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Distinct Roles for LFA-1 and CD28 during Activation of Naive T Cells: Adhesion versus Costimulation

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Summary

Efficient T cell activation requires the engagement of a variety of ligand/receptor molecules in addition to T cell receptor (TCR)–major histocompatibility complex (MHC)/peptide interactions. The leukocyte function antigen 1 (LFA-1) and the CD28 glycoprotein have both been implicated in T cell activation. The present study dissects the roles of LFA-1 and CD28 in the activation of naive virus-specific $CD8^+$ T cells. We demonstrate that LFA-1 facilitates T cell activation by lowering the amounts of antigen necessary for T cell activation. In the absence of LFA-1, 100-fold more antigen was required for T cell–antigen-presenting cell (APC) conjugation and all subsequent events of T cell activation, including TCR down-regulation, Ca^{2+}-flux, T cell proliferation, and lytic effector cell induction. Thus, LFA-1 facilitates the functional triggering of TCRs by promoting adhesion of T cells to APCs but does not affect T cell activation otherwise. In contrast, CD28 played an entirely different role during T cell activation. CD28 reduced the number of TCRs that had to be triggered for T cell activation and allowed activation of T cells by low affinity ligands. CD28 but not LFA-1 prevented induction of T cell unresponsiveness after stimulation of TCRs. These results demonstrate that LFA-1 and CD28 exhibit distinct, nonoverlapping ways to influence T cell activation and suggest that the terms costimulation and signal 2 should be revisited.

Introduction

T cell activation is a complex, multistep process involving a multitude of molecules. For full T cell activation, two signals are necessary: an antigen-specific signal 1, mediated by the interaction of T cell receptors (TCRs) with major histocompatibility complex (MHC)/peptide complexes, and a second signal delivered by costimulatory molecules (Lafferty and Woolnough, 1977; Cohn and Langman, 1990; Schwartz, 1992). The most prominent costimulatory molecule is CD28, but other molecules such as lymphocyte function-associated antigen 1 (LFA-1), intercellular adhesion molecule 1 (I-CAM), or 41BB-L have also been suggested to be important for T cell activation (Springer et al., 1987; Seventer et al., 1990; Goodwin et al., 1993; Lenschow et al., 1996; DeBenedette et al., 1997). It is, however, not clear to what extent the different molecules exhibit overlapping functions and how exactly they facilitate or costimulate T cell activation.

CD28 is a disulphide linked homodimeric glycoprotein which is expressed on murine T cells and up-regulated after T cell activation. CD28 interacts with B7-1 and B7-2 on antigen-presenting cells (APCs) (Lenschow et al., 1996). T cell responses are greatly impaired in the absence of the B7-CD28 interaction and, under some circumstances, T cell anergy rather than T cell activation is induced (Schwartz, 1990; Harding et al., 1992; Linsley and Ledbetter, 1993; Kündig et al., 1996; Lenschow et al., 1996). Moreover, blocking of B7 interactions has been shown to inhibit autoimmunity and graft rejection in vivo (Lenschow et al., 1992; Linsley et al., 1992; Turka et al., 1992; Lin et al., 1993; Finck et al., 1994). Nevertheless, high affinity ligands could activate T cells in the absence of CD28 (Shahinian et al., 1993; Lucas et al., 1995; Bachmann et al., 1996; Kündig et al., 1996).

LFA-1 (CD11a/CD18) is a cell adhesion molecule belonging to the integrin family. It is expressed on T and B cells, granulocytes, and macrophages (Springer et al., 1987). The natural ligands for LFA-1 are ICAM-1, 2, and 3, which are expressed on most leukocytes (Springer, 1990). LFA-1 and ICAM-1 have been shown to play an important role in lymphocyte recirculation and inflammation (Sligh et al., 1993; Springer, 1994; Schmits et al., 1996) as a well as in T cell activation. In particular, LFA-1 has been found to enhance the contact between T cells and APCs and facilitate cytotoxic T lymphocyte (CTL)–target cell interactions (Davignon et al., 1981). Interestingly, stimulation of T cells by receptor cross-linking or phorbol esters transiently increases the affinity of LFA-1 for ICAM-1 (Dustin and Springer, 1989; Lollo et al., 1993; Buckley et al., 1997), which subsequently leads to an up-regulated expression of LFA-1 (Springer et al., 1987). Therefore, it is likely that LFA-1 is important to establish the initial T cell–APC contact and after an activation step to strengthen the contact between T cells and APCs. In addition, LFA-1 has also been suggested to act as a costimulatory molecule for T cell activation (Springer et al., 1987; Seventer et al., 1990; Sligh et al., 1993; Schmits et al., 1996; Cai et al., 1996, 1997).

Costimulation is an important parameter for self/nonself discrimination by lymphocytes. The two-signal theory of lymphocyte activation proposes that lymphocytes receiving a TCR-mediated signal 1 in the absence of an additional, accessory molecule-mediated signal 2 are functionally tolerized (Lafferty and Woolnough, 1977; Cohn and Langman, 1990; Schwartz, 1990). Thus, signal 2 is important not only for full T cell activation but also to prevent the induction of unresponsiveness. However,

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these two aspects of signal 2 are not usually distinguished. This is most likely because they are technically difficult to separate, since TCR-mediated and accessory molecule-mediated signals are integrated intracellularly and are difficult to analyze independently. Thus, it would be desirable to have assay systems that distinguish between the following three types of stimuli: triggering of TCR (signal 1), costimulation of T cell activation (signal 2c, c for costimulation), and tolerance induction (signal 2t, t for tolerance).

