Different Roles of the CD2 and LFA-1 T-Cell Co-receptors for Regulating Cytotoxic, Proliferative, and Cytokine Responses of Human Vγ9/Vδ2 T Cells

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Abstract

Background: Human Vγ9/Vδ2 T lymphocytes recognize nonpeptidic antigens in a manner distinct from the classical antigen recognition by αβ T cells. The apparent lack of major histocompatibility (MHC) restriction and antigen processing allows very fast responses against pathogenic insults. To address the potential functional requirement for accessory molecules, we investigated the roles of the CD2 and lymphocyte function-associated antigen (LFA)-1 T-cell co-receptors in antigen-induced activities of human Vγ9/Vδ2 T-cell clones.

Materials and Methods: Human peripheral blood Vγ9/Vδ2 T lymphocytes were cloned and their cytotoxicity against Daudi lymphoma was measured by a standard ⁵¹Cr-release assay. The responses of Vγ9/Vδ2 T lymphocytes to nonpeptidic antigens were assessed using DNA synthesis and cytokine ELISA assays. Monoclonal antibodies specific for various molecules with potential T-cell accessory functions were utilized in blocking assays.

Results: All of our Vγ9/Vδ2 T-cell clones displayed the Th1 phenotype. The anti-LFA-1 antibody strongly inhibited the cytotoxicity of Vγ9/Vδ2 T cells against Daudi B-cell lymphoma; whereas, it had no influence on the antigen-induced cytokine release or proliferation. In contrast, antibodies against CD2 and LFA-3 had no effect on the lytic activity of Vγ9/Vδ2 T cells, but strongly inhibited the cytokine release and proliferation. However, the CD2-LFA-3 interaction was not an absolute requirement for the cytokine release and the DNA synthetic activity of antigen-stimulated Vγ9/Vδ2 T cells, since the inhibitory effect could be reversed by addition of exogenous interleukin 2 (IL-2).

Conclusions: These novel observations indicate that the signals generated by different accessory molecules and IL-2 can contribute in an integrated fashion to the regulation of Vγ9/Vδ2 T cells. These interactions may be important for the effectiveness of Vγ9/Vδ2 T-cell responses.

Introduction

Human T cells expressing the Vγ9 and Vδ2 chains of the T-cell receptor (TCR) form a small subset of circulating T cells (1). The identified antigens for these cells belong to the group of nonpeptidic phosphoantigens (2) and alkylamines (3). The recognition of nonpeptidic antigens is mediated through the Vγ9/Vδ2 TCR (4). The structure of these antigens is very different from that of the major histocompatibility (MHC)-peptide complex (5) and, consequently, the serial trigger mode of activation of T cells may not be applicable to the Vγ9/Vδ2 subset (6). The recognition of peptide/MHC complex by the TCR is a key element in the activation of αβ T cells (7). However, the crosslinking of the αβ TCRs alone appears to be insufficient for the full development of T-cell response (8) and many accessory
molecules that contribute to the T-cell activation process have been identified (9).

Lymphocyte function-associated antigen (LFA)-1, a member of the integrin family, is expressed on T cells, B cells, granulocytes, and macrophages (10). Studies with αβ T cells show that LFA-1 can mediate cell-to-cell adhesion and stimulate various intracellular processes (10). LFA-1 is important for target-cytotoxic αβ T-cell interactions (11). LFA-1 can also affect other cellular functions of αβ T-cells, such as apoptosis, proliferation, cytokine production and antigen presentation (10). CD2 belongs to the immunoglobulin superfamily and is expressed on all subsets of T lymphocytes, natural killer (NK) and lymphokine-activated killer (LAK) cells (12). The CD2 ligand, LFA-3, is expressed on human lymphoid cells as a transmembrane-integrated form or phosphoinositol-linked form (13,14). The CD2-LFA-3 interaction delivers an important costimulatory signal and strengthens the adhesion between interacting cells (10,15). It has been demonstrated that antibodies against CD2 can inhibit the antigen-induced proliferation of αβ T cells (10) and the cytotoxicity of CD8 αβ T cells (15).

