Review

Unraveling the Mystery of γδ T Cell Recognizing Lipid A

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Traditionally, the materials which are regarded as antigens recognized by γδ T lymphocytes are protein and carbohydrate, not nucleic acid or lipid. Recently, it has been demonstrated that γδ T cells can recognize lipid A and directly induce immune responses that involve CD1 (cluster of differentiation type 1) family and Toll like receptors (TLRs). This is a review about the interacting-mechanism, immunological effect and clinical application of them. *Cellular & Molecular Immunology*. 2005;2(5):359-364.

Key Words: CD1, TLR, anti-bacterial, anti-tumor, anti-virus

Introduction

There are three kinds of antigen receptors on lymphocytes: B cell receptors (antibodies), T cell receptors (TCRs) expressed by αβ T cells, and TCRs expressed by γδ T cells. All three kinds of antigen receptors have the potential to form an enormous number of antigen-binding sites. For antibodies and αβ TCRs, it is borne out they are able to respond to almost any antigen or antigenic peptide bound to major histocom- patibility complex (MHC) molecules. For γδ TCRs, however, few ligands have been identified and definite functions for T cells that bear γδ TCRs remain to be elucidated.

It has been identified that γδ T cell can recognize classical MHC molecules (MHC I/II) or nonclassical MHC molecules, such as nonpolymorphic MHC-related CD1 protein (1) and the closely related MHC class I b molecules T22 (2) by TCR. Specific MHC molecule itself is recognized as antigens, whereas the loaded peptides on the membrane do not play any role as ligands. In addition, γδ T cell can recognize non-MHC molecules, for examples, Isopentenyl Pyrophosphate, heat shock protein (Hsp), MICA/B (3), ULBP3 (4), virus protein directly *via* γδ TCR or cooperatively through antigen specific receptor, such as NKG2D (4) without antigen processing and presentation requirements.

Over a decade ago, the pioneering work of Beckman EM et al. revealed the recognition of lipid antigens by CD1-restricted T lymphocytes (5). This discovery enriched the prevailing antigen spectrum that T lymphocytes recognize and also demonstrated the antigen presenting capability of MHC-like molecules. What's more important was that a new antigen-recognition pathway was found, from which a new area of intense research in immunology began (6). To further clarify the mechanism of the interation of γδ T cells with lipid antigens, we summarize the development of scientific research about lipid A-γδ T cell interactions in recent years and wish to provide evidence for defining the precise function of γδ T cells and γδ T cell-based immunotherapies.

The generalization of lipid A and γδ T cell

Lipid A is the membrane anchor of lipopolysaccharide (LPS) moiety and it is the most conservative part and the main component of endotoxin bioactivity. It is phospholipid and comprises of aminoglucose, fatty acid and pyrophosphate. Its backbone is made of two aminoglucoses which get together through pyrophosphoryl bond at β-1,6 site. Many kinds of long chain fatty acid and pyrophosphate respectively link with disaccharide by lipobond and amide bond (Figure 1). Lipid A is amphipathic because of its hydrophilic head and hydrophobic tail. Lipid A is with potent antigenicity and it can activate many kinds of immunocytes and induce both

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Abbreviations: CAT, chloramphenicol acetyltransferase; CD, cluster of differentiation; CDR, complementarity-determing region; CTL, cytotoxic T lymphocyte; DC, dendritic cell; *E.coli, Escherichia coli*; ET, endotoxin tolerance; HCV-LPs, hepatitis C virus-like particles; HIV-1, human immunodeficiency virus type 1; LPS, lipopolysaccharide; LRR, leucine-rich repeats; MHC, major histocompatibility complex; MPL, monophosphoryl lipid A; MTM, mean tumor masses; TCR, T cell receptor; Th1,T helper type 1; TLR, Toll like receptor; TNF-α, tumor necrosis factor-α.

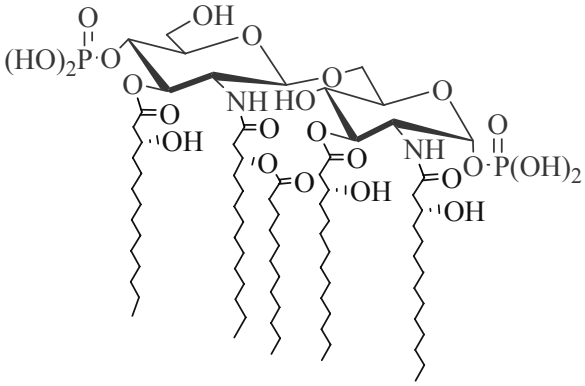

Figure 1. Schematic representation of lipid A structure. The basic structure of lipid A consists of a β-1,6-linked glucosamine disaccharide substituted with two negatively charged phosphates and saturated fatty acids. Different kinds of bacteria are with different length and numbers of saturated fatty acids chains.

