Research article

Human CD81 directly enhances T_h1 and T_h2 cell activation, but preferentially induces proliferation of T_h2 cells upon long-term stimulation

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Abstract

Background: CD81, a cell-surface protein of the tetraspanin superfamily, has been shown to costimulate T cell activation in murine T cells, and is involved in development of Th2 immune responses in mice.

Results: Here it is shown that stimulation of CD81 on human T cells can enhance T cell activation by antigen or superantigen, causing an increase in the early activation marker CD69, and increasing the number of cytokine-producing and proliferating T cells. Interestingly, CD81 costimulates cytokine production by T cells producing both Th1 and Th2 cytokines. Although human CD81 is highly expressed on non-T as well as T cells, CD81 costimulation appears to act directly on T cells. Pre-incubation of purified T cells with anti-CD81 antibody is sufficient to increase T cell activation, while pre-incubation of non-T cells is not. However, long-term polyclonal stimulation of T cells by anti-CD3 antibody, in the presence of CD81 costimulation, biases T cells towards the production of IL-4 and not IFNγ. This is accomplished by a preferential proliferation of IL-4-producing cells.

Conclusion: Thus, signalling through CD81 on T cells costimulates both Th1 and Th2 cells, but increases the number of Th2 cells during long-term activation.

Background

The tetraspanins are a family of cell-surface proteins with four transmembrane domains, two extracellular loops, and conserved cysteine residues at key positions in the second extracellular loop [1]. They facilitate a wide array of functions, including cell activation, differentiation, adhesion, morphological changes, and motility, which may all relate to the promiscuous associations of these molecules with integrins and other signaling proteins within the cell membrane and the cytoskeleton.

CD81, a defining member of the tetraspanin superfamily, is widely expressed on human hematopoietic and other cells [2]. It associates on B cells with a signaling complex that includes CD19 and CD21 [3], as well as associating with MHC class II molecules [4] and other tetraspanins [5,6]. On T cells, CD81 interacts with CD4, CD8, CD82, and selected integrins [7–10].

An anti-CD81 antibody was first isolated for its ability to induce cell death in B cell lines [11]. This is likely dependent upon CD81's association with MHC class II molecules, which can transmit death-inducing signals in B cells [12]. CD81 cross-linking can also induce adhesion in B and T cells, apparently by multiple pathways [10,13,14]. Triggering of the CD19-CD21-CD81 complex on murine

B cells has been shown to lower the threshold for B cell activation via the immunoglobulin receptor [15].

On murine T cells and thymocytes, CD81 costimulates T cell receptor-mediated activation, through a pathway independent of CD28 [16]. On human T cells, CD81 costimulation results in increased IL-2 production and LFA-1-mediated T-B cell adhesion [17]. Murine CD81 also appears to play a role in thymocyte maturation as shown in fetal thymic organ cultures [18].

Finally, CD81 signalling has been shown to have an effect on the Th1/Th2 balance of immune responses. In cell cultures of CD4 T cells and B cells from allergic individuals, addition of anti-CD81 antibody enhances IL-4 production from the T cells [19]. In mice, either complete lack of CD81, or lack of CD81 on B cells, leads to impaired humoral and Th2 immune responses [20,21]. Allergen-induced airway hyperresponsiveness is also decreased in CD81null mice [22]. Finally, lack of CD81 on murine T cells diminishes IL-4 production, with reduced expression of ICOS, GATA-3, STAT6 and phosphorylated STAT6 [23].

In this report, an attempt is made to reconcile the findings of general T cell costimulation versus specific Th2 biasing by CD81 in human T cells. Short-term CD81 cross-linking on normal human T cells is shown to co-stimulate T cell activation (via antigen or superantigen), extending previous findings in mouse splenocytes [16] and human PBMC [17]. The effect appears to be a direct consequence of CD81 triggering on T cells. Of interest, production of both Th1 and Th2 cytokines is augmented by CD81 costimulation. However, during longer-term stimulation of T cells, the presence of CD81 costimulation leads to a disproportionate increase in IL-4-producing cells. This is due to increased induction of proliferation. Thus, CD81 signalling provides short-term costimulation of cells producing Th1 or Th2 cytokines, but results in a disproportionate increase in Th2 cytokine-producing cells during long-term activation.

Results
CD81 cross-linking costimulates CD69 expression and IL-2 induction
Two early events in T cell activation are the induction of CD69 expression and the stimulation of IL-2 production by the T cells. To determine whether costimulation through human CD81 affected these early activation events, peripheral blood cells from normal CMV seropositive donors were incubated for 6 h with a superantigen, SEB, or the viral antigen, CMV, in the presence or absence of an agonist anti-CD81 mAb, 5A6. As seen in Figure 1, over a range of antigen or superantigen concentrations, the addition of 5A6 increased the number of CD69+ cells as well as the number of IL-2-producing cells, measured

by intracellular staining. The effect of anti-CD81 mAb was often greater than that of the classical costimulatory antibody, CD28. The effect of anti-CD81 was also greater for CD4+ T cells (A, B, E, F) compared to CD4- T cells (C, D, G, H). It also appeared to be more dramatic for CD69 (A,C,E,G) than for IL-2 (B,D,F,H). As such, essentially no effect was observed on CD8 (CD3+CD4-) T cell IL-2 production (panels D and H). Stimulation with 5A6 alone, in the absence of SEB or CMV, gave consistently = 0.05% cytokine-positive T cells in such experiments (data not shown). These results confirm and extend the findings of VanCompernolle et al. [17] on CD81 costimulation of SEB-induced CD69 expression.