The signaling through the CD2 molecule is dependent on the TCR-associated ζ chain (16). However, there are interesting differences between αβ T cells and γδ T cells in this regard. For example, at least two monoclonal antibodies specific for different epitopes of CD2 are necessary to induce the activation of αβ T cells (16); whereas, a single CD2 antibody is enough to activate γδ T cells (17). Moreover, the murine γδ TCR associates with a distinct member of the ζ family, the γ chain of the FcεR1 (reviewed in ref. 18) and, therefore, signaling through CD2 may operate differently in γδ and αβ T cells. However, the situation in primates and other species is unclear.

Human Vγ9/Vδ2 T cells recognize a broad spectrum of nonpeptidic antigens (5,19). This recognition requires neither antigen processing nor the expression of MHC or MHC-related molecules (20). Most Vγ9/δ2 T cells can respond to nonpeptidic antigens in the absence of antigen-presenting cells (APCs). However, the presence of APCs can greatly enhance the Vγ9/δ2 T-cell response (20). This suggests that accessory molecules may be involved in these responses. Here we show for the first time that LFA-1 and CD2 have different roles in regulating the Vγ9/δ2 T-cell activities.

Materials and Methods

Cells and Cell Cultures

Cells were cultured in RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. The T-cell clones were generated using the method described elsewhere (21). The clonality was confirmed by gene scan analysis of CDR3 polymorphism (data not shown).

Antibodies, IL-2, and FACS Analysis

The Vδ2-phycocerythrin (PE) antibody (clone B6.1), anti-CD28-PE antibody (clone CD28.2) and their isotype (immunoglobulin G1 (IgG1)-PE, clone MOPC-21) control antibody were purchased from Pharmingen (San Diego, CA). The anti-CD2 (clone TS/18), anti-LFA-3 (clone TS/2/9), anti-LFA-1 (clone TS1/22) and anti-intercellular adhesion molecule (ICAM)-1 (clone P2A4) monoclonal antibodies were purchased from Endogen (Woburn, MA). The unconjugated IgG1 (clone MOPC-21) antibody was also purchased from Pharmingen. All the antibodies used in the blocking study were carrier and preservative-free. In the blocking studies, anti-CD2 antibody and anti-LFA-3 antibody were used at a concentration of 5 μg/ml. The anti-LFA-1 antibody and IgG1 antibody were used at a concentration of 10 μg/ml. In some experiments, recombinant interleukin 2 (IL-2) was used to reverse the inhibitory effects. One hundred units (defined as described; 22) per one ml of culture were used. Analysis of surface antigen expression was performed by flow cytometry (21).

TNF-α Assay

10^4 responder cells were stimulated with the indicated concentrations of isopentenyl pyrophosphate (IPP) purchased from Sigma (Harz, Germany). The supernatants were collected 6 hr later. The concentration of tumor necrosis factor (TNF)-α was determined using the ELISA assay kit from R&D Systems (Minneapolis, MN) following the manufacturer’s instructions. The blocking or control antibodies were added to the culture at the initiation of antigen exposure.

Proliferation Assay

Proliferation assay was performed in 96-well round bottom plates using 10^5/well responder
cells and 10^5/well irradiated LCL721 cells (11,000 rads, ^{137}Cs source) as feeders. 48 hr after IPP stimulation, 1 μCi/well of [methyl-^3H] thymidine (2.6-3.2 TBq/mM, Amersham Pharmacia Biotech, Piscataway, NJ) was added, the cells were harvested after additional 18 hr and DNA synthesis was measured as described (23). The blocking or control antibodies were added to the culture at the initiation of antigen exposure. To assess the produced IL-2 levels, the supernatants were diluted 1:4 with medium, cultured with CTLL-2 cells and their DNA synthetic response was measured as described (23).