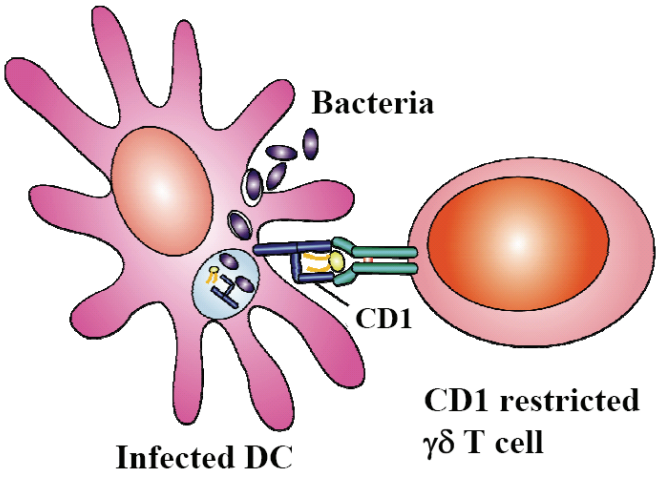

Figure 2. Lipid-CD1 complex interacts with γδ TCR. DCs that are infected with intracellular bacreria present foreign bacterial lipid antigens on the cell surface bound to CD1 molecules. Lipids bind to CD1 molecule by hydrophobic tail chains, exposing the hydrophilic head for interaction with the TCR.

innate and adaptive immune responses.

γδ T cells are a minor population in the peripheral blood compared with αβ T lymphocytes, but constitute a major population among intestinal intraepithelial lymohocytes. γδ T cells are suggested to have a sentinel function by participating in the early host response against bacterial, parasitic and viral infections and in linking the innate and the adaptive immune systems by providing the first barrier until antigen specific αβ T cells have been recruited and expanded. γδ T cells share several features of αβ T cells, natural killer (NK) cells and immunoglobulin, but the way they recognize peptide antigen is more similar to antibodies without MHC-presentation. Futhermore, when interacting with lipid antigens, γδ T cells use similiar mechanisms to that of αβ T cells that need presenting by CD1 molecules on antigen presenting cells (APCs).

The interaction of lipid A and γδ T cells

Lipid A and γδ T cells interact mainly through two pathways. One is CD1/lipid A complex pathway, through which lipid A need presenting by CD1c molecule for γδ T cells recognition; Another is TLR pathway, by which γδ T cells can directly recognize lipid A.

The lipid A/CD1c complex pathway

There are 5 alleles of CD1 in human and their sequences are all different from each other, but the proteins encoded by them possess the same heavy chain spatial structure, i.e., noncovalently connecting with β_2-microglubulin in form of heterodimers. According to the characteristics of structure and tissue distribution, the CD1 family of molecules can be divided into two groups. Group 1 includes CD1a, CD1b and CD1c, while group 2 only consists of CD1d. Group 1 mainly presents foreign lipid/glycolipid antigens to conventional T lymphocytes (5, 7); whereas group 2 CD1 molecule predominantly presents self lipids to specialized NKT cells (8). Lipid A from Gram-negative bacteria can be recognized by CD1c-restricted γδ T lymphocytes. Group 1 CD1-mediated lipid antigen presentation and T-cell activation provide the immune system with a valuable mechanism to efficiently control microbial invasion.

Through analyzing the known crystal structure of CD1 molecules (9-11), the antigen binding domain of CD1 molecule is a pocket consisted of nonpolar or hydrophobic amino radicals which are advantageous for binding lipid antigens and disadvantageous for binding protein antigens. It has been proved that lipids bind to CD1 molecule by hydrophobic alkyle and acyl chains, exposing the hydrophilic sugar, phosphate and other polar functions for interaction with the γδ TCR. γδ TCR comprises three complementarity-determing regions (CDRs). Among them, CDR3 basic residues may form important contacts with the exposed hydrophilic and negatively charged moieties. In turn, the CDR1 and CDR2 loops of γδ TCR are predicted to make contacts with the CD1 helixes, which may act on stabilizing and orienting the TCR diagonally across the longitudinal axis of the CD1 helices, similar to that observed for TCR-MHC complex (11) (Figure 2). Although there is no crystal structure of CD1c-lipidA-γδ TCR interaction complex, from the structural and biochemical character of them we can speculate the similarity.