Similar to results obtained by VanCompernolle et al. [17], CD81 costimulation was also found to increase SEB-specific proliferation, as measured by BrdU incorporation (data not shown).

Effect on Th1 and Th2 cytokines
Since CD81 signaling has been implicated in the mouse to be preferentially important for Th2-dominated responses [21], it was examined whether CD81 costimulation in human T cells preferentially induced Th2 cytokine production over Th1 cytokine production. To examine this, 6 h stimulation with SEB or CMV was carried out in peripheral blood cells of normal human CMV seropositive donors, in the presence or absence of anti-CD81 mAb 5A6, followed by intracellular staining for IL-2, IFNγ, or IL-4. As seen in Figure 2, addition of 5A6 approximately doubled the number of cytokine-producing cells for each cytokine at these doses of SEB (0.1 μg/ml) or CMV (0.5 μg/ml). There was no preferential costimulation of cells producing IL-4 versus IFNγ or IL-2.

CD81 exerts a direct effect on human T cells
To determine whether the effects observed in the above experiments were due to direct stimulation of CD81 on T cells, or whether indirect effects of ligation of CD81 on other cells were involved, the following experiment was carried out. PBMC from a normal human donor were depleted of non-T cells by magnetic bead separation, then T cells and non-T cells were independently incubated with 5A6 anti-CD81 mAb, washed, and re-combined in the presence of SEB. As seen in Figure 3, only when the T cell fraction was incubated with 5A6 was costimulation of CD69 expression and cytokine production observed. Thus, direct ligation of CD81 on T cells was sufficient to induce costimulation of T cell activation.

