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Macrophages express multiple ligands for γδ TCRs

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ABSTRACT

As only a handful of ligands have been identified, the general nature of the ligands recognized by γδ T cells remains unresolved. In this study, soluble multimerized γδ T cell receptors (smTCRs) representing the TCRs of two γδ 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 lipopolysaccharide (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 γδ T cells in mice and humans, expression of these smTCR-defined ligands does not depend on β2-microglobulin, suggesting that they are not MHC class I or class I-like molecules.

1. Introduction

γδ 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 γδ TCR and a coherent theory of ligand recognition remain elusive.

Despite this, some ideas about ligand recognition by γδ T cells and the nature of γδ TCR ligands have gained broad acceptance. Because of structural similarities between the putative ligand-binding-sites of the γδ TCRs and the BCR (Rock et al., 1994), and because several cell surface-expressed ligands for γδ 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 γδ TCRs has been postulated to have features in common with that of immunoglobulin molecules. Conversely, this concept is difficult to reconcile with the observed stimulation of certain human γδ 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 γδ T cells can be stimulated in many different ways (Bonneville and Scotet, 2006). However, recognition of IPP may more resemble antigen recognition by the αβ 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 γδ 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 nonclassical 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 γδ 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 Dδ2 element when expressed in one of three possible reading frames. In contrast, no contributions from the γ chain were seen,

Abbreviation: smTCR, soluble multimeric T cell receptor.

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and only one amino acid was supplied by Vδ, a residue that could alternatively be encoded by Dδ1, or by appropriately placed N or P nucleotides in the rearranged δ gene. The interaction of this γδ TCR with its ligand contrasts markedly to that typical of αβ 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 αβ TCR bound to peptide/MHC complex. Further examples are needed in order to determine whether or not this is typical of γδ 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 γδ TCR.

Towards this end, we have taken the approach of generating soluble multimeric γδ 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 γδ 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 γδ T cells in mice, that many immortalized cell lines express unknown ligands for γδ 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 γδ T cells in mice, suggesting that they express at least two different ligands for γδ T cells, even in untreated mice. Because of changes in the smTCR staining patterns noted during an infection of mice with the bacterium *Listeria 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 γδ T cells do not require expression of β2-microglobulin, which is typically associated with class I and class I-like MHC molecules.

2. Materials and methods

2.1. Mice

Fully backcrossed C57BL/6 background mice homozygous for a genetically inactive TCR-δ constant region gene [B6.TCRδ−/− 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.FcRγ−/−, B6.β2-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.

2.2. 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 h, to allow the macrophages to adhere to tissue culture flasks or plates. Any nonadherent cells were then poured off. Next, the bound macrophage-enriched population was removed from the plate by culturing them for 30 min 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.

2.3. In vivo stimulation of macrophages

Mice were infected with *L. 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.

2.4. In vitro stimulation of macrophages

Fresh peritoneal resident macrophages were isolated and maintained at 37 °C in a 10% CO_2 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 μg/ml. Tripalmitoyl-*S*-glycerylcysteine (Pam3Cys), a TLR2 agonist, was the kind gift of Dr. Ross Kedl, University of Colorado Denver, 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.

2.5. 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 min 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 two flasks per spleen, and incubated for 3 h at 37 °C in a 10% CO_2 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.

2.6. 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 μg/ml of a purified rat monoclonal antibody against mouse CD16/CD32 [clone 2.4G2 (Unkeless, 1979)] prepared in our laboratory, plus 20–40 μg/ml mouse IgG (Jackson ImmunoResearch, West Grove, PA), for 45 min at 4 °C. Affinity-purified γδ smTCRs were generated in our laboratory after the method of Crawford et al. (2004), and used to stain cells as previously described (Aydintug et al., 2004), using a TCR concentration of 30 μg/ml. Unless otherwise indicated in the figure legends, a low-level biotinylated anti-Cδ "core" monoclonal antibody [clone 403A.10 (Itohara 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,