Several assays were used to assess these three types of stimuli. The number of TCRs functionally triggered by MHC/peptide complexes at a given time is a quantitative assessment of the amount of signal 1 received by a cell. It has been shown that functionally triggered TCRs are rapidly internalized, and reduced TCR levels can be measured by FCM (Valitutti et al., 1995b). The number of internalized TCRs was found to correlate strictly with the efficiency of T cell activation (Viola and Lanzavecchia, 1996; Bachmann et al., 1997a). Thus, TCR downregulation reflects the number of triggered TCRs and therefore is an appropriate way to measure signal 1. In addition, the correlation of signal 1 with T cell activation in the presence or absence of different accessory molecules can distinguish between molecules that facilitate generation of signal 1 and true costimulatory molecules that deliver signal 2c. Induction of T cell anergy in the absence of different accessory molecules after antigenic stimulation was used to measure signal 2t.

Using these principles, the present study analyzed the contribution of LFA-1 and CD28 during T cell activation. To study antigen-specific activation of naive T cells in vitro and in vivo, transgenic mice expressing a TCR specific for the glycoprotein of lymphocytic choriomeningitis virus (LCMV) (Pircher et al., 1989) were crossed with mice deficient for CD28 (TCR×CD28−/−) (Shahinian et al., 1993) or LFA-1 (TCR×LFA−/−) (Schmits et al., 1996). The results show that CD28 is a costimulatory molecule that delivers signal 2c by reducing the amount of signal 1 necessary for T cell activation and is essential for signal 2t. In contrast, LFA-1 does not deliver signal 2c nor signal 2t but facilitates generation of signal 1 in particular at low antigen concentrations by promoting adhesion of T cells to APCs. Thus, CD28 delivers signal 2c by modulating signal 1 and delivers signal 2t while LFA-1 assists T cell activation by facilitating the T cell–APC interaction.

Results

Differential Affects of Ligand Quality versus Ligand Concentration on LFA-1– or CD28-Mediated T Cell Activation

To analyze the importance of LFA-1 and CD28 to induce proliferation of naive T cells, spleen cells from TCR-transgenic mice deficient for LFA-1 (TCR×LFA−/−), CD28 (TCR×CD28−/−), or control littermates (TCR) were stimulated in vitro with splenic APCs pulsed with graded doses of peptide p33. Proliferation was assessed 2 days later (Figure 1A). While TCR×CD28−/− T cells proliferated similarly to control cells, TCR×LFA−/− T cells failed to proliferate at low peptide doses, and the dose response curve was shifted by about 100-fold. Proliferation of TCR×LFA−/− T cells was, however, similar to controls at high peptide concentrations. Similar findings were observed for the induction of cytolytic effector cells (Figure 1B).

When the peptide A4Y, a weak ligand for the transgenic TCR, was used for stimulation, LFA-1–deficient T cells again required approximately 100 times more peptide for similar levels of T cell proliferation (Figure 1C). This response is clearly different from CD28-deficient T cells, which failed to proliferate upon stimulation with peptide A4Y even at high peptide concentrations (Figure 1C) (Bachmann et al., 1996).

Since LFA-1 is expressed both on T cells and APCs, the experiments were repeated with peritoneal macrophages from LFA-1–deficient mice. Absence of LFA-1 on APCs had only a minor effect on T cell proliferation (data not shown). Thus, expression of LFA-1 on T cells was more critical for T cell activation than LFA-1 expression on APCs.

These results show that the absence of LFA-1 and CD28 have significantly different effects on T cell activation. CD28 alters the range of peptides to which a given T cell can respond, while LFA-1 changes the minimal number of ligands required to induce T cell proliferation.

LCM Virus-Specific T Cell Responses in the Absence of LFA-1

LFA-1–deficient mice have been reported to mount normal LCMV-specific CTL responses (Schmits et al., 1996). To evaluate whether LCMV-infected APCs were able to stimulate naive TCR-transgenic T cells in vitro in the absence of LFA-1, spleen cells from LFA-1–deficient mice were stimulated with LCMV-infected macrophages. LFA-1–deficient T cells proliferated efficiently albeit somewhat less compared with control T cells (Figure 2A). This indicated that sufficient LCMV peptide was present on LCMV-infected macrophages to confer T cell proliferation in the absence of LFA-1. To determine whether LCMV infection could stimulate LFA-1–deficient transgenic T cells in vivo, adoptive transfer experiments were performed (Moskophidis et al., 1993; Zimmermann et al., 1996). One million transgenic T cells were transferred into nonirradiated C57BL/6 recipient mice and subsequently infected with LCMV. One week later, splenocytes were isolated and analyzed for the presence of TCR transgenic T cells. Both LFA-1–deficient and control T cells had dramatically expanded and represented the majority of $CD8^+$ T cells in recipient mice (Figure 2B). In addition, lytic activity of transgenic T cells was comparable on EL-4 cells pulsed with the peptide MB6, which is recognized by transgenic T cells but not by endogenous CTLs of the recipient (Bachmann et al., 1997b; Figure 2C). Thus, LFA-1–deficient TCR-transgenic T cells responded strongly to LCMV infection both in vitro and in vivo, since ligand densities achieved during LCMV infection are sufficiently high for LFA-1–independent T cell activation.

