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Coordinate Regulation of T Cell Activation by CD2 and CD28

Jonathan M. Green, Vladimir Karpitskiy, Stephanie L. Kimzey, and Andrey S. Shaw

T cell activation requires co-engagement of the TCR with accessory and costimulatory molecules. However, the exact mechanism of costimulatory function is unknown. Mice lacking CD2 or CD28 show only mild deficits, demonstrating that neither protein is essential for T cell activation. In this paper we have generated mice lacking both CD2 and CD28. T cells from the double-deficient mice have a profound defect in activation by soluble anti-CD3 Ab and Ag, yet remain responsive to immobilized anti-CD3. This suggests that CD2 and CD28 may function together to facilitate interactions of the T cell and APC, allowing for efficient signal transduction through the TCR.

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Materials and Methods

Mice

CD28-deficient mice backbred five generations to C57BL/6 have previously been described (10). CD2-deficient mice were generously provided from N. Killeen (University of California, San Francisco) via J. Bromberg (University of Michigan, Ann Arbor). The mice were crossed to generate mice homozygous for the null alleles at CD2 and CD28. Littermate were bred to either CD2- or CD28-deficient mice to generate control mice. Genotypes were determined by PCR and confirmed by Southern blotting. CD2/CD28-deficient mice described above were then crossed to CD28-deficient D011.10 mice (provided by S. Reiner and C. Thompson, University of Chicago). Pups deficient at both CD2 and CD28 were then screened for the presence of the TCR transgene by flow cytometry using the clonotypic mAb KJ1-26. MHC haplotype was also determined by flow cytometry of peripheral blood. Mice were 6–12 wk of age for all experiments, and were age and sex matched within each experiment. All mice are housed in specific pathogen free environments maintained by the Department of Comparative Medicine at the Washington University School of Medicine.

Antibodies

Anti-CD3 (145-2c11, hamster IgG) was provided by J. Bluestone, (University of Chicago). The clonotypic mAb KJ1-26 was generously provided by K. Murphy (Washington University, St. Louis, MO). All other Abs were purchased from PharMingen (San Diego, CA). Flow cytometric analysis was performed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson Corporation, Mountain View, CA).

Proliferation assays

Lymph node cells or splenocytes were isolated, and single cell suspensions were prepared following standard protocols. Cultures were stimulated with the indicated doses of anti-CD3 mAb and pulsed for the final 8 h of a 48-h culture with 1.0 μCi/well tritiated thymidine ([3H]Tdr; ICN, Costa Mesa, CA). Each condition was plated in quadruplicate and the mean ± SD
shown. Exogenous IL-2 (100 U/ml) was added at the start of the culture as indicated in the experiments. Purified T cells were prepared from lymph node cells by negative selection with magnetic bead conjugated anti-B220 and anti-MHC class II mAb according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Cells prepared in this manner were >99% Thy1.2 positive by FACS analysis and unresponsive to Con A stimulation (data not shown). Purified lymph node T cells were stimulated with plate immobilized anti-CD3 following standard methods. For stimulation of cells in the D011.10 background, 1.5 x 10⁵ splenocytes were stimulated with the OVA₃₂₃-₃₃₉ peptide in round-bottom microtiter plates. Proliferation was determined by [³H]Thymidine incorporation. Thymidine incorporation was measured at 48, 72, and 96 h. All experiments were repeated a minimum of three times, and representative data are presented.

**Conjugate assay**

The murine B cell line A20 that expresses I-A<sub>d</sub> was pulsed with the indicated dose of Ag for 18 h and then labeled with Cell Tracker Orange (0.5 μM; Molecular Probes, Eugene, OR) per the manufacturer's protocol. Lymph node cells were isolated and labeled with Cell Tracker Orange (0.5 μM; Molecular Probes). The ability to form T cell/APC conjugates was assessed as previously described (16). Briefly, T cells and APC were resuspended in cold DMEM following labeling at 5 x 10⁶ cells per ml. Twenty-five microliters of each were then placed into a 0.2 ml tube and the cells briefly pelleted at 4°C and then incubated at 37°C for 6 min. The cells were then resuspended in 2 ml of PBS containing 1% BSA and gently mixed. The presence of T cell/APC conjugates was assessed using two-color flow cytometric analysis on a FACSCalibur flow cytometer. Data are expressed as the percentage of conjugates relative to wild type, where the wild type is set at 100%. The mean of two independent experiments is presented.