Effect of CD81 ligation on long-term T cell activation
Another possible way that CD81 could affect T cell cytokine production is by directing T cells towards Th2 cytokine production during long-term stimulation. Other investigators have shown (in mouse systems) that
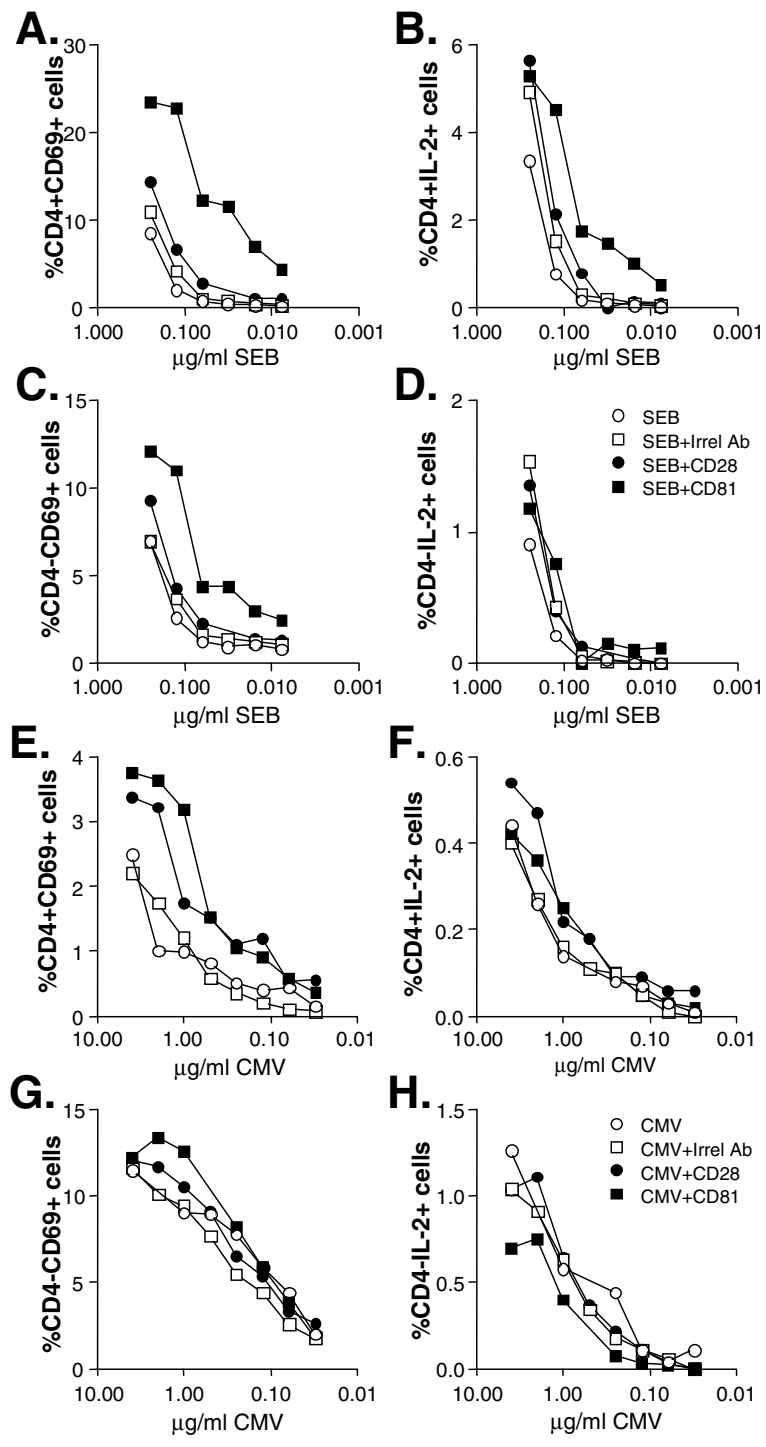
Figure 1
Costimulatory effect of CD81 stimulation on CD69 expression (A, C, E, and G) and IL-2 production (B, D, F, and H), measured by cytokine flow cytometry. Whole blood from a CMV seropositive donor was stimulated for 6 h with varying concentrations of SEB (A-D) or CMV (E-H) in the presence of the activator alone (open circles), an irrelevant iso-type control antibody (open squares), 5 μg/ml anti-CD28 mAb (closed circles), or 5 μg/ml 5A6 anti-CD81 mAb (closed squares). CD69 and IL-2 were then quantitated by intracellular staining on CD3+CD4+ cells (A, B, E, F) or CD3+CD4- cells (C, D, G, H). Results are representative of three experiments.
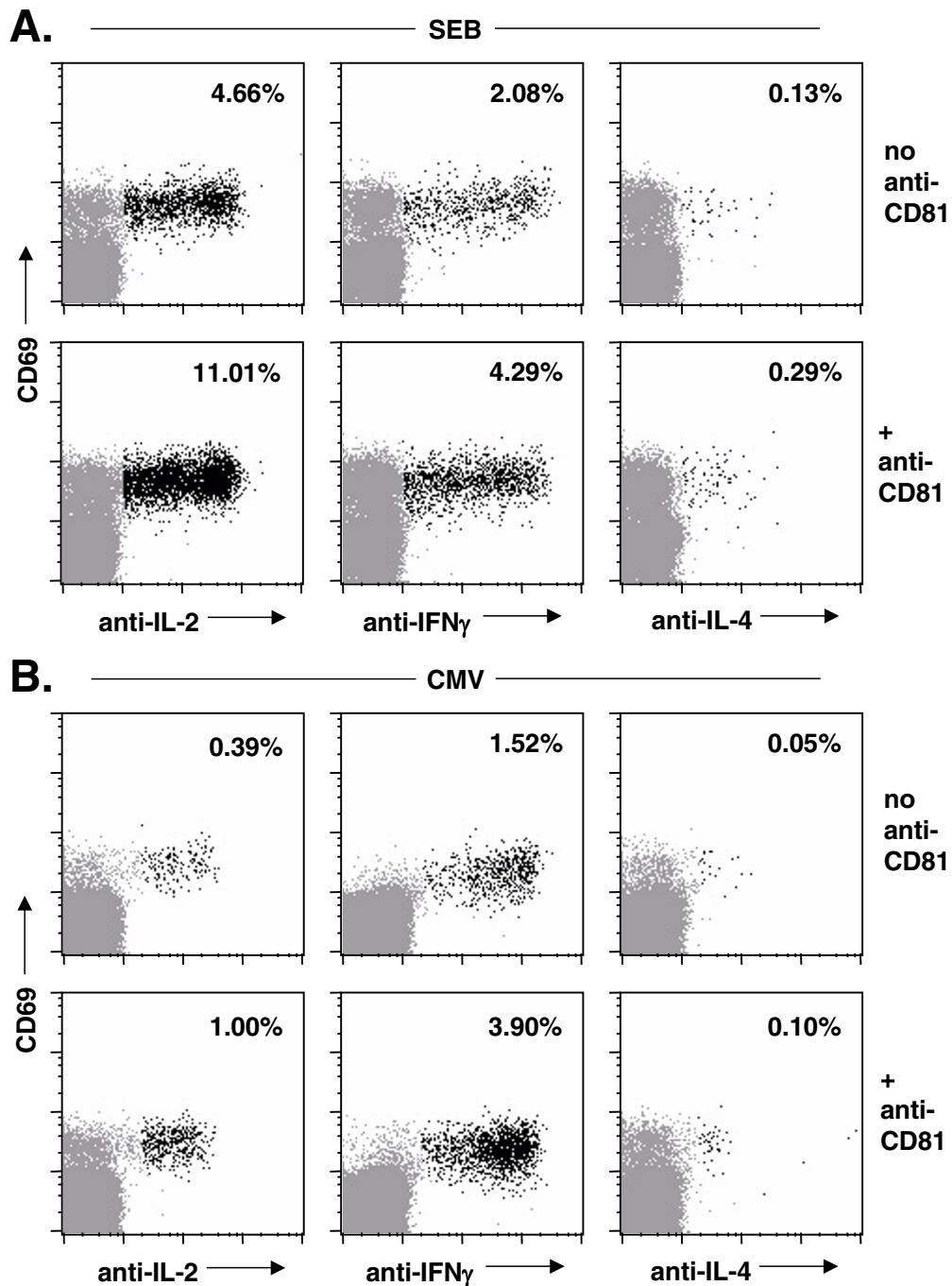
Figure 2
Effect of CD81 stimulation on production of Th1 and Th2 cytokines. (A) Cytokine flow cytometry data from 0.1 µg/ml SEB stimulation of whole blood in the absence (top panels) or presence (bottom panels) of 5 µg/ml 5A6 anti-CD81 mAb. (B) Similar experiment using 0.5 µg/ml CMV stimulation in the absence (top panels) or presence (bottom panels) of 5A6. Production of all three cytokines was equally increased in the presence of CD81 costimulation. Results are representative of three experiments.
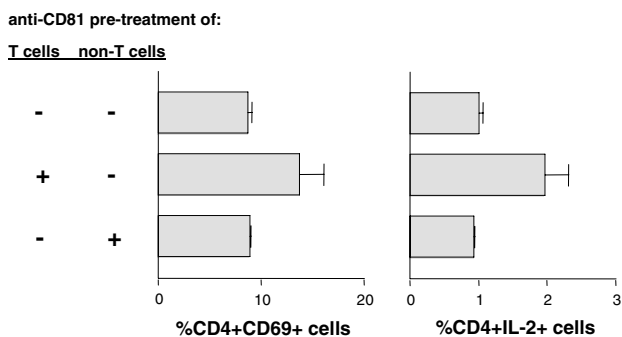
anti-CD81 pre-treatment of:

T cells   non-T cells

Figure 3
CD81 costimulation occurs via direct action on T cells. PBMC were treated with magnetic beads for depletion of non-T cells, then T cells or non-T cells were treated for one hour on ice with 10 μg/ml 5A6 anti-CD81 mAb, washed twice, and re-mixed. The cultures were then stimulated for 6 h in the presence of 0.1 μg/ml SEB, and CD69 and IL-2 expression were quantitated by intracellular staining. Note that pre-treatment of T cells, but not non-T cells, resulted in augmentation of CD69 expression and IL-2 production. Error bars represent S.D. Results are representative of three experiments.

commitment to Th1 or Th2 cytokine production by naïve T cells requires prior cell proliferation [24]; and reversal of Th1 or Th2-committed cells requires at least several days of proliferation [25]. When normal human PBMC were stimulated for 7 days with SEB, the presence of 5A6 anti-CD81 mAb resulted in a modest increase in IL-4-producing T cells, when those cells were restimulated with PMA and ionomycin (Figure 4A). No significant change in IL-2- or IFNγ-producing T cells was seen. Similar results were seen when PBMC were stimulated with plate-bound anti-CD3 mAb, rather than SEB, for 7 days in the presence of 5A6 anti-CD81 mAb (Figure 4B). These results were more dramatic when CD45RO+ cells were first isolated, by magnetic bead separation, and then subjected to 7 d stimulation by anti-CD3, in the presence or absence of 5A6 anti-CD81 mAb. As seen in Figure 4C, the percentage of CD4+ T cells producing IL-4 upon PMA+ionomycin restimulation was increased more than 6-fold when 5A6 anti-CD81 mAb had been added to the initial anti-CD3 culture. No significant change in IL-2 or IFNγ-producing cells was seen. A less dramatic increase in IL-4-producing cells was seen for CD45RO- cells (Figure 4D). The results for CD8+ T cells mirrored those for CD4+ T cells, except that the overall number of IL-4-producing CD8+ T cells was lower (data not shown).

Because the effect of CD81 on IL-4-producing T cells was most dramatic with CD45RO+ cells (Figure 4C), it was hypothesized that CD81 costimulation was affecting the number of IL-4-producing memory T cells over time. To determine the effect of CD81 costimulation over time on the absolute number of IL-4- or IFNγ-producing T cells, the experiment of Figure 5 was carried out. CD45RO+ T cells were stimulated with anti-CD3 ± anti-CD81 mAb as in Figure 4, but cells were restimulated with PMA + ionomycin after 1, 3, or 7 d of culture. The absolute number of IL-4- and IFNγ-producing T cells was then quantified using intracellular staining and TruCount™ beads. As seen in Figure 5, the number of IL-4-producing CD4 T cells increased much more significantly over time in cultures incubated with anti-CD81 mAb than in those without. The number of IFNγ-producing CD4 T cells increased similarly in cultures incubated with or without anti-CD81 mAb. Thus, CD81 costimulation appeared to preferentially induce the accumulation of IL-4-producing T cells over time.

To further test whether CD81 costimulation was inducing preferential proliferation of IL-4-producing memory cells, experiments with CFSE labeling were carried out. As seen in Figure 6A, the percentage of IL-4-producing cells increased preferentially over time in long-term anti-CD3 stimulations that received CD81 costimulation, relative to stimulations in the absence of anti-CD81 mAb. Although CFSE content was equal in all cells on day 0 (Figure 6B), the mean level of CFSE fluorescence of IL-4-producing cells became lower over time in the presence of CD81 costimulation versus anti-CD3 stimulation alone (Figure 6B and 6C). This indicates that IL-4-producing cells had divided, on average, more times with CD81 costimulation than without. The difference was apparent after one day of stimulation, and did not increase over time, consistent with a greater initial induction of proliferation of IL-4-producing cells in the presence of anti-CD81. There was no such difference in CFSE levels for IFNγ-producing cells in the presence or absence of anti-CD81 (Figure 6D). However, anti-CD28 costimulation induced lower levels of CFSE in all cells, including IFNγ-producing cells, after 3 days of anti-CD3 stimulation (p < 0.001, data not shown). Thus, CD81 costimulation appears to uniquely induce greater proliferation of IL-4-producing cells relative to other memory T cell populations.