In Vivo Activation of T Cells in the Absence of LFA-1 or CD28

In vitro experiments performed thus far had suggested that the absence of CD28 does not influence the amount

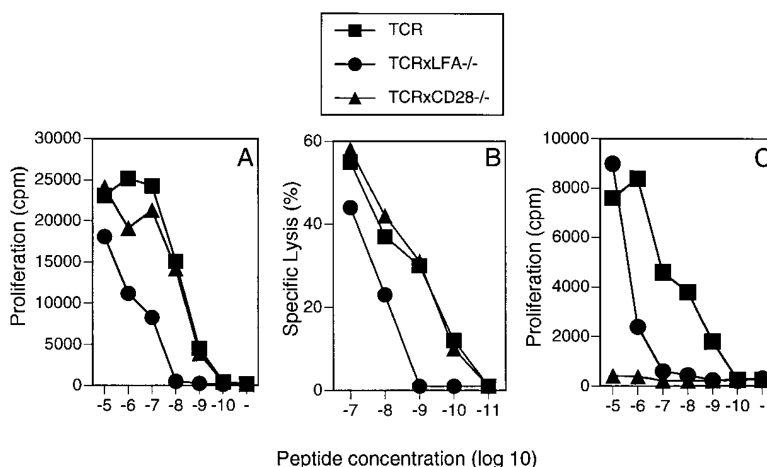

Figure 1. TCR×LFA−/− T Cells Fail to Proliferate at Low Ligand Density While TCR×CD28−/− T Cells Fail to Proliferate after Stimulation with a Low-Affinity Ligand

Spleen cells from control TCR, TCR×LFA−/−, or TCR×CD28−/− mice were stimulated with APCs pulsed with various concentrations of peptide p33.

(A) Proliferation was assessed 48 hr later by [^{3}H]thymidine incorporation.

(B) Or the presence of lytic effector cells was assessed 3 days later on p33-pulsed EL-4 cells.

(C) Proliferative responses of T cells from TCR, TCR×LFA−/−, and TCR×CD28−/− mice were analyzed after stimulation with the low-affinity ligand A4Y. One representative experiment of five (A and B) or three (C) is shown.

of ligand needed for T cell activation, while about 100 times greater p33 concentrations were required for T cell activation in the absence of LFA-1. To extend these observations in vivo, the mutant mice were injected with various doses of p33. Twenty-four hours later, spleen cells were isolated and CD69 expression was assessed on $CD8^+$ T cells expressing the transgenic TCR (Vα2). About ten times more peptide was required for similar CD69 expression levels in cells lacking LFA-1 (Figure 3A). If CD69 expression was assessed in TCR×CD28−/− mice, it was also slightly reduced. Overall, the effects on CD69 expression were limited. However, the induction of lytic effector cells evaluated in the same experiment demonstrated that at least 1000-fold more peptide p33 was required to induce CTL in TCR×LFA−/− mice, while T cells from TCR×CD28−/− mice responded normally (Figure 3B). This confirms earlier data that expression of activation markers such as CD69 can be uncoupled from T cell proliferation and effector cell induction (Bachmann et al., 1996).

LFA-1 but Not CD28 Facilitates Generation of Signal 1

The in vitro and in vivo experiments suggested that LFA-1 but not CD28 altered the peptide concentration required for T cell activation. However, these studies did not address whether LFA-1 directly altered TCR-mediated signals. Functionally triggered TCRs are internalized shortly after stimulation (Meuer et al., 1984; Valitutti et al., 1995b). TCR down-regulation therefore can be used to assess the number of functionally triggered TCRs and thus the amount of signal 1 (Viola and Lanzavecchia, 1996; Bachmann et al., 1997a). To assess whether LFA-1 altered the intensity of signal 1, splenocytes from TCR×LFA-1−/− or TCR×CD28−/− and control TCR mice were incubated with peptide-pulsed macrophages. Expression levels of TCRs were assessed 5 hr later (Figure 4). As described previously, strong TCR down-regulation occurred on control T cells (Bachmann et al., 1997a). The extent of down-regulation was largely independent of the peptide concentrations between 10^{-6} and 10^{-9} M; at 10^{-10} M, down-regulation was less pronounced and was not detectable at lower concentrations. Identical results were obtained with CD28-deficient T cells, indicating that the absence of CD28 does not alter TCR internalization. Interestingly, LFA-1–deficient T cells failed to modulate TCR expression at low peptide concentrations. This indicated that LFA-1 was essential for a functional signal 1 at low ligand density. In contrast LFA-1 was dispensable for efficient TCR down-regulation at high peptide concentrations. Absence of CD28 had no influence on the kinetics of TCR down-regulation. If assessed with APCs pulsed with a high concentration of p33, absence of LFA-1 also did not alter the kinetics of TCR internalization (data not shown).

Absence of CD28 Does Not Interfere with TCR Down-Regulation after Stimulation with Low-Affinity Ligands

TCR×CD28−/− T cells fail to proliferate after stimulation with the low affinity ligand A4Y (Figure 1B) (Bachmann et al., 1996). To assess whether A4Y failed to stimulate TCR internalization and hence signal 1 in the absence of CD28, T cells from the different mouse strains were activated with A4Y. Interestingly, the peptide A4Y-mediated TCR internalization in CD28-deficient T cells was as efficient as with control T cells (Figure 5). This demonstrated that the failure of A4Y to induce proliferation of CD28-deficient T cells was not due to a failure to trigger TCR down-regulation. Although TCR down-regulation was markedly reduced in LFA-1–deficient T cells, it was nevertheless clearly observable at high A4Y concentrations (Figure 5) and sufficient to stimulate T cell proliferation (Figure 1).