Cytotoxicity Assay
Daudi cells were labeled with 100 μCi Na_2[^51]CrO_4 (7.4–18.5 GBq/mg Cr, Amersham Pharmacia Biotech.) for 1 hr at 37°C. After three washes with phosphate-buffered saline (PBS) Daudi cells (10^4/well) were incubated in 96-well round bottom plates with Vγ9/Vδ2 T cells at the indicated effector:target (E:T) cell ratios. The cells were incubated for 6 hr at 37°C, and the specific lysis was determined as described (24). The blocking or control antibodies were added to the Daudi cells just before mixing with the effector Vγ9/Vδ2 T cells.

Analysis of TCR Downregulation
The Vγ9/Vδ2 T-cell clones were stimulated with the indicated concentrations of IPP. Twenty-four hours later, cells were harvested and washed three times with ice-cold PBS before staining. Cells were stained with Vδ2-PE on ice for 30 min, washed three times with ice-cold PBS and analyzed by flow cytometry (FASCan® Flow Cytometer, Becton Dickinson, San Jose, CA) using the Quantum Simple Cellular kit following the instructions of the manufacturer (Flow Cytometry Standards Corp., San Juan, Puerto Rico). Dead cells were excluded by propidium iodide (PI) staining. Data analysis was performed using CELLQuest (Becton Dickinson).

Evaluation of Results
The error bars in the figures indicate calculated standard errors (SE) of the measurement. The statistical significance of differences was calculated by Student’s t-test. A p value of less than 0.05 was considered significant.

Results
Antigen Presenting Cells Are Required for Optimal Vγ9/Vδ2 T-Cell Responses to Antigen
Human Vγ9/Vδ2 T cells recognize nonpeptidic antigens in a MHC-independent manner. Previous studies showed that some Vγ9/Vδ2 T cells can respond to IPP in the absence of APCs (20). To examine the APC requirements in our Vγ9/Vδ2 T-cell clones, six clones were stimulated with IPP in the presence or absence of APCs (irradiated LCL721 cells). Four out of six clones responded to IPP in the absence of APCs (Fig. 1A). Nevertheless, the presence of APCs
greatly enhanced their responses. However, two out of six clones absolutely required the presence of APCs for the antigen-induced DNA synthetic response to occur (Fig. 1B). This observation indicates that, at least in some Vγ9/Vδ2 T cells, surface molecules other than the TCR may be involved in the activation process.

Subsequently, we examined the expression of several of costimulatory molecules, namely CD28, CD2, LFA-1 and LFA-3, by flow cytometry. None of the tested clones express CD28 (Fig. 2), an observation compatible with the results of Gramzinski et al. (25), suggesting that CD28 may not be involved in Vγ9/Vδ2 T-cell responses. All tested clones expressed CD2, LFA-1 and LFA-3 (Fig. 2). Since CD2 and LFA-1 were expressed on all these clones, we investigated their potential roles in Vγ9/Vδ2 T-cell responses.

LFA-1 Is Required for Vγ9/Vδ2 T-Cell Cytotoxicity Against Daudi B-Cell Lymphoma

Vγ9/Vδ2 T cells are able to kill lymphoma targets such as Daudi cells (21). It has been shown that both LFA-1 and CD2 are involved in the cytotoxic activity of CD8 positive T cells and NK cells (10). To determine the role of LFA-1 and CD2 in the lysis of target cells by Vγ9/Vδ2 T cells, we performed standard cytotoxicity assays in the presence of specific antibodies. The anti-LFA-1 antibody effectively inhibited the lysis of Daudi lymphoma targets by Vγ9/Vδ2 T cells (Fig. 3A). However, neither anti-LFA-3 (Fig. 3B) nor anti-CD2 (not shown) had any significant influence on the target lysis.

