TCRs in cells which have not been stressed or triggered by inflammation is likely a common property rather than a peculiarity of immortal or transformed cell lines. We also showed that the levels of ligands for these two TCRs are increased on macrophages from *Listeria*-infected mice, and that expression of the V γ 6V δ 1 ligand is increased on peritoneal macrophages following in vitro culture with LPS. These data therefore suggest that host-derived endogenous ligands for $\gamma\delta$ T cells, already expressed under steady state conditions, can be induced to higher levels of expression during the course of an infection. However, it must be kept in mind that, because their molecular structure remains unknown, the smTCR-detected ligands on the cell populations that express them may instead vary in form under different conditions. That the same ligands are expressed at different levels seems more likely,

especially in the case of the ligand detected by the V γ 6V δ 1 smTCR, because it is naturally invariant and probably recognizes an equally invariant ligand.

This study also provides evidence that the ligands detected by two smTCRs are different molecules, because they are distinct in abundance and expression kinetics in normal cells, and because of the absence of crossblocking between the smTCRs. Finally, neither smTCR-defined ligand appears to be an MHC class I or a class I-like molecule, because nearly all such molecules require β_2 -microglobulin to form a stable structure on the cell surface (Rodgers and Cook, 2005). This was a somewhat surprising finding, since most of the known ligands for both mouse and human $\gamma\delta$ TCRs are nonclassical class I or class I-like molecules. Because the TCRs we focused on in these experiments represent common $\gamma\delta$ TCR subsets, this finding may indicate that many $\gamma\delta$ TCR ligands, perhaps even the majority, are not MHC class I-like molecules.

We and others have previously noted *in vivo* responses of $\gamma\delta$ T cells bearing both V γ 6V δ 1 and V γ 1, particularly V γ 1V δ 6.3+ cells, during *Listeria* infection (Belles et al., 1996; Dalton et al., 2003; O'Brien et al., 2000; Roark et al., 1996). Our discovery here that ligands for both V γ 6V δ 1 and V γ 1V δ 6.3 TCRs are induced on peritoneal macrophages during *Listeria* infection fits well with these earlier findings. Additionally, *in vivo* evidence has been published that V γ 6V δ 1+ $\gamma\delta$ T cells, expressing the canonical TCR, respond during a peritoneal *E. coli* infection (Mokuno et al., 2000), and that the response is LPS-dependent, whereas V γ 1+ $\gamma\delta$ T cells do not appear to respond in this system. Our finding in this study that, despite strong induction of the V γ 6V δ 1 ligand, the V γ 1V δ 6.3 ligand does not appear to be induced by *in vitro* culture in LPS, further suggests that this differential subset-specific response is in fact driven by the peripherally induced expression of the V γ 6V δ 1 ligand. We were, however, surprised to find that ligands for both the V γ 6V δ 1 and V γ 1V δ 6.3 TCRs are generally detectable on peritoneal macrophages freshly isolated from untreated mice. Although the cell preparation procedure could potentially induce ligands for $\gamma\delta$ T cells, it seems more likely that the peritoneal macrophages express these ligands due to some degree of continuous “background” stimulation. Indeed, culturing macrophages in medium only, without any deliberate inflammatory stimuli, leads to decreased staining with both the V γ 6V δ 1 and V γ 1V δ 6.3 smTCRs, perhaps due to the lack of normal background stimulation (Fig. 3). Interestingly, established macrophage cell lines differ in their ability to stain with these soluble $\gamma\delta$ TCRs. Specifically, J774 cells stained weakly with the V γ 1V δ 6.3 smTCR but not with the V γ 6V δ 1, V γ 5V γ 1, or KN6-derived TCR smTCRs, whereas RAW-264 cells stained with both the V γ 6V δ 1 and the V γ 1V δ 6.3 smTCR, but only very weakly. In contrast, the monocyte line WEHI-3.5 showed no staining with any of these smTCRs (data not shown). Although the lack of staining of these cell lines might be taken to suggest that macrophages require some sort of stimulation to express ligands for the V γ 6V δ 1 and V γ 1V δ 6.3 TCRs, this does not appear to be so at least for the V γ 1V δ 6.3 TCR, because F4/80 monocytes derived from peripheral blood stained well with this smTCR (Fig. 5B).