LFA-1 Is Required for Ca^{2+}-Flux at Low Ligand Density

LFA-1 was essential for induction of TCR down-regulation at low ligand concentrations. Since TCR down-regulation is an important parameter for T cell activation, one would predict that an absence of LFA-1 would interfere with early events of T cell activation, in particular at low ligand densities. Therefore, we investigated the importance of LFA-1 for the induction of Ca^{2+}-flux. Peptide p33 induced long-lasting Ca^{2+}-flux in control TCR-transgenic T cells at concentrations as low as 10^{-10} M (Figure 6A). In contrast, Ca^{2+}-flux was absent in TCR×LFA−/− T cells at low peptide concentrations (Figure 6B). Approximately 100 times more p33 was required

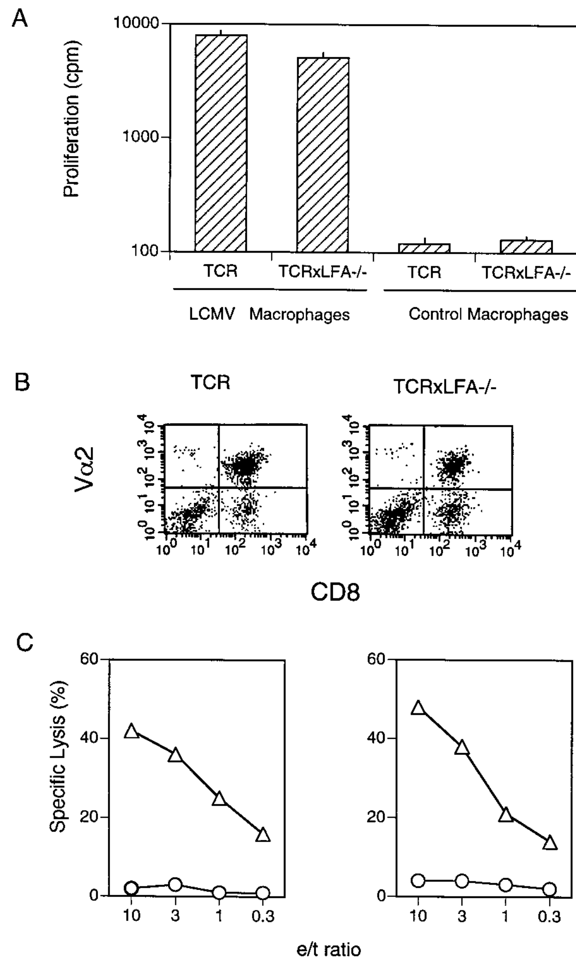

Figure 2. Functional LCMV-Specific T Cell Responses in the Absence of LFA-1

(A) Spleen cells from control TCR or TCR×LFA−/− mice were stimulated with LCMV-infected macrophages. Proliferation was assessed 48 hr later by [^{3}H]thymidine incorporation.

(B) One million spleen cells from control TCR or TCR×LFA−/− mice were adoptively transferred into normal, nonirradiated C57BL/6 mice, which were subsequently immunized with LCMV (200 pfu). Seven days later, the presence of transgenic CD8$^+$ T cells expressing Vα_2 was assessed in recipient mice by FCM analysis.

(C) Presence of TCR-transgenic effector cells was assessed in recipient mice by a ^{51}Cr-release assay. Peptide MB6 (triangles), which is specifically recognized by TCR-transgenic T cells but not by endogenous T cells of recipient mice, or a control peptide (circles) was used to pulse the target cells. One representative experiment of two is shown.

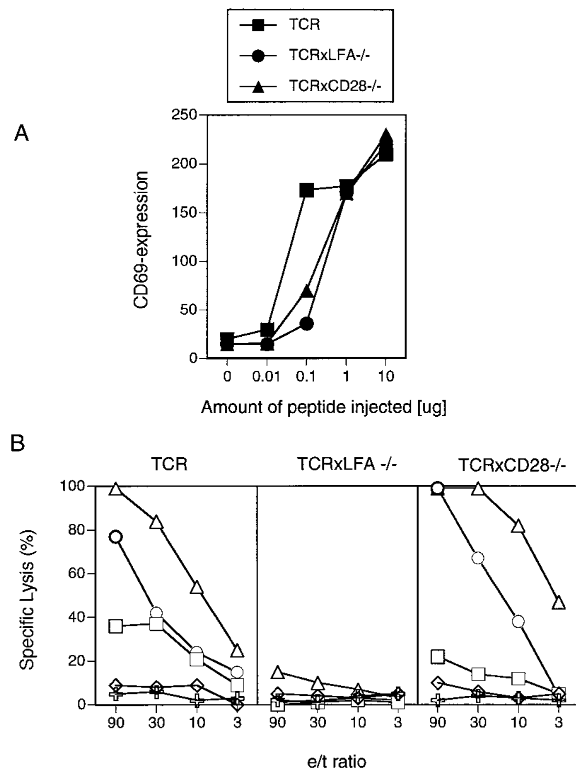

Figure 3. In Vivo Activation of T Cells in the Absence of LFA-1 or CD28

Control TCR, TCR×LFA−/−, and TCR×CD28−/− mice were immunized with various doses of peptide p33.