The CD2-LFA-3 Interaction Is Required for the Vγ9/Vδ2 T-Cell Response to IPP

Some CD2 antibodies can strongly inhibit the αβ T-cell antigenic response (10). We examined whether the relevant antibodies can block the response of Vγ9/Vδ2 T cells to IPP. First, we determined optimal concentrations of anti-CD2 and anti-LFA-3 for blocking (Fig. 4). Vγ9/Vδ2 T cells were stimulated with IPP in the presence of anti-CD2 or anti-LFA-3. Both antibodies strongly inhibited the antigen-induced clones were also stained directly with anti-CD28-PE (solid line) and the corresponding isotype-PE control (dotted line). Very similar results were obtained with all tested clones. The given data illustrate the staining of clone RO1.
Fig. 3. LFA-1 is required for Vγ9/Vδ2 T cell cytotoxicity. The results represent the lysis of Daudi cells by clone RO5. The assays were performed at the described E:T (effector to target cell) ratios in the presence of indicated antibody. (A) Anti-lymphocyte function-associated antigen 1 (LFA-1) inhibited the killing of target cells; whereas, anti-LFA-3 (B) had no influence ($p > 0.05$). For example, at the E:T ratio of 5, the statistical significance of the inhibition by anti-LFA-1 was at the level of $p < 0.03$, when compared with “no antibody present (No AB)” and $p < 0.004$ in comparison with nonspecific control immunoglobulin G1 (IgG1). Very similar results were obtained in repeated experiments with three different clones (RO5, 12.3.4 and 12.3.7). Similar to anti-LFA-3, the presence of anti-CD2 also did not influence the target lysis (not shown). ICAM-1, intercellular adhesion molecule 1.
DNA synthetic response (Fig. 5A). Since TNF-\(\alpha\) is a predominant cytokine produced by antigen-stimulated V\(\gamma\)\(9/\delta\)2 T cells (19), in parallel experiments, we investigated the influence of anti-CD2, anti-LFA-3 and anti-LFA-1 on the production of TNF-\(\alpha\). The TNF-\(\alpha\) production was strongly inhibited by anti-CD2 and anti-LFA-3 (Fig. 5C). In contrast, anti-LFA-1 had negligible influence on both the proliferative response of V\(\gamma\)\(9/\delta\)2 T cells (Fig. 5B) and the TNF-\(\alpha\) production (Fig. 5D).

Similar to \(\alpha/\beta\) T cells, \(\gamma/\delta\) T cells have been categorized as Th1 or Th2 cells according to their cytokine profile (26). We analyzed the secretion of interferon (IFN)-\(\gamma\) by V\(\gamma\)\(9/\delta\)2 T-cell clones. All tested V\(\gamma\)\(9/\delta\) T-cell clones released high levels of IFN-\(\gamma\) upon stimulation with IPP (data not shown), which is consistent with the Th1 phenotype (26).

IL-2 Can Restore the V\(\gamma\)\(9/\delta\)2 T-Cell Response to IPP

There was a possibility that the CD2/LFA-3 inhibitory effect was due to a decreased production of proliferative cytokines, in particular of IL-2. V\(\gamma\)\(9/\delta\)2 T cells were stimulated with IPP in the presence of the anti-CD2 or control antibody. Twenty-four hours later, the supernatants from the cultures were removed and used to culture IL-2-dependent CTLL-2 cells. The supernatants from anti-CD2-treated cells added to the culture at the indicated concentration. The V\(\gamma\)\(9/\delta\) T-cell antigenic response was assessed by measuring DNA synthesis. For example, anti-CD2 effectively inhibited the V\(\gamma\)\(9/\delta\) T-cell response (> 90% inhibition) at a concentration as low as 0.5 \(\mu\)g/ml (\(p < 0.0001\)).