Discussion
In this study, it was shown that CD81 ligation on human T cells costimulates the induction of CD69 expression, the production of cytokines, and the proliferation of both CD4+ and CD4- human T cells. Greater effects were seen on CD4+ T cells compared to CD4- T cells. The costimulatory effects were documented using two antigenic systems: a superantigen (SEB), and a conventional viral antigen
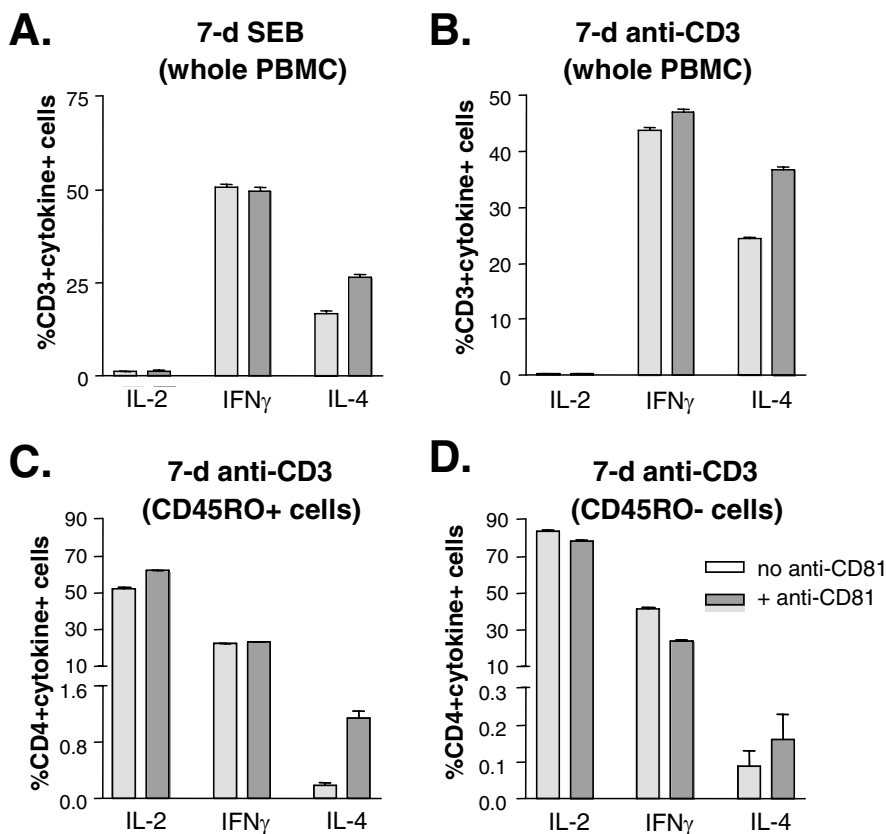
Figure 4
Long-term stimulation in the presence of 5A6 anti-CD81 mAb biases towards IL-4-producing T cells. (A and B) Unfractionated PBMC were cultured for 7 days with either 1 μg/ml SEB (A), or plate-bound anti-CD3 mAb (B) in the presence or absence of 5 μg/ml 5A6 anti-CD81 mAb (added once at the beginning of the culture period). After 7 days the cells were removed and restimulated for 4 h with PMA+ionomycin, and cytokine-producing T cells were enumerated by intracellular staining. (C and D) CD45RO+ cells (C) or CD45RO- cells (D) were isolated from PBMC by magnetic beads, then plated and stimulated for 7 days with plate-bound anti-CD3 as in panel (A). Cells were then removed and restimulated as above, and CD4+ or CD8+ T cell cytokine production measured by intracellular staining. Error bars represent S.D. Results are representative of four similar experiments.

(CMV). The effects were determined to be a direct consequence of CD81 ligation on T cells, as anti-CD81 mAb pre-treatment of purified T cells, but not non-T cells, induced the costimulatory effects.

In a previous study, Van Compernolle et al. showed that the enhancing effect of CD81 on SEB-induced activation was inhibited by antibody to LFA-1 [17]. The current results are consistent with the model proposed by VanCompernolle et al., whereby CD81 ligation induces an increase in LFA-1 avidity for ICAM-3 on accessory cells, resulting in an indirect activation effect on the T cells. However, in the current study, anti-LFA-1 was found to only partially block cytokine production induced by either SEB or SEB+anti-CD81 (data not shown). This suggests that CD81's effects on cytokine production may not be uniquely mediated by LFA-1. In fact, other investigators have shown that anti-CD81 mAb can directly costimulate purified murine T cells [16], in a presumably LFA-1-independent manner.