(A) Expression of the activation marker CD69 was assessed 24 hr later, and the mean fluorescence intensity is shown for CD8$^+$ T cells.

(B) Presence of effector cells was assessed 24 hr after injection of 10 μg (triangles), 1 μg (circles), 0.1 μg (squares), 0.01 μg (diamonds), or no peptide p33 (crosses) on p33 pulsed target cells. Each line represents the mean of two mice. One representative experiment of two is shown.

to induce Ca^{2+}-flux in TCR×LFA−/− T cells, which is consistent with the results of the TCR down-regulation assays (Figure 4). Thus, LFA-1–deficient T cells required about 100 times more peptide for TCR down-regulation, Ca^{2+}-flux, and T cell proliferation. This finding suggests that the LFA-1/I-CAM interaction is essential for the T cell APC interaction at low ligand densities and for the generation of a functional signal 1, but absence of LFA-1 can be compensated for by increasing the ligand density.

To assess whether LFA-1 may exert its effect by increasing T cell–APC adhesion at low peptide concentrations, the percentage of T cells forming complexes with APCs during the Ca^{2+} experiment was determined. Again about 100 times more peptide p33 was required in the absence of LFA-1 to induce an increase in T cell–APC complex formation (Figure 6C). This result demonstrates that LFA-1 exerts its accessory function for T cell activation primarily by facilitating cell adhesion.

CD28 but Not LFA-1 Is Essential for Signal 2t

The two-signal theory of T cell activation predicts that T cells receiving a functional signal 1 in the absence of signal 2 undergo anergy. As shown here (Figure 7) and previously in the LCMV model (Kündig et al., 1996), CD28-deficient T cells stimulated in vivo with peptide become anergic, confirming that CD28 is an important mediator of the second signal (Harding et al., 1992; Linsley and Ledbetter, 1993). To determine whether LFA-1 plays a similar role, TCR transgenic mice deficient for LFA-1 or CD28 were injected with peptide p33 (1 μg). Three days later, spleen cells were isolated and stimulated in vitro with peptide-pulsed APCs in the presence or absence of exogenous IL-2 (ConA supernatant). Proliferation was assessed 2 days later (Figure 7). p33 primed control and LFA-1–deficient T cells proliferated

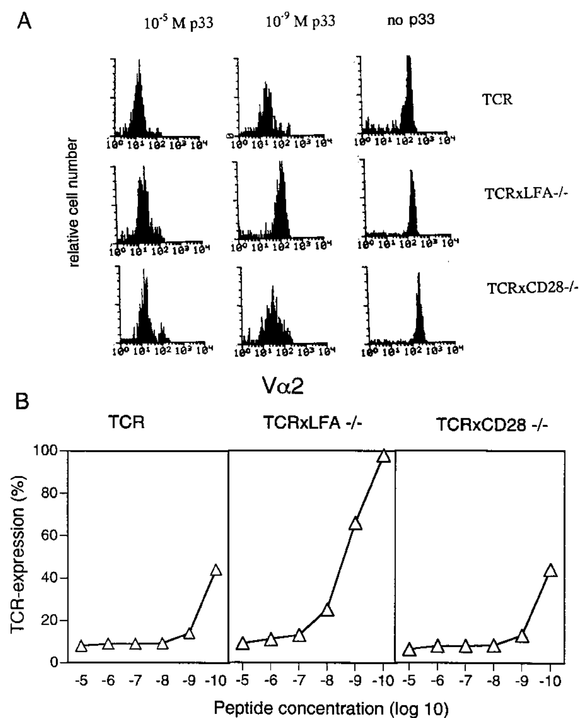

Figure 4. Reduced Concentration-Dependent TCR Down-Regulation after Peptide Stimulation in the Absence of LFA-1 but Not CD28

Spleen cells from control TCR, TCR×LFA−/−, or TCR×CD28−/− mice were stimulated with macrophages pulsed with various concentrations of peptide p33, and TCR expression was assessed 5 hr later for CD8+ T cells by FCM.

(A) Representative profiles are shown for the different T cells.

(B) TCR expression is shown as percent of control as a function of peptide concentration used to pulse the macrophages. One representative experiment of three is shown.

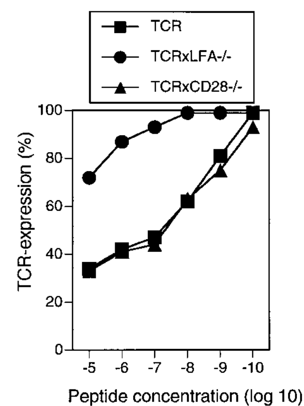

Figure 5. Absence of CD28 Does Not Alter TCR Down-Regulation Mediated by a Weak Agonist

Spleen cells from control TCR, TCR×LFA−/−, or TCR×CD28−/− mice were stimulated with macrophages pulsed with various concentrations of peptide A4Y, and TCR expression was assessed 5 hr later for CD8+ T cells. TCR expression is shown as percent of control as a function of the peptide concentration used to pulse the macrophages. One representative experiment of three is shown.

comparably to unprimed control T cells. In contrast, CD28-deficient T cells failed to proliferate in the absence of IL-2. Addition of IL-2, however, rescued proliferation to control levels, characterizing the observed unresponsiveness as anergy (Figure 7). Thus, CD28, but not LFA-1, was crucial for a functional signal 2t.

