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CD80 expressed on the surface of APCs provides a positive costimulatory signal to naive CD4+ T cells via CD28 during activation. However, CD80 is also expressed on the surface of activated CD4+ T cells, and cross-linking CD80 on the surface of CD4+ T cells activated in the presence of Th1-promoting cytokines induces a direct up-regulation of T-bet, IFN-γ, and Belr expression in primary CD4+ T cells. The present data show that naive CD4+ T cells activated in Th1-promoting conditions in the presence of anti-CD80 mAb increase the level of IFN-γ produced by increasing the rate of IFN-γ mRNA transcription, which is supported by an increase in the level of T-bet phosphorylation and T-bet binding to the third intronic enhancer in the IFN-γ locus. Furthermore, anti-CD80 mAb-induced increase in IFN-γ expression and T-bet phosphorylation is dependent upon the activation of a Ca2+-dependent pathway as shown by anti-CD80 mAb-induced intracellular Ca2+ flux following CD80 cross-linking. These findings indicate a novel regulatory role for CD80-mediated intracellular signals in CD4+ T cells and have important implications for disease therapies using anti-costimulatory mAbs as use of an intact CD80 mAb may lead to CD80 cross-linking on activated T cells and enhanced proinflammatory cytokine production. The Journal of Immunology, 2009, 182: 766–773.

According to the two-signal hypothesis for the activation of naive CD4+ T cells, the first signal necessary for naive CD4+ T cell activation is Ag-specific TCR binding of antigenic peptide-MHC II complex on an APC. The second signal, a costimulatory signal, is largely provided via CD28 expressed on the CD4+ T cell surface binding one of the B7 family members (CD80 or CD86) expressed on the surface of activated APCs (1–4). Although this model of CD4+ T cell activation and differentiation is over simplistic, the two-signal hypothesis has been the basis of multiple therapeutic strategies for treatment of autoimmune disease. For example, it has been hypothesized that blockade of these costimulatory signals may serve as an effective immunotherapy in relapsing-remitting experimental autoimmune encephalomyelitis (R-EAE) (5, 6), diabetes (7), and transplantation (8, 9). As such, multiple studies have detailed the effects of anti-CD80 mAb treatment during the course of R-EAE. Depending on the time of treatment, varying effects on disease outcome have been reported (10). These findings suggest that the blockade of CD80 interacting with its ligand is able to modulate T cell, B cell, and dendritic cell function both in a positive or negative manner (11–13). Although the signaling events following CD28-B7 interACTION have been studied for the CD28 signaling cascade in CD4+ T cells, the ability of CD80 cross-linking to induce an alteration in the function of “classic” CD80-expressing cell types, such as B cells and dendritic cells (1, 2), as well as primary CD4+ T cells (11) has not been thoroughly elucidated.

Besides APCs, activated CD4+ T cells also express CD80 and CD86 (11, 14, 15), and these costimulatory molecules potentially signal to a CD4+ T cell directly following cross-linking with mAb (11). Because CD4+ T cells express CD80 following activation, and CD80 cross-linking induces a putative intracellular signal, the possibility exists that CD80 stimulation of CD4+ T cells may affect T cell effector function. Previous work from our laboratory has shown that cross-linking of CD80 on CD4+ T cells induces an increase in IFN-γ and T-bet transcripts and protein expression (11). The transcriptional regulation of IFN-γ has been widely studied. DNase I hypersensitivity mapping of the IFN-γ locus regulatory regions in Th1 cells have identified three main regulatory regions for the IFN-γ locus. Of these three regions, the distal enhancer sequence within the third intron appears to contain the highest level of cis-regulatory activity in transfection assays (16). Initiation and maintenance of transcriptional control at the IFN-γ locus is primarily via T-bet (17).

