Hierarchical Costimulator Thresholds for Distinct Immune Responses: Application of a Novel Two-Step Fc Fusion Protein Transfer Method

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*J Immunol* 2000; 164:705-711; doi: 10.4049/jimmunol.164.2.705

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Hierarchical Costimulator Thresholds for Distinct Immune Responses: Application of a Novel Two-Step Fc Fusion Protein Transfer Method

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Activation of T cells is dependent upon coordinate engagement of Ag and costimulator receptors on their surfaces. In the case of the Ag receptors (TCRs), activation thresholds have been defined, with the number of TCRs that must be triggered to stimulate cytokine secretion by individual activated T cells differing for the various cytokines. In the present study, we have determined whether comparable activation thresholds exist for the costimulator receptors on T cells. To facilitate this type of quantitative costimulator analysis, we developed a novel two-step protein transfer approach that permits delivery of graded amounts of proteins to APC surfaces. By adding a human B7-1·Fcγ1 (Fc domain of human IgG1) fusion protein to cells precoated with palmitated protein A, fine titration of the B7-1 extracellular domain was achieved. The B7-1·Fcγ1 reincorporated into cell membranes by this method retained costimulator function, as measured by an in vitro proliferation assay. The degree of proliferation was dependent on the surface density of B7-1·Fcγ1. Significantly, the threshold B7-1·Fcγ1 density required for cytokine production differed between IFN-γ and IL-2 and mirrored the hierarchy (IFN-γ < IL-2) described previously for the TCR activation threshold. Hence, this study invokes a novel protein transfer strategy to establish that the levels of surface costimulator on APCs can dictate both the magnitude and the quality of evoked T cell responses. The notion of costimulator receptor activation thresholds emerges.


A-activation of T cells is dependent upon coordinate signaling through Ag and costimulator receptors on their surfaces (reviewed in Refs. 1–3). An “activation threshold model” has been proposed to explain various phenomena associated with antigenic signaling through the TCR (4–10). There are three essential aspects to this model. First, T cell activation is elicited once a threshold number of TCRs have been triggered. Second, a hierarchy of TCR thresholds are operative even at the single-cell level, with differing T cell cytokine outputs and functional capabilities resulting from the triggering of differing numbers of TCRs. Third, TCR activation thresholds can be lowered via costimulation (8) or elevated by at least one anti-inflammatory protein.3

Little is known about quantitative aspects of costimulator signaling, and the possibility of “costimulator activation thresholds,” that might parallel TCR activation thresholds, has not been systematically evaluated. There are two related questions in this regard. First, at any given Ag density, are there a threshold number of costimulator receptors that must be triggered to activate T cells? Second, if costimulator thresholds do indeed exist, is there a hierarchy of such thresholds within single cells, with different T cell outputs pegged to different costimulator inputs? Evidence in favor of such costimulator thresholds comes from recently reported studies by several groups that point to the importance of APC costimulator levels on elicited T cell responses (8, 11, 12).

The quantitative study of costimulation has been limited by technical factors. Gene transfer strategies do not permit fine control of costimulator densities at APC surfaces. As an alternative, cell-free systems have been invoked by some investigators. For example, differing concentrations of costimulators can be immobilized on plastic substrates (13–15) or on cell-sized latex microspheres (16). Such cell-free systems, however, by definition ignore contextual molecular determinants at the APC surface.

Protein transfer offers an alternative means for titrating costimulator densities at APC surfaces. We (17, 18) and others (19, 20) have described one costimulator protein transfer method that invokes chimeric GPI-modified costimulators as membrane-reincorporable “protein paints.” However, scaling up the production of these protein derivatives is complicated by the need to purify them from transfectant cell membranes. In the present study, we report a unique protein transfer strategy that bypasses the need to work with recombinant membrane proteins. This strategy builds upon work by Kim and Peacock (21) who described a two-step approach for binding Abs to cell surfaces. According to their approach, protein A, after derivatization with palmitate, is first incorporated into cell membranes, and in turn, this membrane-associated palmitated protein A (pal-prot A)4 is used as a trap for secondarily added IgG molecules. Building upon this finding, the present study demonstrates that Fc fusion proteins can be similarly delivered to pal-prot A precoated cell surfaces with preservation of trans-signaling protein function. Moreover, we proceed to utilize costimulator·Fcγ1 (Fc domain of human IgG1) protein transfer to establish the existence of costimulator activation thresholds within T cells.