Anti-CD2 and anti-LFA-3 Antibodies Do Not Influence the Antigen-induced TCR Downregulation

The antigenic activation of T cells is accompanied by a significant downregulation of the cell surface TCRs (27). This TCR downregulation process can be modulated by the signal delivered through costimulatory molecules (28). Since anti-CD2 or anti-LFA-3 inhibited the response of V\(\gamma\)\(9/\delta\)2 T cells to IPP, we investigated whether or not the antibodies also influenced the IPP-induced downregulation of TCRs. The level of TCR downregulation in the
Fig. 5. Anti-CD2 and anti-LFA-3 inhibit the Vγ9/Vδ2 T-cell response to a range of IPP concentrations. Vγ9/Vδ2 T cells (clone RO18) were stimulated with the indicated concentration of IPP using irradiated LCL721 cells as antigen-presenting cells. Anti-CD2 or anti-LFA-3 (A, C) strongly inhibited proliferation ($p < 0.02$) and TNF-α release ($p < 0.02$); whereas, anti-LFA-1 (B, D) had no significant effect ($p > 0.05$). The experiments were repeated with 4 different clones (12.3.15, 12.3.12, RO5, RO18).

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presence of anti-CD2 or anti-LFA-3 was comparable to the control (Fig. 8). This indicates that the blocking of CD2-LFA-3 interactions, followed by the functional inhibition of Vγ9/Vδ2 T cells had no substantial influence on the antigen induced TCR downregulation.

Discussion
Human γδ T lymphocytes display potent responses to many antigenic entities of bacterial, protozoal, viral and tumor origin (19,29–31), but the specifics of their protective function and the mechanisms of their regulation remain unknown. The positive effect of APCs on the γδ T-cell response to IPP suggests that accessory molecules may influence the response. The present experiments provide insights into the regulation of Vγ9/Vδ2 T-cell activities and address the role of specific accessory molecules.

It has been shown that both LFA-1 and CD2 are required for CD8+ αβ T cells to kill target cells (11,15). LFA-1 appears to be the primary molecule that mediates the effector-target cell contact and CD2 additionally mediates the adhesion among effector and target cells (32). The data in this study strongly sug-
gest that, similar to αβ T cells, the LFA-1 molecule is important in the contact between the target cell and the cytotoxic Vγ9/Vδ2 T cells. In contrast to αβ T cells, the potential accessory role of CD2 in Vγ9/Vδ2 effector-target interactions is dispensable, since anti-CD2 or anti-LFA-3 have practically no detectable effect on Vγ9/Vδ2 T-cell cytotoxicity against Daudi cells.

Although LFA-1 seems to play an important role in the mechanism of Vγ9/Vδ2 T-cell cytotoxicity, it is not required for Vγ9/Vδ2 T cells to respond to IPP. In contrast, in αβ T cells, LFA-1 can effectively enhance the antigenic response (28). The cell-mediated cytotoxicity is usually dependent on stable effector-target conjugates that require the LFA-1 function (11). Our results imply that stable cell-cell conjugates may not be required for the IPP response of Vγ9/Vδ2 T cells.

Fig. 6. Anti-CD2 treated Vγ9/Vδ2 T cells show decreased secretion of IL-2. Vγ9/Vδ2 T cells (Clone RO5) were stimulated with 20 μM of isopentenyl pyrophosphate (IPP) in the presence of anti-CD2 or the isotype control antibody. Twenty-four hours later, the supernatants were used to stimulate CTLL-2 cells. Anti-CD2 blocked the interleukin-2 (IL-2) secretion (p < 0.0002). CTLL-2 cells cultured with 100 U/ml IL-2 served as a positive control. Two other tested clones (12.3.15, RO18) provided similar results (not shown).