Discussion

To dissect the underlying mechanisms that govern efficient T cell activation, we compared the roles of LFA-1 and CD28 during T cell stimulation. Our results indicate that CD28 and LFA-1 exhibit different, nonoverlapping roles for T cell activation.

The Role of LFA-1 for T Cell Activation: Facilitation of Signal 1

It has been suggested that the LFA-1/I-CAM interaction is critical for full T cell activation (Davignon et al., 1981; Springer et al., 1987; Seventer et al., 1990; Sligh et al., 1993; Cai et al., 1996, 1997). This view is in contrast with the finding that LFA-1–deficient mice mounted normal LCMV-specific immune responses (Schmits et al., 1996). Our results offer an explanation for the discrepancy. Using LCMV-infected APCs or high concentrations of the natural peptide of LCMV presented on APCs, efficient T cell proliferation and CTL-effector function could be induced in vivo and in vitro. The extent of proliferation and the efficiency of target cell lysis by LFA-1–deficient T cells was similar to control cells. At lower ligand densities, however, LFA-1 proved pivotal. Under these conditions, T cells failed to proliferate and differentiate to effector cells in vitro in the absence of LFA-1. Stimulation with low affinity peptides yielded similar results. Thus, LFA-1 shifted the dose response curve of T cells; LFA-1 was essential at low ligand concentrations, but normal T cell responses could be observed at high ligand concentrations both with high and low affinity ligands. This finding differs from a recent report, where LFA-1 was found to be crucial for the induction of T cell proliferation, independent of the ligand density (Cai et al., 1997). Although there is not an obvious explanation for this discrepancy, the use of I-CAM–transfected insect cells rather than macrophages for T cell stimulation in the earlier study may be responsible for the difference. During physiological T cell activation, additional adhesion molecules such as CD2 are present that may assist LFA-1 during the T cell–APC interaction (Davis and vanderMerve, 1996). These additional molecules were probably missing on the transfected insect cells, possibly explaining the absolute requirement for LFA-1 during T cell activation in this system.

To analyze the role of LFA-1 during T cell activation in more detail, TCR internalization and induction of Ca^{2+}-flux was assessed after peptide specific stimulation. At high peptide concentrations, the extent and kinetics of TCR internalization and increases in free intracellular Ca^{2+} were not affected by the absence of LFA-1. In contrast, at low ligand density, both functions were strongly impaired, indicating that the TCR-mediated signal 1 is reduced at low ligand density in the absence of LFA-1. Together with our finding that LFA-1 is critical for the formation of T cell–APC complexes at low peptide densities (Figure 6), these results suggest that LFA-1

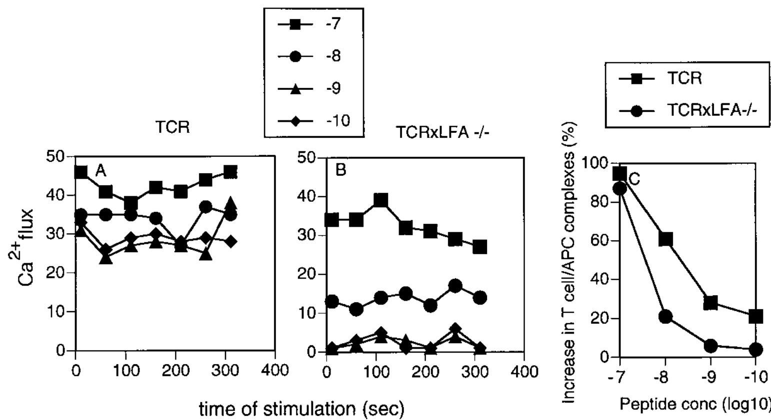

Figure 6. LFA-1–Deficient T Cells Fail to Increase Intracellular Ca^{2+} and to Form Complexes with APCs Pulsed with Low Concentrations of p33

(A and B) Spleen cells from TCR-transgenic control (A) or LFA-1–deficient (B) mice were centrifuged with macrophages pulsed with various concentrations of peptide p33. Three minutes later, cells were resuspended and free intracellular Ca^{2+} was measured (by the Indo-1 405/485 ratio) for the indicated time.

(C) The percentage of $CD8^{+}$ T cells forming complexes with APCs was assessed during the experiment shown in (A and B). One representative experiment of two is shown.

facilitates generation of signal 1 by enhancing T cell–APC adhesion. Thus, LFA-1 largely contributes to T cell activation by physicochemical rather than biochemical parameters.

The Role of CD28 for T Cell Activation: Modulation of Signal 1

Induction of T cell proliferation after stimulation with p33 was only marginally affected by the absence of CD28. However, unlike LFA-1–deficient T cells, the CD28-deficient T cells failed to proliferate after stimulation with the low-affinity ligand A4Y regardless of the concentration. Thus, the roles of LFA-1 and CD28 during T cell activation are distinct. LFA-1 is essential for T cell activation at low ligand densities, while the affinity of the peptide for the TCR plays a minor role. In contrast, CD28 is critical for T cell activation at low ligand affinity, independent of the ligand density. Thus, LFA-1 is critical for low avidity T cell–APC interactions, while CD28 is pivotal for low affinity TCR-MHC/peptide interactions.