Abbreviations used in this paper: pal-prot A, palmitated protein A; Fcγ1, Fc domain of human IgG1.

Received for publication July 12, 1999. Accepted for publication November 4, 1999.

Acknowledgments

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1 This work was supported in part by Grants RO1 CA-74958 and RO1 AI-31044 from the National Institutes of Health.

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4 Abbreviations used in this paper: pal-prot A, palmitated protein A; Fcγ1, Fc domain of human IgG1.
Materials and Methods

Palmitation of protein A

Recombinant protein A (Calbiochem, La Jolla, CA) was derivatized with the N-hydroxysuccinimide ester of palmitic acid (Sigma, St. Louis, MO) as described (21). The lipid-derivatized protein A was purified as described (22) on a 30-ml Sephadex G-25 (Sigma) column. The protein product was quantitated using a bichinonic acid kit (Bio-Rad, Richmond, CA), filter sterilized, and stored at 4°C until use.

Membrane incorporation of pal-prot A

Cells (3–7 × 10⁶/ml) were resuspended in RPMI 1640 medium (BioWhittaker, Walkersville, MD) after three washes with this same medium. Varying concentrations of pal-prot A (or nonderivatized protein A as negative control) were added to the cell suspension, and the mixture was incubated at 4°C for 2 h with constant mixing. To assay the incorporation of pal-prot A onto cell surfaces, cells were washed twice in buffer (0.25% BSA/0.01% sodium azide/PBS) and then incubated on ice for 1 h with 100 µl of 100 µg/ml FITC-human IgG (Sigma) diluted with the same buffer. Cells were washed twice in the buffer and analyzed on a FACStar (Becton Dickinson, Mountain View, CA).

Preparation of recombinant B7-1·Fcγ1

The expression plasmid pCDM8/B7lg, encoding the complete human B7-1 extracellular domain linked in-frame to the Fcγ1, was obtained from the American Type Culture Collection (Manassas, VA). The sequence encoding B7-1·Fcγ1 was mobilized from pCDM8/B7lg by digesting with XbaI, filling-in with Klenow fragment, and subsequently digesting with HindIII. The mobilized fragment was subcloned into the EDV episomal expression vector pREP7β (Invitrogen, San Diego, CA) with HindIII and filled-in BamHI sites. The plasmid was transfected into 293 cells (human kidney cell line; American Type Culture Collection), and hygromycin B-resistant colonies were selected in serum-free UltraCulture medium (BioWhittaker) supplemented with 10 mM glutamine, penicillin/streptomycin, and 200 µg/ml hygromycin B. Secreted B7-1·Fcγ1 was purified from conditioned medium by protein A-agarose (Life Technologies, Walkersville, MD) after three washes with this same medium. Varying concentrations of pal-prot A (or nonderivatized protein A as negative control) were added to the cell suspension, and the mixture was incubated at 4°C for 2 h with constant mixing. To assess the incorporation of pal-prot A onto cell surfaces, cells were washed twice in buffer (0.25% BSA/0.01% sodium azide/PBS) and then incubated on ice for 1 h with 100 µl of 100 µg/ml FITC-human IgG (Sigma) diluted with the same buffer. Cells were washed twice in the buffer and analyzed on a FACStar (Becton Dickinson, Mountain View, CA).

Preparation of recombinant B7-1·Fcγ1 protein transfer

Cells precoated with pal-prot A were washed once and resuspended in RPMI 1640 medium (3–7 × 10⁶ cells/ml) at 4°C for 1 h with constant mixing. To monitor protein delivery, 10⁶ cells in 100 µl of buffer. Cells were washed once and immunostained (on ice for 1 h with 100 µl of 100 µg/ml hygromycin B. Secreted B7-1·Fcγ1 was purified from conditioned medium by protein A-agarose (Life Technologies, Germantown, MD) af-finity chromatography and verified by SDS-PAGE.