**Results**

**T cell development in CD2/CD28-deficient mice**

To assess the role of CD2 and CD28 in T cell development, we bred mice singly deficient in either CD2 or CD28 to obtain mice deficient in both genes. The genotype of each mouse was determined by PCR analysis using primers specific for the wild-type alleles (data not shown). In addition, surface expression of CD2 and CD28 was assessed by flow cytometry for each experiment. Analysis of peripheral T cell subsets demonstrated no significant alterations in cell number or distribution (Fig. 1). Thymocyte cellularity and subsets were also similar to wild-type mice. The percentage of T cells and B cells in the lymph nodes as assessed by Thy1.2 and B220 staining were similar in all genotypes examined (data not shown). Thus, T cell development was not grossly altered in mice lacking both CD2 and CD28.

**CD2/CD28 double-deficient T cells fail to proliferate in response to soluble anti-CD3 but respond to immobilized anti-CD3**

To determine whether T cells lacking both CD2 and CD28 were able to respond to TCR engagement, we stimulated bulk lymph node cells with soluble anti-CD3 and measured proliferation by thymidine incorporation (Fig. 2A). Wild-type mice or mice deficient in only CD2 or CD28 mounted a robust proliferative response to anti-CD3 stimulation. In contrast, T cells from mice deficient in both CD2 and CD28 were virtually unresponsive to CD3 engagement by soluble Ab. However, T cells from all genotypes proliferated strongly to stimulation with PMA and ionomycin (Fig. 2B). Similar results were obtained in experiments performed with splenocytes or lymph node cells and when examined at 24, 48, and 72 h. No reproducible differences were observed in the response of mice homozygous or heterozygous for the wild-type alleles (data not shown).

Surprisingly, when purified T cells from the CD2/CD28 double-deficient mice were activated using immobilized anti-CD3, near normal proliferative responses were measured (Fig. 2C). This demonstrates that signal transduction pathways initiated through the TCR are functional, and suggests that the defect may involve interactions between the T cell and the APC. In support of this idea, the CD2/CD28-deficient mice failed to respond to Con A treatment, which is also dependent on interactions with accessory cells (Fig. 2B).

We also examined the expression of the activation markers CD69 and CD25 following stimulation of bulk lymph node cells with soluble anti-CD3. Expression of both CD69 and CD25 were decreased in the double knockout mice (Table I). Consistent with the failure to induce CD25 expression, addition of exogenous IL-2 did not restore the proliferative response of the CD2/CD28-double deficient T cells (Fig. 2D).

CD28 has been demonstrated to influence cell survival following stimulation (17). To determine of the proliferative defect was solely due to increased cell death, we assessed cell viability following stimulation with anti-CD3 (Table I). Viability was >90% for all genotypes at the initiation of the culture (data not shown). At 48 h following activation, there was a decrease in cell survival for both the CD28 and CD2/CD28-deficient cells. However, the
The magnitude of the difference is insufficient to account for the differences in proliferation suggesting that induction of cell death alone cannot explain the lack of proliferative response.

Ag-specific proliferation of CD2/CD28-deficient T cells is impaired in a dose- and time-dependent manner

To examine the proliferative response following stimulation in a more physiologically relevant context, we bred double-deficient mice to the TCR transgenic mouse expressing the DO11.10 TCR. The DO11.10 mouse expresses a transgenic TCR specific for the OVA323–339 peptide in the context of I-A^d (18). Expression of the TCR transgene as assessed by flow cytometry with a clonotype-specific Ab revealed similar levels of TCR expression in all genotypes (data not shown). Stimulation of CD2/CD28 double-deficient splenocytes with OVA323–339 peptide demonstrated a dose- and time-dependent impairment in the proliferative response (Fig. 3).