Kinetics and the extent of TCR down-regulation after p33 stimulation were normal in the absence of CD28, consistent with the observed normal T cell activation. Peptide A4Y induced a reduced, but nevertheless significant, TCR down-regulation on normal T cells. Although A4Y failed to trigger T cell proliferation in the absence of CD28, TCR down-regulation after stimulation with peptide A4Y was similar in the presence or absence of CD28. Thus, CD28 does not directly alter signal 1. However, it apparently influences the number of TCRs that need to be triggered for T cell activation: the reduced number of triggered TCRs with the low-affinity peptide A4Y induced T cell activation in the presence, but not in the absence, of CD28. These observations using naive T cells are consistent with an earlier study using T cell clones, where CD28 was found to influence the minimal number of TCRs necessary for activation of the T cell clones (Viola and Lanzavecchia, 1996). Interestingly, p33-induced T cell proliferation was independent of CD28 also at low ligand concentrations. This observation is explained by the finding that even very low concentrations of p33 induce massive TCR down-regulation that is apparently sufficient for CD28-independent T cell activation.

CD28 thus does not change the amount of signal 1 generated during the T cell–APC interaction but modulates the amount of signal 1 required for T cell activation. CD28 may therefore be considered a true costimulatory molecule that delivers signal 2c by modulating signal 1.

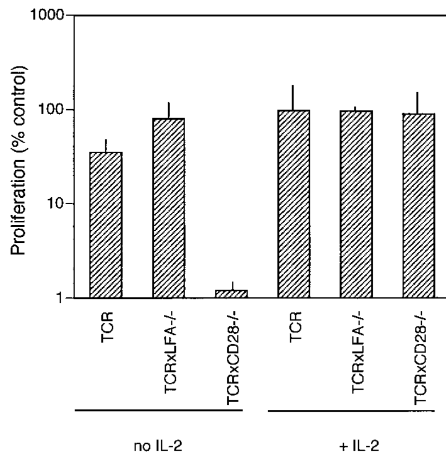

Figure 7. Absence of LFA-1 Does Not Lead to T Cell Anergy In Vivo

Control TCR, TCR×LFA−/−, or TCR×CD28−/− mice were injected with the peptide p33 (1 μg). Three days later, spleen cells were isolated and stimulated with p33-pulsed APCs in the presence or absence of IL-2. Proliferation was assessed 48 hr later. Proliferation is expressed as percent of proliferation of spleen cells from uninjected mice. One representative experiment of two is shown.

CD28 but Not LFA-1 Is Critical for Signal 2t

According to the two-signal theory of T cell activation, T cells receiving an antigen-specific signal 1 in the absence of the second signal are rendered unresponsive (Lafferty and Woolnough, 1977; Cohn and Langman, 1990; Schwartz, 1990). It is important to distinguish this type of signal from others influencing T cell activation. If p33 was injected into TCR×CD28−/− mice, the specific T cells became functionally unresponsive within 3 days, confirming earlier observations (Kündig et al., 1996). TCR×LFA−/− T cells, however, were not anergized at this time point and responded comparably to T cells from uninjected TCR×LFA1−/− mice. This indicated that a functional signal 2t could be generated in the absence of LFA-1 but not in the absence of CD28. It is important to point out that CD28 may not always

be absolutely necessary for signal 2t. Immunization of CD28-deficient mice with LCMV or vaccinia virus does not induce T cell unresponsiveness but leads to long-term T cell memory (Kündig et al., 1996). Thus, during a viral infection but not following peptide injection, an alternative interaction may generate signal 2t and prevent induction of long-lasting unresponsiveness. One possibility for signal 2t replacement may be a sustained signal 1 after viral infection (Kündig et al., 1996). In addition, induction of inflammatory cytokines probably also plays a role.

T Cell Activation as a Multistep Process

T cell activation may be divided into at least three phases. First, the T cells and APCs make contact. In a second phase, TCRs get triggered and are internalized. In a third phase, which may overlap with the second phase, T cells proliferate, differentiate to various effector cells, and/or are tolerized. Different accessory molecules contribute to T cell activation during these three phases. In the first phase, adhesion molecules such as LFA-1 and CD2 are of critical importance, in particular at low ligand concentrations. After the initial contact has been made, costimulatory molecules influence T cell activation by delivering signal 2c. They may change the number of TCRs that have to be triggered for T cell activation, as shown here for CD28. Other costimulatory molecules may alter the kinetics of TCR-triggering during the second phase. This is not the case for CD28 but it is possible that such a role will be found for 41BB or yet unknown molecules. During the third phase, different parameters will influence the outcome of T cell activation. The total number of triggered TCRs will influence the types of effector functions induced, and the presence or absence of signal 2t will decide whether or not T cell unresponsiveness is induced. Thus, adhesion molecules are critical for the first phase, costimulatory molecules may modulate the second phase, and molecules delivering signal 2t are crucial for the third phase.

Conclusion

T cell activation generally requires accompanying signals in addition to the antigen-specific TCR-MHC/peptide interactions. Molecules that deliver these additional stimuli are generally termed costimulatory molecules and are often considered equal to signal 2 of the two-signal theory. This study demonstrates that this view is a massive oversimplification and that different accessory molecules alter T cell activation in distinct, nonoverlapping ways. While the functional effect of the additional stimuli may be similar, e.g., increased proliferation, the mechanism by which this effect is achieved is fundamentally different. The two molecules studied here, LFA-1 and CD28, have three distinct effects on T cell activation. LFA-1 facilitates generation of signal 1, in particular at low ligand densities, but does not deliver signal 2c nor signal 2t. In contrast, CD28 modulates signal 1 by reducing the minimal number of TCRs that have to be triggered for T cell activation and is essential for a functional signal 2t.