Furthermore, the molecular interaction of γδ T cell with lipid A, might come down to various adhesion and costimulatory molecules present in both T cells (LFA-2, ICAM-1, and NKG2D) and APCs (LFA-3, LFA-1 and MICA). Together these signals stimulate proliferation, cytokine secretion, cytotoxic lysis, granulysin secretion, and other γδ T cell effector functions (12, 13).

The TLR pathway

Several lines of evidence suggest that some kind of TLR is the cell-surface receptor for LPS, the prototypical activator of NF-κB and other pro-inflammatory responses. Furthermore, several other kinds of LPS receptors have been found, such as CD14, CD11/CD18 integrins, P-selectin and L-selectin. However, CD14 lacks a transmembrane domain, and cytoplasmic domains of CD11/CD18 integrins do not appear to be necessary for the transduction of signals to activate NF-κB in response to LPS. Thus, these receptors may function to transfer LPS to a second receptor that transduces the signal.

Toll is first identified as a protein controlling dorsoventral pattern formation in the early development of *Drosophila* and is shown to participate in anti-microbial immune responses. All the members in Toll family are class I transmembrane proteins, which have parallel extracellular domain including 18-31 leucine-rich repeats (LRR) and the similar intracellular domain consists of 200 amino acids, the latter resemble IL-1R cytoplasmic domain, so it is called Toll/IL-1R homology region. Recently, several mammalian Toll homologues have been identified. TLR genes have diversity and species discrepancy, based on phylogenesis and genetic comple-mentation technology. Now 10 human TLRs with highly conservative structures and functions have been found taking part in innate immune response.

TLR2 and TLR4 are responsible for LPS responses. TLR2 recognizes several atypical types of LPS from *Leptospira interrogans* and *Porphyromonas gingivalis*, in contrast to TLR4, which recognizes LPS from enterobacteria such as *Escherichia coli* and *Salmonella spp.* The properties of the atypical LPS recognized by TLR2 differ structurally and functionally from the enterobacteria LPS recognized by TLR4. In particular, the two types of LPS differ structurally in the number of acyl chains in the lipid A component (Figure 1). TLR2 and TLR4 may differentially recognize these structural variations in LPS (14-16).

TLR4 is a critical component of the heterodimeric receptor complex that transduces signals delivered by LPS of Gram-negative bacteria. CD14, a molecule selectively expressed by monocytes and granulocytes, and MD-2 are also involved in LPS-mediated signaling. C3H/HeJ mice which bear mutations in the *Tlr4* gene exhibit defective LPS signaling, and TLR4 knockout mice are selectively impaired in their ability to recognize Gram-negative bacteria (14). TLR2 plays a pivotal role in the recognition of Gram-positive bacteria and *Mycobacteria*. Pathogen recognition by TLR2 is strongly enhanced by CD14. TLR2 knockout mice are selectively impaired in their ability to recognize Gram-positive bacteria. But Yasuji Mokuno found that the purified γδ T cells from C3H/HeN and C3H/HeJ mice expressed a canonical TCR repertoire encoded by Vγ6-Jγ1/Vδ1-Dδ2-Jδ2 gene segments and proliferated in response to the native lipid A derived from *E.coli* in a TCR-independent manner (17). The lipid A-reactive γδ T cells bearing canonical Vγ6/Vδ1 expressed TLR2 mRNA and TLR2-deficient mice show an impaired increase of the γδ T cells following injection of native lipid A. So γδ T cell also recognize lipid A derived from *E.coli* by TLR2.

The proliferating experiment of γδ T cell induced by lipid A with or without anti TCR-γδ mAb showed that the γδ T cells exhibited a significant proliferative response even without TCR stimulation, and the proliferative response was augmented by TCR stimulation. Furthermore, they found that γδ T cells stimulated with LPS produced a small amount of IFN-γ in the absence of TCR stimulation, compared with those in the presence of TCR stimulation. So, even though there is no confirmed evidence yet, it is possible that CD1d molecules in mice were capable to present lipid antigen for γδ TCR to recognize, which provide evidence for the "traffic hypothesis" of CD1 evolution (18, 19).