At low Ag doses, no significant proliferative response was generated in the double-deficient T cells. Proliferation was detected only at the highest peptide dose used (3 \( \mu g/\mu l \)). In addition, the impaired response of T cells from the double knockout mice was most significant at later time points. Mice lacking CD28 alone also demonstrated a time- and dose-dependent response to peptide/MHC, but unlike the double knockout T cells, this impairment was overcome at higher doses of Ag and was less significant at later time points. CD2-deficient mice proliferate at levels similar to wild-type mice (Ref. 15 and data not shown). No significant differences were observed in stimulation of lymph node cells or splenocytes (data not shown). Thus, T cells lacking both CD2 and CD28 exhibit a profound impairment to Ag activation.

**Decreased T cell/APC conjugate formation in the absence of CD2 and CD28**

As both CD2 and CD28 bind to ligands expressed on APC, both proteins may be involved in facilitating interactions between T cells and APC. We tested this hypothesis by measuring the ability of double-deficient cells to form T cell:APC conjugates. DO11.10 T cells were co-incubated with Ag-pulsed APC and assessed for conjugate formation by flow cytometry. As shown in Fig. 4, T cells lacking both CD2 and CD28 had a marked reduction in the number of stable conjugates formed as compared with either wild-type T cells or T cells from mice deficient in only CD2 or CD28. At the highest dose of Ag examined, no difference was observed between genotypes, consistent with the observation that some proliferative response can be elicited at these Ag doses. This suggests that both molecules work together to facilitate T cell interactions with APC.

**Discussion**

To determine whether there is redundancy in the function of the accessory molecules CD2 and CD28, we generated mice deficient in both proteins. In contrast to T cells lacking only CD2 or CD28, we found that T cells deficient in both proteins were severely impaired in their ability to be activated and proliferate in response to stimulation by either soluble anti-CD3 Ab or peptide Ag. T cells from CD2- and CD28-deficient mice had a profound defect in response to soluble anti-CD3 stimulation. This was not due solely to impaired IL-2 secretion, as addition of exogenous IL-2 had little effect. Consistent with this finding, we found that double

**Table I. Viability and activation marker expression**

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<thead>
<tr>
<th>CD2</th>
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<th>% IL-2R</th>
<th>% Viable</th>
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<td>+/−</td>
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<td>13</td>
<td>11</td>
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* Bulk lymph node cells were stimulated with soluble anti-CD3 (1.0 mg/ml), and expression of CD69 and CD25 was determined after 18 and 48 h, respectively, by flow cytometry. The percentage of cells positive for each marker is shown. Viability was determined at 48 h by propidium iodide exclusion, and the percentage of live cells is shown.
knockout mice failed to efficiently up-regulate the early activation markers, CD69 and CD25. This suggests that the signaling defect involves the earliest steps in T cell activation.

Surprisingly, we found that purified T cells from the double-deficient mouse could be activated with immobilized anti-CD3. Differences in the mechanism by which soluble vs immobilized anti-CD3 activates T cells may therefore provide insights into how CD2 and CD28 function. Stimulation of T cells using soluble anti-CD3 relies on the presence of Fc-bearing cells to cross-link the TCR. In contrast, immobilized anti-CD3 results in activation in the absence of any accessory cells as the solid phase substrate on which the Ab is immobilized provides for high degrees of cross-linking. That immobilized anti-CD3 activates double-deficient T cells demonstrates that the intrinsic signaling capabilities of the TCR are intact. The discrepancy in the ability of soluble vs immobilized anti-CD3 to activate double-deficient T cells suggests that CD2 and CD28 may function primarily by stabilizing the interaction of the T cell with the APC. In the absence of CD2 and CD28, interactions with Fc-bearing B cells and macrophages might be impaired, resulting in ineffective TCR cross-linking.

Breeding the double knockout mouse to a TCR transgenic mouse enabled us to examine T cell activation by peptide Ag. Similar to soluble anti-CD3 stimulation, we found that Ag-induced T cell proliferation was significantly impaired. Given that the TCR is competent to signal in these cells, the most compelling explanation for the defect is the inability of T cells to form stable contacts with the APC. In confirmation of this, direct assay of the ability of T cells to form conjugates with APC demonstrated a marked reduction in T cell:APC pairs, particularly at submaximal Ag stimulation. While both CD2 and CD28 contribute to the formation of the T cell:APC contact, this does not exclude other mechanisms, such as activation of intracellular signaling cascades, as potential mechanisms for either CD2 or CD28 function. Our data suggest that ligation of either CD2 or CD28 is required for the initial activation of T cells. However, their mechanism of action may be either similar or distinct, but in the absence of both, the T cell is severely impaired in its ability to respond to Ag.