Fig. 7. IL-2 can counteract the CD2/LFA-3 inhibition of the Vγ9/Vδ2 T-cell response to IPP. Vγ9/Vδ2 T cells were stimulated with 20 μM isopentenyl pyrophosphate (IPP) in the presence of indicated antibody. The addition of 100 U/ml IL-2 could restore the secretion of tumor necrosis factor-alpha (TNF-α) in the presence of inhibitory anti-CD2 (A; p < 0.04) or anti-LFA-3 (B; p < 0.006). No detectable levels of TNF-α were induced by the addition of IL-2 alone in the absence of antigenic stimulation.
The mechanism of the inhibition by anti-CD2 antibodies has been studied in αβ T cells, resulting in a suggestion that anti-CD2 inhibits the expression of the functional IL-2 receptor (33). This is clearly not the case in our experiments with Vγ9/Vδ2 T-cell clones. Our results indicate that functional IL-2 receptors are expressed on Vγ9/Vδ2 T cells after stimulation in the presence of anti-CD2. The possible explanation that the expression of IL-2 receptors was caused by in vitro culture of Vγ9/Vδ2 T cells is unlikely, because the Vγ9/Vδ2 T cells could only respond to IL-2 after the IPP stimulation. Thus the anti-CD2 inhibitory effect on the proliferation of IPP-stimulated Vγ9/Vδ2 T cells was more likely to be due to an inadequate secretion of proliferative cytokines. This was further supported by the finding that exogenous IL-2 could reverse the anti-CD2 inhibitory effect on Vγ9/Vδ2 T-cell proliferation (data not shown).

The anti-CD2 antibody used in this study does not induce any intracellular phosphorylation when binding to the CD2 molecule and its effects are attributable solely to the deficit of CD2 signaling caused by the disrupted interactions between CD2 and LFA-3 (34). The ability of IL-2 to restore the TNF-α response of Vγ9/Vδ2 T cells in the presence of anti-CD2 suggests that the CD2 signals induced by the interaction with LFA-3 can be successfully substituted by signaling through the IL-2 receptor to proceed with the antigen-induced cytokine production. Neither the signaling through CD2 nor the signals through the IL-2 receptor are fully understood. Interestingly, both of them can activate PI3 kinase (35,36), which in turn, activates transcription factors important for entering cell cycle (36). Some accessory molecules mediate costimulatory signals that can modulate the αβ TCR downregulation induced by antigenic exposure (28). However, our experiments with Vγ9/Vδ2 T-cell clones indicate that the blocking of the CD2 signal important for the DNA synthetic and cytokine responses does not influence the process of antigen-induced TCR downregulation in these cells.

The interactions of accessory molecules (such as LFA-3 or ICAMs) with their T-cell co-receptors, such as CD2 or LFA-1, appear to check the plasticity of Vγ9/Vδ2 T-cell activation, allowing exquisite control of effector functions. Our study shows that the accessory costimulation of T cells stimulated through the TCR is regulated in a somewhat different fashion in γδ T cells, compared with αβ T cells. Addressing these differences in detail in future experiments may bring a better understanding of the mechanisms by which a successful co-

**Fig. 8. Anti-CD2 or anti-LFA-3 does not influence the antigen-induced T-cell receptor (TCR) downregulation.** Vγ9/Vδ2 T cells were stimulated with the given concentration of isopentenyl pyrophosphate (IPP) using LCL721 cells as antigen-presenting cells. The number of TCRs remaining on the cell surface after stimulation with IPP was quantified with Quantum Sim-
An ordered immune response is induced. The costimulatory signals are most likely to be involved in the expansion phase of the immune response when proliferative cytokines such as IL-2 are the main limiting factors for generating high numbers of antigen-specific T cells. In addition, similar (but negative) signals may be important for the termination phase of the immune response, when the downmodulation of T-cell immunity is necessary to prevent immunopathology or the development of autoimmunity. Thus, the exact knowledge of unique pathways activated by individual classes of accessory molecules and T-cell co-receptors, their cross talk and the regulation of their expression may be crucial when designing novel strategies for vaccination, immunotherapies and the treatment of autoimmune diseases.

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