CD81 costimulation increased in equal proportion the number of T cells producing IL-2, IFNγ, and IL-4. Using CD81[null] mice, CD81 was found to be required for
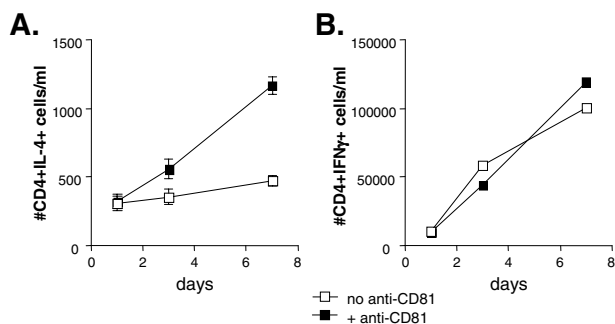
Figure 5
CD81 costimulation results in the accumulation of IL-4-producing CD4 T cells over time. CD45RO+ cells were stimulated with plate-bound anti-CD3 mAb in the presence or absence of 10 μg/ml 5A6 anti-CD81 mAb for 1, 3, or 7 days. They were then restimulated for 4 h with PMA+ionomycin, and cytokine-producing T cells were enumerated by intracellular staining. (A) The absolute number of IL-4-producing CD4 T cells increases more dramatically over time in the presence of anti-CD81 mAb than in its absence. (B) The absolute number of IFNγ-producing CD4 T cells is similar over time in cultures incubated with or without anti-CD81 mAb. Error bars represent S.D. Results are representative of two similar experiments.

effective Th2, but not Th1, immune responses [20–23]. But CD81 has also been found to generally costimulate T cell responses in mouse [16] and human [17]. Thus, the costimulation of T cell cytokine production by CD81 need not necessarily be biased towards Th2 cytokines. The current results bear this out.

More recent results have shown that CD81 expression on T cells is indeed important for their induction of IL-4 production [23] in a mouse system. The current study complements these results by demonstrating that CD81 directly influences the number of IL-4-producing T cells in cultures of stimulated human memory T cells. The effect of anti-CD81 mAb was to increase the absolute number of IL-4-producing T cells over time relative to cultures not costimulated with anti-CD81 mAb. Furthermore, the CD81 costimulation appeared to cause a greater induction of proliferation of IL-4-producing cells, relative to that seen in the absence of CD81 mAb.

The effect of CD81 costimulation on the number of IL-4-producing T cells could occur through several different mechanisms. First, it is possible that CD81 induces re-

cruitment of naïve cells to the Th2 pathway. This did not appear to be the case, as the effect of CD81 was most prominent on CD45RO+ cells, which do not include naïve T cells. However, CD81 signalling could induce the switching of some IFNγ-producing (Th1) T cells to IL-4-producing (Th2) T cells over time. This is a difficult mechanism to test, due to the low number of IL-4-producing T cells in human PBMC relative to IFNγ-producing T cells. Experiments attempting to deplete IL-4-producing T cells prior to anti-CD3 stimulation were attempted, but could not be accurately interpreted, since depletion of 100% of potential IL-4-producing cells could not be documented (data not shown). It was thus impossible to rule out this mechanism as possibly contributing to the accumulation of IL-4-producing cells over time, although it did not appear to be the major mechanism (see below).

Second, it is possible that CD81 costimulation preferentially rescues IL-4-producing T cells from apoptosis, and thus they accumulate to a greater degree over time. This did not appear to be the case, as IL-4-producing T cells were no more likely to be apoptotic than IFNγ-producing or IL-4-negative T cells in long-term stimulations (data not shown).