Most current models of T cell activation subscribe to the two-signal hypothesis which proposes that two signals are required for T cell activation (3). The first is transduced by the TCR, and the second by a costimulatory molecule. Although many molecules have been implicated as costimulatory molecules, the best studied is CD28. CD28 is thought to transduce a required and specific biochemical signal that integrates with the signal initiated by the TCR to effect T cell activation. As T cells lacking CD28 can still be activated, either the signal transduced by CD28 can be delivered by another molecule on the surface of the T cell- or TCR-mediated signals alone may be sufficient.

Evidence is accumulating to support the latter possibility. These data suggest that CD28 may function mainly to enhance and amplify signals transduced by the TCR. Because CD28 is an adhesion molecule, it may function in concert with other adhesion molecules such as CD2 to stabilize the formation of a close membrane contact between the T cell and the APC. CD28 engagement also promotes a cytoskeletal mechanism that recruits lipid rafts to the T cell/APC contact (19, 20). These adhesive and cytoskeletal functions of CD28 result ultimately in promoting and stabilizing T cell interactions with APCs. This interaction is critical as TCR recognition of Ag is dependent upon formation of a stable and organized contact structure known as the immunological synapse (13, 21).

In support of this model, CD28 has been shown to be particularly important for Ags with a short half-life. CD28-deficient mice cannot respond to Ags that are rapidly cleared from the mouse, but can mount normal responses to Ags that are present in the mouse for several days (22, 23). These data suggest that one mechanism by which CD28 may regulate T cell proliferation is by enhancing TCR sensitivity and length of engagement rather than transducing a unique signal.

Like CD28, CD2 is thought to play an important role in facilitating interactions between the T cell and the APC. CD2 binds specific ligands expressed on a wide range of APC. In humans, the principle ligand for CD2 is CD58, also known as LFA-3 (24). In rodents, the related molecule CD48 is the most active natural ligand for CD2 (12). Although the affinity of CD2 for its ligands in vitro is relatively low (10⁻⁵), recent evidence suggest that this affinity is physiologically relevant (25).

As CD2 ligation can enhance signaling mediated by the TCR, it has been considered by some to be a costimulatory molecule (26, 27). CD2 also shares some structural features in common with CD28. Both are members of the Ig superfamily, and each contains Ig repeats in the extracellular domain. Bound to their ligands, they
span the same distance, 15 nm, similar to the distance spanned by the TCR bound to peptide/MHC. Lastly, both contain relatively long cytoplasmic domains that contain potential sites of binding of Src homology 3 (SH3) domain containing proteins. Both molecules are likely to be involved in facilitating cell-cell contact between the T cell and the APC.

As mice lacking CD2 are relatively normal, it seemed likely that the role of CD2 in T cell biology was not essential and could be substituted by other molecules on the surface of the T cell (15). In this paper we present data supporting a model where CD2 and CD28 share some functions. We have shown previously that CD2 clustering plays an essential role in helping to form a specialized cell contact called the immunological synapse (21). Preliminary experiments suggest that CD28 can also induce protein clustering and segregation and thus may function in a manner similar to CD2 (S. Bromley and M. Dustin, personal communication). Furthermore, it is also possible that the cytoplasmic tails of CD2 and CD28 interact with the same or similar intracellular proteins. Although the precise mechanism by which CD2 and CD28 coordinately regulate the process of T cell activation remains to be defined, the data presented in this manuscript demonstrate that each may function to facilitate the interaction with APCs. The binding of CD2 and CD28 with their ligands would allow for efficient TCR engagement, especially in the setting of limiting Ag. Thus, the engagement of the accessory molecules CD2 and CD28 may effectively set the threshold for T cell activation by Ag.

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