Although induction of both smTCR-defined ligands was evident by day 4 of *Listeria* infection, a difference was seen in the duration of the induced expression levels, with faster kinetics for the ligand for the V γ 6V δ 1 TCR. The kinetics of the response of V γ 6V δ 1+ cells following *Listeria* infection depends to some degree on the dose given, but was at its highest in liver and spleen at day 5 for a fairly heavy systemic infection (Roark et al., 1996). In contrast, an early and a late biphasic response of V γ 1V δ 6.3 cells during *Listeria* infection has been previously noted (Belles et al., 1996). Therefore, there appears to be a positive correlation between the expression of the smTCR-defined ligands and the cellular responses of the corresponding $\gamma\delta$ T cell subsets, with ligand-expression preceding the cellular responses. Although we have yet to show that the molecules detected on macrophages by these $\gamma\delta$ smTCRs are functional ligands, this correlation suggests that they are indeed ligands which act to stimulate $\gamma\delta$ T reactivity *in vivo*.

Our finding that macrophages deficient in $\beta 2$ -microglobulin stain with both the V $\gamma 6$ V $\delta 1$ and V $\gamma 1$ V $\delta 6.3$ smTCRs equivalently to macrophages from wildtype mice strongly suggests that the ligands these TCRs recognize are not MHC class I or class I-like molecules. This was unexpected because several class I-type molecules have previously been shown to act as ligands for both mouse and human $\gamma\delta$ TCRs, including MICA (Groh et al., 1998), T10 and/or T22 (Shin et al., 2005), and CD1c (Spada et al., 2000). This may indicate that class I molecules are not typically $\gamma\delta$ TCR ligands, and indeed, one potential ligand described recently for human V $\gamma 9$ V $\delta 2+$ cells is neither class I nor class II-like (Scotet et al., 2005). Because $\gamma\delta$ TCRs do not generally recognize MHC class II molecules either, this would represent a major difference between $\alpha\beta$ and $\gamma\delta$ T cells. However, nearly all of the ligands thus far described for $\gamma\delta$ TCRs are inducible by stress or inflammation, including natural “phosphoantigens” for human V $\gamma 9$ V $\delta 2+$ cells (Bonneville and Scotet, 2006), a characteristic shared by the ligands we detected in this study using the mouse V $\gamma 6$ V $\delta 1$ and V $\gamma 1$ V $\delta 6.3$ smTCRs. A better understanding of these ligands awaits their structural characterization, but this study shows that even without knowing their structure, a considerable amount of information can be gained by detecting and tracking the ligands with soluble $\gamma\delta$ TCR multimers.

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Abbreviations used in this paper

smTCR, soluble multimeric T cell receptor.

Fig. 1.

A. Freshly isolated peritoneal cells from B6.TCR $\delta^{-/-}$ mice: two main peritoneal cell types can be distinguished by forward/side scatter properties (left panel), which also stain differentially with F4/80 (middle panel), a pan macrophage marker. All F4/80-positive cells stain brightly with the V γ 1/V δ 6.3 smTCR, and whereas most stain weakly with the V γ 6/V δ 1 smTCR, a small percentage of the macrophages stain brightly with this smTCR as well. The F4/80-negative peritoneal cells (largely lymphocytes) did not stain with the V γ 6/V δ 1 smTCR, but stained weakly with V γ 1/V δ 6.3 smTCR (mean fluorescence values obtained with this TCR are indicated, right panel). Neither the F4/80-positive or negative population stained appreciably with either the KN6-derived or V γ 5V δ 1 canonical smTCR. Note that staining here was carried out with double the concentration of smTCR used in the other experiments in this study, in an attempt to reveal any weak staining. **B.** Pretreatment of the V γ 1V δ 6.3 smTCR with a monoclonal antibody specific for V δ 6.3, but not with rat IgG, inhibits its ability to stain peritoneal macrophages. **C.** Peritoneal macrophages from both B6.FcR $\gamma^{-/-}$ and C57BL/6 (B6) wildtype mice show staining profiles with the V γ 6V δ 1 and V γ 1V δ 6.3 smTCRs similar to those of B6.TCR $\delta^{-/-}$ peritoneal macrophages.

Fig. 2.

Time course of ligand expression on peritoneal macrophages. B6.TCR $\delta^{-/-}$ mice were injected intraperitoneally with *Listeria monocytogenes* (2×10^4 CFU). The mice were sacrificed postinfection after the number of days indicated under each profile, for analysis. Freshly isolated peritoneal macrophages were identified by F4/80 staining, and any dead/dying cells in this population (propidium iodide-positive) were excluded. Mean fluorescence values for the entire peak stained with V γ 1V δ 6.3 smTCR are indicated above the peak, and above the indicated gate for cells stained with the V γ 6V δ 1 smTCR. Note that unstained and KN6 smTCR stained cells are shown as the negative control, rather than anti-C δ tetramer, because the peritoneal cells from infected mice often show high background staining with anti-C δ tetramer.