The immunological effect of lipid A-reactive γδ T cells

The interaction of lipid A-reactive γδ T cells with immature dendritic cells (DCs)

Immature myeloid DCs express only low levels of major histocompatibility complex (MHC) class II molecules, but express high levels of CD1a, b and c antigen-presenting molecules at the cell surface. γδ T cells can recognize CD1 on the surface of immature DCs in the absence of a specific foreign antigen and stimulate immature DCs to undergo maturation. Upon recognization of CD1c, γδ T cells secrete TNF-α and other factors (20). During host infection, microbial antigens such as LPS provide critical innate signals for DC maturation *via* cell surface TLRs (21) and the resulting mature DC has an inflammatory (interleukin-12 producing) phenotype (22). So in the presence of LPS and CD1-restricted γδ T cells, immature DCs matured and produced bioactive heterodimeric interleukin-12p70. CD1-restricted γδ T cell recognition of immature DCs provides human immune system with the capacity to rapidly generate a pool of mature DCs early during microbial invasion. As antigen presenting cells, DCs may mediate the activation of antigen-specific naïve T cells and stimulate the subsequent adaptive immune response. So the host acquires the ability to successfully defend against microbial products (23).

The interaction of lipid A-reactive γδ T cells with macrophages

The secretion of TNF-α from macrophages is regulated by both priming and triggering signals. Macrophages from mice lacking γδ T cells, which lack the gene encoding the δ chain, produced only small amounts of TNF-α in response to LPS and showed a reduced level of expression of CD14. Pre-incubation of macrophages from TCRδ$^{-/-}$ mice with γδ T cells from their TCRδ$^{+/-}$ littermates restored their capacity to produce TNF-α in response to LPS. Collectively, these results suggest that γδ T cells play a role in priming macrophages to a steady state of activation, which allows macrophages to produce TNF-α when exposed to LPS. The stimulus-function of γδ T cell is performed by producing IFN-γ and TNF-α at the very early phases of infection (24).

Thus, effector function of activated γδ T cells already takes place before they start proliferation.

One striking feature of CD1 molecules is that their cellular localization is not significantly different in immature and mature DCs. Antigen presentation to CD1-restricted T cells by immature and mature DCs seems equally efficient, suggesting that foreign antigen-specific CD1-restricted T cell responses are initiated earlier in the course of an infection than MHC class II-restricted response, and that stimulation of CD1-restricted T cells may occur at peripheral sites of inflammation as well as in lymphoid tissues (25). The data further support the notion that γδ T cells are important mediators of natural immunity against bacterial infection and may bridge the gap between innate and acquired immunity.

Therapeutic significance of lipid A/lipid A-reactive γδ T cells

Based on the increasing understanding of the lipid A recognition and activation mechanisms of γδ T cells, new perspectives for the development of lipid A/lipid A-reactive γδ T cell-based immunotherapies are opened. These are some application researches of them.

Anti-bacteria: In human, large expansions of γδ T cells during infections suggest their importance. γδ T cells are increased from normal levels of 4% of all circulating T cells to a mean of 12%, 14%, 29%, and 57% of all circulating T cells during infection with *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Brucella melitensis* and *Ehrlichia chaffeensis*, respectively.

Both the native lipid A (diphosphoryl lipid A) derived from *E.coli* and MPL (monophosphoryl lipid A) can induce the increase of γδ T cells in mice (17, 26). Administration of MPL results in the redistribution of fully mature DCs in the T cell area of the spleen and enhances the ability of macrophages and B cells to sensitize naïve T cells by inducing the development of Th1 and Th2, which suggests that MPL may induce an antigen-specific primary immune response by provoking the migration and maturation of DCs with the cooperation of cytokines secreted by γδ T cells in the presence of bacterial components (26). Pajak B et al. found that the novel adjuvant OM-174, a lipid A analog, also has the capacity to induce the migration from the periphery to the T cell areas of lymphoid organs and the maturation of murine DCs *in vivo*, which is considered as the initiation of the adaptive immune response (27).

Anti-tumor: Despite the wide range of available therapies, human cancers remain difficult to cure. Evidence for efficient anti-tumoral immune responses to be raised is now widely accepted, and numerous strategies exploiting the host immune systems have been developed. A treatment based on the lipid A-derivative OM-174 has been developed in Larmonier CB's laboratory (28). OM-174 induces the rejection of tumors established by injection of PROb colon cancer cells in syngenetic BDIX rats. Their immunohistochemistry study demonstrated that OM-174 treatment is associated with tumor cell apoptosis. The kinetics of tumor cell apoptosis induced by OM-174, as well as the showed-up order of immune cells in the tumor nodules, were compatible with cell activation and the development of immune response.