Analysis of intracellular cytokine production

A total of 10⁵ T cells was incubated with 5 × 10⁶ processed K562/REPβ7 cells (B7-1·Fcγ1, positive or negative) in 48-well plates using either plate-bound HIT3a or PHA as a source of first signal. Supernatants were collected after 48 h, and ELISAAs for human IFN-γ and IL-2 were performed using a commercial ELISA kit according to manufacturer’s protocol (Genzyme, Cambridge, MA).
observed, indicating the dependence of Fc protein attachment on protein A lipid anchoring (Fig. 2B). Next, the feasibility of quantitatively painting B7-1·Fcγ1 onto pal-prot A precoated cells was established. When K562 cells were precoated with excess amounts of pal-prot A (33 μg/ml), surface levels of B7-1·Fcγ1 were dependent on the concentrations of applied B7-1·Fcγ1 (Fig. 2C). Surface B7-1 epitope levels started to plateau at 33 μg/ml, and the epitope density was similar to that on B7-1-transfected K562 cells (data not shown).

Painted B7-1·Fcγ1 costimulates T cells

In vitro proliferation assays were performed to determine whether membrane-tethered B7-1·Fcγ1 can costimulate T cells. In these assays, PHA and B7-1·Fcγ1-coated K562/REP7β cells (i.e., K562 cells stably transfected with the pREP7β EBV episomal expression vector) were used to provide first and second signals, respectively, to T cells. K562/REP7β cells lack detectable B7-1 (data not shown) and provide a suitable negative control for experiments with K562/B7-1 cells (i.e., K562 cells stably transfected with a pREP7β vector containing human B7-1 cDNA sequence). Surface B7-1 levels on K562/B7-1-transfected cells and B7-1·Fcγ1-coated K562/REP7β cells were determined by immunostaining, and the mean fluorescence intensities were 550 and 450, respectively. As shown in Fig. 3A, in the presence of suboptimal PHA concentrations (, 0.5 μg/ml), B7-1·Fcγ1-coated K562/REP7β cells, but not K562/REP7β cells, significantly enhance T cell proliferation. The costimulatory effect was comparable to that of K562/B7-1 cells.
achieved with K562/B7-1-transfected cells. The B7-1 · Fcγ1/palprot A-dependence of the observed costimulation was verified by showing that cells treated with a combination of (nonderivatized) protein A and B7-1 · Fcγ1, or with a combination of pal-prot A and control CD8 · Fcγ1, did not enhance T cell proliferation. In the presence of higher PHA concentrations (>1 μg/ml), K562/REP7

Table I. Painting of B7-1 · Fcγ1 onto K562 cells

<table>
<thead>
<tr>
<th>B7-1 · Fcγ1 (μg/ml)$^a$</th>
<th>No. of B7-1 · Fcγ1/Cell (mean ± SD)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.033</td>
<td>460 ± 240</td>
</tr>
<tr>
<td>0.33</td>
<td>9,900 ± 1,200</td>
</tr>
<tr>
<td>3.3</td>
<td>92,000 ± 8,300</td>
</tr>
<tr>
<td>33</td>
<td>460,000 ± 34,000</td>
</tr>
</tbody>
</table>

$^a$ The final concentration of B7-1 · Fcγ1 present during the painting procedure.

$^b$ Values were determined as described in Materials and Methods. Specific activity of 125I-labeled B7-1 is 2.1 × 10^6 cpm/μg.

FIGURE 3. Costimulation of T cell proliferation by B7-1 · Fcγ1-painted K562/REP7β. K562/REP7β cells were incubated with 33 μg/ml of either protein A or pal-prot A, and these cells were then incubated with 33 μg/ml of either B7-1 · Fcγ1 or control · Fcγ1. For the proliferation assay, 10^5 purified human T cells were cocultured with 4 × 10^5 mitomycin C-treated K562/REP7β processed as described above in combination with the indicated amounts of PHA (A) or plate-bound anti-human CD3 mAb HIT3a (B). Effective depletion of accessory cells was documented in all T cell preparations by demonstrating the lack of a response to PMA or PHA in the absence of a source for costimulation. All cultures were incubated for 60 h and pulsed with 1 μCi of [3H]thymidine/well for the last 16 h of the incubation period before determining [3H]thymidine incorporation. Points are the means and SEs of triplicate samples. The data are representative of at least three independent experiments with similar results.