Finally, it is possible that CD81 induces preferential proliferation of IL-4-producing T cells relative to other T cells. This indeed appears to be the case, as seen by proliferation experiments using CFSE (see Figure 6). As cells divide, this cytosolic dye is diluted, such that cells express lower levels of CFSE with each successive division. Indeed, after just one day of stimulation, IL-4-producing cells in the presence of CD81 costimulation had lower mean levels of CFSE than did IL-4-producing cells in the absence of CD81 costimulation. This was not true for IL-4-negative or IFNγ-producing cells. The relative amount of CFSE in CD81-costimulated cultures versus non-costimulated cultures did not change further over time. Thus, it did not appear that IL-4-producing cells were continually proliferating faster in the presence of CD81 costimulation. Rather, they appeared to be more readily induced into proliferation, as the maximum difference in CFSE levels was already observed after one day of stimulation. Thus, the primary mechanism by which CD81 costimulation induces Th2 bias in long-term stimulation appears to be a greater induction of proliferation in IL-4-producing cells. This complements the findings of Deng et al. [23], in which the lack of CD81 in a murine culture system correlated with decreased expression of signalling molecules critical for activation and IL-4 production. This effect of CD81 is not shared by other classical costimulatory molecules, such as CD28, which has been shown to induce the proliferation of both Th1 and Th2 cells (data not shown, and references [26–29]).
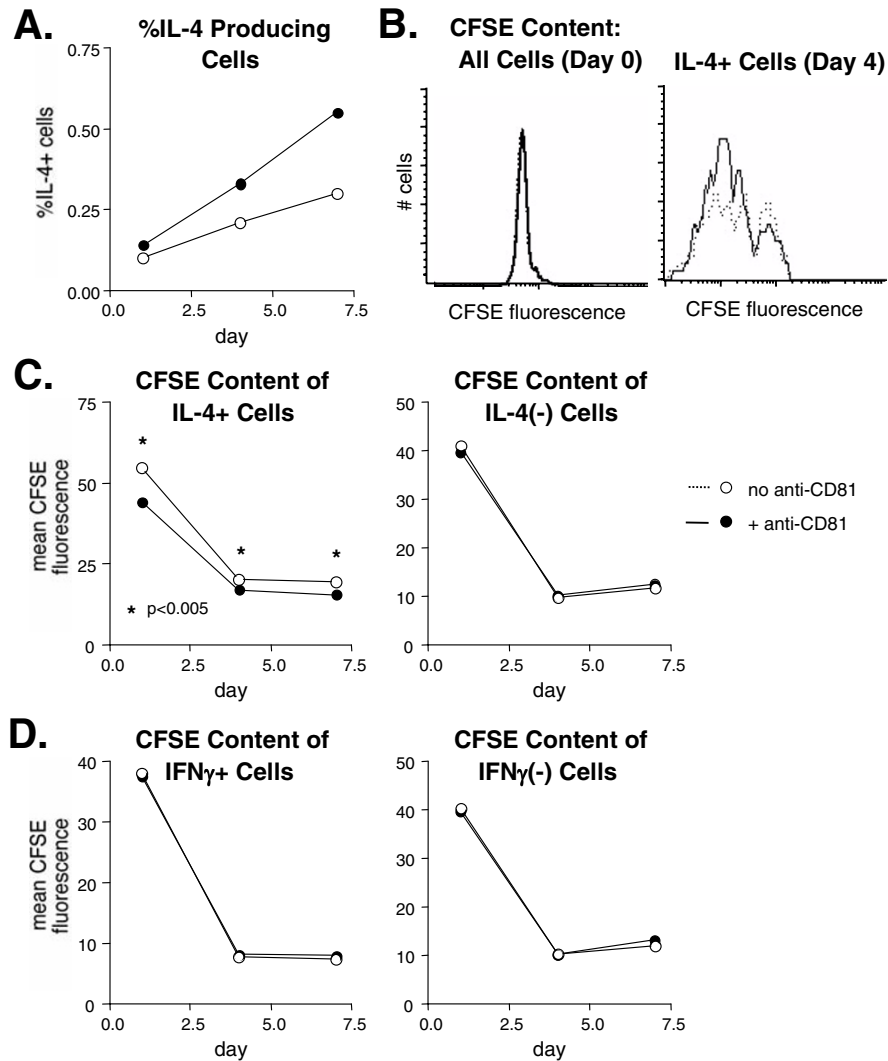
Figure 6
CD81 costimulation induces preferential proliferation of IL-4-producing cells. CD45RO^{+} PBMC were labeled with CFSE as described in Materials and Methods. They were then stimulated with plate-bound anti-CD3 mAb in the presence or absence of 5 μg/ml 5A6 anti-CD81 mAb for 1, 4, or 7 days. At these time points, cells were restimulated for 4 h with PMA+ionomycin, and cytokine-producing T cells were enumerated by intracellular staining. (A) The percentage of IL-4-producing cells increased faster over time in anti-CD81 mAb-treated cultures. (B) CFSE content of all cells at day 0 (left panel) was equal in the presence of anti-CD81 mAb (solid histogram) or in its absence (dotted histogram). The CFSE content of IL-4-producing cells (right panel) was lower in the presence of anti-CD81 mAb (solid histogram) than in its absence (dotted histogram), after 4 days of stimulation. (C) The mean CFSE content of IL-4-producing cells was lower in the presence of anti-CD81 mAb than in its absence (left panel); there was no such difference for IL-4-negative cells (right panel). (D) The mean CFSE content of IFNγ-producing or IFNγ-negative cells did not differ in the presence or absence of anti-CD81 mAb. Thus, CD81 costimulation preferentially induces proliferation of IL-4-producing cells. Results are representative of two similar experiments.

It should be noted that the present study, due to limitations of working with human PBMC, could not address the results of withdrawing endogenous CD81 signalling. Rather, CD81 co-stimulation could only be increased over basal levels by application of an agonistic mAb, 5A6. Because endogenous CD81 costimulation could still occur in cultures without 5A6 added, the differences due to addition of 5A6 might actually underestimate the full potential of CD81's effect. This caveat of the experimental system also implies that there could be considerable donor-to-donor variability in the effect of anti-CD81 mAb, as different donors could have different levels of endogenous CD81 costimulatory activity. The natural ligand for CD81 has never been identified.

Conclusions

In conclusion, CD81 costimulation appears to have multiple effects on T cells, including costimulation of Th1 and Th2 cytokine production in short-term assays, and preferential induction of Th2 cell proliferation in long-term cultures of human memory T cells. These results expand upon and help to explain the Th2-biasing effects of CD81 in mouse [21–23] and human [19] systems.

Methods

Cells and cell separation

Peripheral blood from normal human donors was collected under informed consent in accordance with an institutionally-approved and physician-supervised protocol. Blood was collected in heparinized Vacutainer™ tubes or heparinized CPT tubes (BD Vacutainer, Franklin Lakes, NJ). CPT tubes were centrifuged as directed by the manufacturer to obtain peripheral blood mononuclear cells (PBMC). These were resuspended for stimulation in the autologous plasma. For the experiments of Figures 3,4,5,6, PBMC were further fractionated using magnetic beads (Milltenyi Biotec, Auburn, CA) to deplete non-T cells, and/or to select for CD45RO$^+$ versus CD45RO$^-$ cells. Cell separation was performed on an AutoMACS system (Milltenyi) according to the manufacturer's instructions. For Figure 4, separated cells were then aliquoted and treated (or not) with 5 μg/ml 5A6 anti-CD81 mAb ([11], a gift of Shoshana Levy, Stanford University) for one hour on ice. Cells were then washed twice with medium, and resuspended in 0.5 ml medium. T cells (treated or not) were recombined with non-T cells (treated or not) for stimulation with SEB as described below. Medium consisted of RPMI-1640 + 10% fetal bovine serum and antibiotics.