Experimental Procedures

Mice and Viruses

Transgenic mice expressing a T cell receptor specific for peptide LCMV $GP_{33\text{-}41}$ presented in association with $H\text{-}2D^b$ (Pircher et al., 1989) and gene-targeted mice lacking expression of LFA-1 (Schmits et al., 1996) and CD28 (Shahinian et al., 1993) have been described previously. The LCMV isolate WE was provided by Dr. R. M. Zinkernagel, Zürich, Switzerland and grown on L cells at a low multiplicity of infection.

Peptides

Peptides p33 (KAVYNFATM), A4Y (KAVANFATM), and MB6 (KAVVNIATM) were generated at the Amgen Institute (Boulder, CO) by a solid phase method using the Fmoc/tBu-based protocol on an ABI-431 instrument. The crude product was purified by high performance liquid chromatography (HPLC). p33 defines the major CTL epitope on the LCMV GP in the $H\text{-}2^b$ haplotype (Pircher et al., 1990). To prevent disulphide bonds, the C-terminal C has been replaced by an M (Pircher et al., 1993).

Induction of LCMV-Specific Primary In Vitro T Cell Responses

Spleen cells (1×10^5 cells/well) from naive mice expressing a transgenic receptor specific for LCMV GP (p33) were stimulated in vitro with macrophages or splenic APCs (10^4/well or 5×10^4/well, respectively) pulsed with various amounts of peptides in 96-well plates. Proliferation was assessed 48 hr later by means of [^{3}H]thymidine incorporation. To assess induction of lytic effector cells, spleen cells from TCR-transgenic mice (3×10^6/ml) were stimulated with peptide-pulsed macrophages (10^5 cells/well). Three days later, spleen cells were resuspended in 1/2 ml of medium per culture well, and serial 3-fold dilutions of effectors were performed (results are shown for a 1/3 dilution) and tested in a conventional ^{51}Cr release assay using p33 pulsed (10^{-5} M) EL-4 cells as target cells (Bachmann, 1997).

In Vivo Activation of T Cells

TCR-transgenic mice were injected intravenously with various doses of peptide p33. Twenty-four hours later, spleen cells were tested in ^{51}Cr-release assays on p33-pulsed target cells. Cells were also stained for expression of CD8 (FITC, Cedarlane, Ontario, Canada), $V\alpha 2$ (PE, Pharmingen, San Diego, CA), and CD69 (Biotin, Pharmingen followed by Streptavidin-RED670, GIBCO, BRL, Grand Island, NY) and analyzed by FCM.

In Vivo Expansion and Effector Cell Induction

Spleen cells from TCR-transgenic LFA-1 or CD28–deficient and control mice (10^6 cells) were adoptively transferred into normal C57BL/6-recipient mice. One hour later, mice were challenged with LCMV. At the indicated time points, spleen cells were harvested and stained for CD8 (PE, Pharmingen) and transgenic $V\alpha 2$ expression (FITC, Pharmingen), and the presence of TCR-transgenic cells was assessed by FCM analysis.

To assess cytolytic effector function, spleen cells were tested in ^{51}Cr-release assays using peptide-pulsed EL-4 target cells. To distinguish endogenous CTL activity of the C57BL/6 recipient mice from CTL activity of the transferred TCR-transgenic T cells, EL-4 cells pulsed with peptide MB6 were used as target cells. MB6 is only recognized by the TCR-transgenic T cells but not by the polyclonal C57BL/6 T cells (Bachmann et al., 1997b).

Ca^{2+}-Flux

Spleen cells were loaded with Indo-1 (10 μM) for 1 hr at 37°C in Iscove's modified Dulbecco's medium supplemented with 2% FCS. Cells were stained for CD4 and B220 expression, and $\text{Indo-1}^+ \text{CD4}^- \text{B220}^-$ cells exhibiting a large forward scatter corresponding to T cell–APC duplexes, were analyzed with a FACS Vantage (Becton Dickinson) and Cell Quest-Software, using a ion laser (Innova Enterprise, Coherent, Santa Clara, CA) optimized for UV argon ions, set for 355-nm excitation at a power setting of 50 mV. For stimulation of T cells, peritoneal macrophages were pulsed with various concentrations of p33 for 1 hr. Macrophages (4×10^6/ml) were mixed with

spleen cells (1×10^7 cell/ml) at 4°C, centrifuged, and warmed up to 37°C for 3 minutes. Cells were gently resuspended and immediately analyzed. Results are expressed as mean channel of 405/485 nm (violet/blue) ratio less the mean channel of violet/blue ratio of T cells stimulated with unpulsed APCs (Viola and Lanzavecchia, 1996). To determine the number of T cells forming complexes with APCs, the frequency of Indo-1^+CD4^-B220^- cells with a large forward scatter (corresponding to the forward scatter of the T cells conjugated to APCs) was determined (Valitutti et al., 1995a).

Induction of TCR Down-Regulation

Spleen cells from TCR-transgenic mice (10^5/well) were mixed with peptide-pulsed macrophages (2×10^5/well), centrifuged, and incubated at 37°C (5% CO_2) in IMDM supplemented with 10% FCS in round bottom 96-well plates. At the indicated time points, cells were harvested and stained for CD8 (PE, Pharmingen) and Vα2 (FITC, Pharmingen), and Vα2 expression is shown for CD8^+ T cells.

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