FIGURE 4. B7-1 threshold concentration for T cell proliferation. A total of 10^5 purified T cells was cocultured with 4 × 10^5 K562/REP7β cells painted with 33 μg/ml pal-prot A and subsequently with the indicated amounts of B7-1 · Fcγ1 along with PHA (A) or plate-bound anti-human CD3 mAb HIT3a (B). Proliferation assays were performed as described in the Fig. 3 legend.

Concentration-dependence of cell-associated B7-1 · Fcγ1’s costimulatory activity

With an effective costimulator protein transfer method in hand, quantitative aspects of B7-1 costimulation were evaluated. To this end, T cell proliferation assays were performed using K562/REP7β cells painted with variable concentrations of B7-1 · Fcγ1. The concentration dependence of B7-1 · Fcγ1-mediated costimulation could be readily demonstrated when a fixed suboptimal concentration of anti-CD3 mAb (<10 μg/ml) was used as a source of first signal (Fig. 4A). For example, in the presence of 0.5 μg/ml PHA, T cell proliferation was observed once a threshold B7-1 · Fcγ1 concentration (0.1 μg/ml) was reached, and the level of proliferation continued to rise with increasing B7-1 · Fcγ1 concentrations until reaching a plateau at ~3.3 μg/ml. In the presence of a lower concentration of PHA (0.25 μg/ml), T cell proliferation was observed when a higher threshold B7-1 concentration (1 μg/ml) was reached, indicating that costimulator thresholds can be modulated by the strength of the first signal.
Having documented that B7-1 levels can modulate the extent of T cell proliferative responses, we next determined whether B7-1 levels can also dictate the quality of immune responses by altering the ratios of cytokines produced by activated T cells. ELISA was used to measure T cell cytokine secretion in response to varying painted B7-1 concentrations and fixed suboptimal primary stimulus concentrations. At a fixed PHA dose, the B7-1 · Fcγ1 concentrations eliciting minimal and maximal cytokine responses differed with 0.75 µg/ml PHA (A) or 3.3 µg/ml anti-human CD3 mAb HIT3a (B). The cells were cultured at 37°C for 48 h, and the media were then tested by ELISA for secreted human IFN-γ (Fig. 5). A similar hierarchy for the cytokine responses was observed, only after a threshold B7-1 · Fcγ1 concentration was reached, and a further dose-dependent increase in proliferation was also seen. Hence, in the presence of a suboptimal first signal (whether PHA or anti-CD3 mAb), a threshold B7 level is required for T cells to proliferate and the extent of T cell proliferation is dictated by the costimulator level.

**A hierarchy of B7-1 costimulator thresholds for distinct cytokine responses**

Evidence for hierarchical costimulator thresholds for cytokine responses at the single-cell level

To substantiate the ELISA findings with bulk T cell populations, multiparameter flow cytometric analyses were performed to assess intracellular IFN-γ and IL-2 levels within individual cells. At low B7-1 · Fcγ1 concentrations, the T cell response is dominated by IFN-γ-only producers; however, at higher B7-1 · Fcγ1 concentrations, substantial numbers of IFN-γ and IL-2 double producers emerge (Fig. 6). Relatively few IL-2-only producers were observed, even at the highest B7-1 · Fcγ1 concentrations. These findings are consistent with the bulk T cell cytokine response data, showing that an IFN-γ response requires less B7-1 costimulation than does an IL-2 response.