Antigens

Human cytomegalovirus (CMV) lysate was obtained from Advanced Biotechnologies (Columbia, MD), aliquoted and stored at -80°C, and used at the doses indicated in the figures. Staphlococcal enterotoxin B (SEB), a superanti-

gen, was obtained from Sigma Chemical Co. (St. Louis, MO), and used at the doses indicated in the figures.

Short-term stimulations

0.2–1 ml of whole blood or PBMC was stimulated in 15 ml conical polypropylene tubes (Falcon, BD Discovery Labware, Bedford, MA) or 2 ml microtubes (Sorenson Bio-Science, Salt Lake City, UT). For experiments examining cytokine production and CD69 induction, whole blood or PBMC were incubated with antigen or superantigen for 6 h at 37°C, with addition of 10 μg/ml Brefeldin A (Sigma) at 2 h. For experiments examining proliferation, PBMC were incubated with SEB for 48 h at 37°C, with addition of 60 μM BrdU (Sigma) and 10 μg/ml Brefeldin A for the last 6 h. Whole blood was incubated in upright tubes, while PBMC were incubated in tubes slanted to 5° above horizontal. Incubations were carried out in the presence or absence of 5 μg/ml purified 5A6 anti-human CD81 mAb.

Long-term stimulations

For stimulation with anti-CD3 mAb, 24-well or 6-well tissue culture plates (Falcon) were coated with purified anti-CD3 mAb (clone JE6 [30]). A 10 μg/ml solution of JE6 in PBS was added to each well and incubated for 2 h at 37°C. Wells were then washed several times with PBS prior to addition of cells. PBMC, or fractions of PBMC isolated by magnetic bead separation, were resuspended in RPMI medium + 10% fetal bovine serum (Sigma), supplemented with L-glutamine and antibiotics, and plated at approximately 10^6 cells per ml. The cells were stimulated either with plate-bound anti-CD3 mAb (coated as described above) or soluble SEB superantigen (Sigma, at 1 μg/ml final concentration), in the presence or absence of 5–10 μg/ml 5A6 anti-CD81 mAb (added only at the initiation of the culture). After 1–7 days, cells were stimulated for 4 h with 10 ng/ml PMA + 1 μg/ml ionomycin (both from Sigma), in the presence of 10 μg/ml Brefeldin A.

Cell preparation and staining

Cells were prepared for cytokine flow cytometry as described previously [31–33]. Briefly, cells were incubated 15 min with 2 mM EDTA to remove adherent cells, then treated with 10 volumes of FACS Lysing Solution (BD Biosciences, Immunocytometry Systems, San Jose, CA) for 10 min at room temperature. Cells were washed, aliquoted into 12 × 75 mm polystyrene tubes (Falcon), and treated with 0.5 ml FACS Permeabilizing Solution 2 (BD Immunocytometry Systems) for 10 min at room temperature. After washing once more, cells were stained (30–60 min at room temperature) for flow cytometry, typically with anti-cytokine FITC/CD69 PE/CD4 PerCP-Cy5.5/CD3 APC. For proliferation experiments, cells were stained with anti-BrdU FITC/anti-cytokine PE/CD4 PerCP (all antibodies from BD Immunocytometry Systems).

After an additional wash, cells were fixed with 1% paraformaldehyde for flow cytometry analysis.

Flow cytometry

Samples were collected on a FACSCalibur flow cytometer using CellQuest software (BD Immunocytometry Systems). In general, 20,000–100,000 CD3-gated (or, in some experiments, CD4-gated) lymphcytes were acquired, using a logical gate on forward versus side scatter and CD3 (or CD4) versus side scatter. For experiments examining cytokine production and CD69 induction, a "response region" was defined for CD69+cytokine+ cells using a positive control such as SEB stimulation, on a CD4-gated dot plot. Background responses in the presence of 5A6 anti-CD81 antibody alone were less than 0.05% in all experiments shown.

Absolute cell counts

Absolute numbers of CD4+ T cells producing IL-4 or IFNγ were calculated by spiking samples, processed and stained as above, with 10 µl of TruCount™ High control beads (BD Immunocytometry Systems). Samples were then acquired with a threshold on FL4 (CD3), rather than forward scatter, and the number of beads per sample were calculated by gating on the bead population in a forward versus side scatter dot plot. The number of cytokine-producing cells per ml were then calculated as: [(number of gated cells)/(number of gated beads)] × [number of beads per sample].

CFSE labeling

PBMC magnetically enriched for CD45RO+ cells were labeled with 5(6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Sigma) as follows. A 0.5 mM stock solution of CFSE in DMSO was aliquoted and stored at -20°C. Just prior to use, an aliquot was thawed and 2 µl of stock solution were diluted into 10 ml of PBS. CD45RO+ cells were washed once in PBS and resuspended in 1 ml of the CFSE/PBS solution. After incubating 2 min at room temperature, the cells were washed once with 10 ml of RPMI medium + 10% fetal bovine serum (supplemented with L-glutamine and antibiotics). They were then plated in wells of a 24-well plate as under "Long term stimulations" above.

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