Fig. 3.

Macrophages were isolated from the peritoneum of B6.TCR $\delta^{-/-}$ mice. Staining results with these cells obtained on the day of isolation are shown (left panel), and compared to results obtained following in vitro culture. Macrophages cultured for 4 days without LPS express no or only very low levels of ligands for any of the smTCRs tested (middle panel). However, following 4 days of in vitro activation with LPS (right panel), the ligand for the V γ 6V δ 1 smTCR is strongly induced on over half of the cells. Ligands for the KN6-derived, the V γ 5V δ 1, and the V γ 1V δ 6.3 smTCRs in contrast show no induction.

Fig. 4.

Irradiated XB-2 cells were cultured with either Vγ6Vδ1 transgenic T cells (left) or αβ T cells (right), in the presence of tritiated thymidine, at the effector:target ratios indicated. The white bar shows as a positive control the response of the T cells when incubated in a well pre-coated with the appropriate anti-TCR monoclonal antibody.

Fig. 5.

A. Differential staining of XB-2 cells with two different $\gamma\delta$ smTCRs. The smTCRs in this experiment were prepared using histidine-tagged soluble TCRs together with an anti-histidine tag monoclonal antibody tetramer core. Mean fluorescence values obtained with each smTCR are indicated above the relevant peak. **B.** Pretreatment of XB-2 cells with an excess of unlabeled (cold) Vγ6Vδ1 monomer fails to block subsequent staining with labeled Vγ1Vδ6.3 smTCR. For cells pre-blocked with Vγ6Vδ1 monomer, staining with Vγ1Vδ6.3 smTCR is undiminished compared to that of untreated cells. Note that in this experiment, to prevent cross-recognition, the labeled Vγ1Vδ6.3 smTCR octamer was prepared using an anti-Cγ4 core monoclonal antibody [clone 2.11 (Pereira and Boucontet, 2004)], which cannot recognize the

V γ 6V δ 1 TCR. The ability of the “cold” V γ 6V δ 1 monomer to bind to the XB-2 cells was verified by showing that cells incubated with the V γ 6V δ 1 monomer only can be detected with a labeled anti-C δ tetramer secondary reagent [clone GL3 (Goodman and Lefrancois, 1988)]. **C.** Control experiment verifying that a soluble TCR can block itself. Compared to untreated cells, pre-treatment of XB-2 cells with cold V γ 6V δ 1 monomer substantially reduces their subsequent ability to stain with V γ 6V δ 1 HisTag monomer, whose binding is detected using anti-HisTag tetramer as a secondary reagent. **D.** Double staining of macrophages (top row) and XB-2 cells (bottom row) with differentially labeled V γ 6V δ 1 and V γ 1V δ 6.3 smTCRs. The smTCRs for this experiment were prepared with core monoclonal antibodies that recognize only the bound TCR: an anti-C γ 4 monoclonal antibody for the V γ 1V δ 6.3 smTCR (Pereira and Boucontet, 2004), and an anti-histidine tag monoclonal antibody for the V γ 6V δ 1 smTCR (which carries a C δ -terminal histidine tag not present on this V γ 1V δ 6.3 TCR). Mean fluorescence values obtained for each peak are indicated (shown as FL4 for the V γ 6V δ 1 smTCR and FL1 for the V γ 1V δ 6.3 smTCR). The smTCRs when used to double-stain either cell type (right panel) are as bright in mean fluorescence as when used to single-stain (left two panels), indicating that they do not cross-block one another during double-staining.

Fig. 6.

A. Freshly isolated peritoneal macrophages from $B6.TCR\delta^{-/-}$ vs. $B6.\beta2m^{-/-}$ mice were stained with either the $V\gamma1V\delta6.3$ or $V\gamma6V\delta1$ smTCR. Macrophages, first identified by staining with F4/80, were then analyzed for staining with each smTCR, as shown. **B.** Freshly isolated blood monocytes from $B6.TCR\delta^{-/-}$ mice were identified by F4/80 staining and any dead cells were excluded by propidium iodide staining; this population was then examined for co-staining with the indicated smTCR. For splenic macrophages, plastic-adherent spleen cells from C57BL/6 mice were cultured overnight before staining. F4/80 positive, propidium iodide negative cells were then analyzed for co-staining with smTCRs as indicated.