Discussion

In the present study, we have introduced a novel approach for quantitatively delivering costimulators to cell surfaces, and, in turn, this method has been used to look for B7-1 costimulation thresholds. Specifically, a B7-1 · Fcγ1 fusion protein was “painted” onto tumor cells that had been precoated with chemically lipided protein A, and the influence of varying levels of painted protein on T cell activation was monitored. Key findings included the following: 1) B7-1 · Fcγ1 can be quantitatively titrated onto protein A-precoated cell surfaces. 2) Painted B7-1 · Fcγ1 retains costimulator function. 3) At a specified level of TCR triggering, there is a threshold level of costimulator trans-signaling required for T cell activation. 4) There are distinct costimulator thresholds at the single-cell level that are each coupled to different T cell outputs, and the hierarchy of observed T cell cytokine responses parallels that previously documented for TCR activation thresholds.

Previous studies bear upon the question of costimulator thresholds. Itoh and Germain (8) reported that at a given Ag density, APC expressing high levels of B7-1 and ICAM-1 stimulated more...
IL-2-producing cells than did APC with low levels of the costimulators. In another study, increasing LFA-3 density on melanoma cells was shown to enhance cytokine production by melanoma-specific CTL clones (11). Recently, Murtaza et al. (12) reported that the level of costimulation has a significant effect on responses to an Ag, and that a strong costimulatory signal can convert a weak agonist into a full agonist and an agonist into a superagonist. Although these studies point to the importance of APC costimulator levels on elicited T cell responses, and thus support the conclusions of the present study, they were limited in two ways by the experimental systems employed. First, all of these studies used transfected and/or supertransfected sublines that were selected on the basis of low vs high costimulator expression. Although the sublines were carefully examined to ensure comparable expression of several immunoregulatory molecules, such as I-EK (8), HLA-A2, and ICAM-1 (11), it remains possible that observed functional differences might stem from variable expression of yet other immunoregulatory molecules. Second, the costimulator levels on the transfected sublines could not be tightly controlled, and, consequently, the functional impact of low vs high, but not graded, expression of costimulators was examined in those studies. In contrast, the protein transfer approach of the present study enabled titration of costimulators at the cell surface, while avoiding the confounding effects of subline selection and consequent molecular heterogeneity.

Protein transfer offers a number of advantages over gene transfer for engineering APC. These include the suitability of the former to poorly transfected cells (for example, biopsy-derived tumor cells), the simplicity of expressing multiple proteins on the same cell surface, and the relative ease and rapidity of the procedure (reviewed in Ref. 23). Previously, we (17, 18, 24, 25) and others (19, 20) have reported the successful application of protein transfer to costimulator and MHC proteins using their recombinant GPI-modified derivatives. However, one limitation of the GPI protein transfer strategy is in scaling up the purification of GPI proteins from membranes of the transfected host cells. The protein transfer approach presented here circumvents this problem, since Fc fusion proteins, involving lipidated protein A with the cells. The extension of Ab protein transfer to anti-CD28 mAbs, and hence costimulation, was reported recently (26). In that report, an alternative multistep approach was employed in which cells were first chemically biotinylated, protein G-biotin was linked to the biotinylated cells using avidin as a bridge, and, finally, mAb was attached to the anchored protein G. The limitation of this latter protein transfer procedure, as compared with the original one advocated by Kim and Peacock and our own, is in its dependence on chemical biotinylation of cells and the associated perturbation of resident cell surface molecules.

The principle finding of the present study is that levels of costimulators can not only tune the magnitude of an evoked immune response, but can also dictate the quality of the immune response. Taken together with the TCR activation threshold model (4–10), this argues that T cell responses at the single-cell level are quite plastic and can be driven in qualitatively different ways by altering the levels of both antigenic and costimulatory inputs. The interplay between these antigenic and costimulatory inputs is likely to be of considerable interest and physiological significance, especially once insights into costimulator cooperativity emerge. One class of tumor cell vaccines consists of immunogenic tumor cells engineered via gene or protein transfer to express lymphoid costimulators (27). Given its simplicity and effectiveness, our protein transfer method offers an attractive new option for tumor vaccine engineering. Flexible use of costimulator·Fc proteins, invoking diverse costimulator combinations that are tailored to individual tumor types, may provide significant therapeutic advantages.

Acknowledgments

We thank Joseph Manupello for his assistance with labeling of the protein with 125I, Pan Tao and Drs. John Fayen, Jacob Rachmilewitz, and J.-H. Huang for helpful discussions, and Susan Brill for her assistance.

References