To analyze the anti-tumoral effect of LPS upon glioblastoma, Won EK et al. have done some experiments using mice (BALB/c, nude or SCID) which were implanted *s.c.* with DBT glioblastomas and then were treated with LPS (with or without dexamethasone) or with lipid A (29). That was concluded that the LPS-mediated anti-tumoral response against glioblastoma was dependent upon the lipid A subunit of LPS, partially dependent upon T lymphocytes, independent of B lymphocytes, unaffected by dexamethasone and provided partial protection against subsequent challenges with glioblastoma.

ONO-4007 is a new synthetic lipid A analog with low endotoxic activities. It can induce the production of TNF-α in rat hepatoma KDH-8 tumor tissues and bring about the regression of transplanted KDH-8 cells. The production of TNF-α is associated with macrophages.

Anti-virus: Coadministration of liposomal influenza vaccine with MPL resulted in enhanced $CD8^+$ CTL (cytotoxic T lymphocyte) response and IFN-γ production among old mice. These results demonstrate that MPL stimulates CTL and Th1 cytokines (IFN-γ) in aged mice and may serve to reverse age-related $CD8^+$ CTL deficiency and reduce severe influenza disease in elderly human populations.

Immunization with HCV-LPs (hepatitis C virus-like particles), generated in insect cells, elicited both humoral and cellular immune responses in mice (30). To further characterize the HCV-LPs as a vaccine candidate, the effects of adjuvant AS01B (the complex of MPL and QS21), CpG 10105, and the combination of the 2 adjuvants on the immunogenicity of HCV-LPs in AAD mice (transgenic for HLA-A2.1) were evaluated. After injection intramuscularly, all HCV-LP-immunized mice (with or without adjuvant) developed high titers of anti-HCV E1/E2 antibodies. However, antibody titers in mice immunized with HCV-LP plus AS01B, or plus CpG 10105, or plus the combination of AS01B and CpG 10105 were 4, 3, and 10 times higher, respectively, than that of HCV-LP alone. In conclusion, HCV-LP is a promising vaccine candidate against HCV infection and the adjuvants used are potent immune enhancers for this approach.

To enhance immunity induced by DNA vaccination against human immunodeficiency virus type 1 (HIV-1), Sasaki S et al. evaluated the efficacy of MPL (31). BALB/c mice were intramuscularly injected with immunogenic DNA, encoding the *env* and *rev* genes of the HIV-1 (IIIB) strain, formulated with MPL dissolved in different vehicles (MPL in stable emulsion and MPL in aqueous formulation). The sera from mice immunized with the two preparations of MPL revealed 6 to 9 times higher HIV-1-specific IgG titers than the sera from mice immunized without MPL. In virus neutralization tests for IIIB, by p24 assay and antifusion assay of infected MOLT-4 cells, MPL tends to elicit antibody more protective than antibody elicited without adjuvant. MPL also elicited stronger delayed-type hypersensitivity and CTL activity against IIIB compared to DNA alone. HIV-1-

specific IgG subclass analysis showed that MPL tended to facilitate IgG2a production, suggesting enhancement of a predominant Th1 response, and this enhancement may facilitate protective-antibody induction. Furthermore, a chloramphenicol acetyltransferase (CAT) assay was employed to determine whether MPL affected the gene expression process. Interestingly, both MPL preparations reduced CAT activity in the muscle injected with CAT expression vector but increased anti-CAT antibody production. These results indicate that MPL acts as an effective adjuvant for immunogenic DNA injection despite reduced expression of encoding protein in muscle. It can be concluded that MPL has a strong adjuvant effect on DNA vaccination against HIV-1.

Conclusion

Lipid A is the main component of endotoxin bioactivity and the most conservative part of LPS. Lipid A can be recognized by γδ T lymphocytes and induce immune responses which is associated with CD1 family and TLRs. The relationship between CD1 pathway and TLRs pathway has not been clearly known. The CD1c-reactive γδ T cells were cytotoxic and used both perforin- and Fas-mediated cytotoxicity. Moreover, they produced granulysin, an important anti-microbial protein. They induce antigen-specific primary immune response by provoking the migration and maturation of DC, as well as they can prime macrophages to a steady state of activation, which allows them to produce TNF-α when exposed to LPS. Lipid A and lipid A analogs are efficient adjuvants for vaccines to enhance protective immune responses, so they are perspective drugs for tumor and virus-infecting diseases. Furthermore, γδ T cells in inflammatory setting are useful targets for manipulation in vaccine research and immunotherapy (32-35).

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