Our previous results demonstrated that anti-CD80 mAb treatment induces an increase in IFN-γ and T-bet, without altering the number of IFN-γ secreting cells (11). Considering the aforementioned finding that the third intronic enhancer regulates transcriptional activity at the IFN-γ locus, we hypothesized that cross-linking CD80 on the surface of a CD4+ T cell activated in Th1-promoting conditions induces an increase in T-bet phosphorylation and binding to the third intronic enhancer. In this study, we show that CD80 cross-linking increases the level of IFN-γ by increasing the rate of IFN-γ transcription, and binding of T-bet to the third intronic enhancer. Further, we show that cross-linking CD80 on CD4+ T cells induces the activation of a Ca2+-dependent signaling pathway. The present data help to illustrate the complexity of using intact mAbs for therapeutic use in vivo, given that bivalent mAbs may bind and cross-link the same surface molecules on multiple cell types leading to diverse functional outcomes.
Materials and Methods

Mice and CD4⁺ Th cell-promoting culture conditions

Female SJL (Harlan Sprague Dawley), C57BL/6 (The Jackson Laboratory), and PD-L1 knockout (provided by L. Chen at Johns Hopkins University, Baltimore, MD) were housed under specific pathogen-free conditions in the Northwestern University Animal Facility. Naïve CD4⁺ T cells were purified using AutoMacs Magnetic Bead cell separation technology (Miltenyi Biotec) from total lymph node cells isolated from unprimed mice. The purity of the isolated naïve CD4⁺ T cells (L-selectin⁺CD4⁺ cells) was routinely found to be 98–99.9%. For in vitro activation, 3–5 × 10⁵ naïve CD4⁺ T cells were activated in the presence of 5–25 × 10⁵ latex beads coated with 1 µg of anti-CD3 and/or 1 µg anti-CD28 in neutral (IL-2 at 200 U/ml, Th1-driving (IL-2 at 200 U/ml; IL-12 at 40 U/ml; anti-IL-4 at 10 µg/ml), or Th2-driving (IL-2 at 200 U/ml; IL-4 at 500 U/ml; anti-IFN-γ at 50 µg/ml) (Armenian Hamster IgG (eBioscience)) or intact anti-CD80 Ab (clone 16-10A1 (Bio X Cell)).

ELISA and Ca²⁺ flux

Naïve CD4⁺ T cells were isolated and activated as described above. After 24 h in culture, the cells were collected and labeled with 1 µM Indo-1 (Invitrogen) for 15 min at 37°C followed by repeated washes and continued incubation at 37°C for an additional 2 h. In some experiments cells were treated with cell signaling inhibitors (Wortmannin (50 nM), GF109203X (20 nM), Go-6967 (2.3–20 nM), p38 MAPK inhibitor (35 nM), SB202190 (30 nM), or U73122 (1.5 µM) (BioSource International)) during the final 30 min before analysis in the presence of either Control Ig or anti-CD80 mAb. Cells were sampled by an LSRII for 30 s before the addition of an increasing concentration of either isotype control, anti-CD3, antiCD80 mAb, and/or anti-CD80 Fab. Sample analysis was immediately continued following Ab addition for an additional 3 min. Data are presented as the ratio of 398 nm (Indo-1 bound to Ca²⁺) / 482 nm (unbound Ca²⁺) in CD4⁺ T cells over a period of 3.5 min.

Nuclear run-on

The rate of IFN-γ transcription was determined by nuclear run-on, as described in detail elsewhere (18, 19). In brief, 20 × 10⁵ naïve CD4⁺ cells were activated as described above, collected on day 3 following activation. Nuclear run-on and RNA isolation were performed in the presence of unprimed mice. The purity of the isolated naïve CD4⁺ T cells (L-selectin⁺CD4⁺ cells) was routinely found to be 98–99.9%. For in vitro activation, 3–5 × 10⁵ naïve CD4⁺ T cells were activated in the presence of 5–25 × 10⁵ latex beads coated with 1 µg of anti-CD3 and/or 1 µg anti-CD28 in neutral (IL-2 at 200 U/ml, Th1-driving (IL-2 at 200 U/ml; IL-12 at 40 U/ml; anti-IL-4 at 10 µg/ml), or Th2-driving (IL-2 at 200 U/ml; IL-4 at 500 U/ml; anti-IFN-γ at 50 µg/ml) (Armenian Hamster IgG (eBioscience)) or intact anti-CD80 Ab (clone 16-10A1 (Bio X Cell)).

IFN-γ transcript stability and real-time PCR

On day 3 following the initial activation of naïve CD4⁺ T cells in Th1-promoting conditions, 20 µg/ml actinomycin D (Sigma-Aldrich) was added to each culture to stop the further production of mRNA transcripts. T cells were collected from the cultures over a 16 h time course following the addition of actinomycin D, cell viability was analyzed by trypan blue exclusion, and total mRNA isolated. Total mRNA was isolated with TRIzol Reagent (Invitrogen) and was reversed transcribed into cDNA using random hexamer primers. In brief, a common master mix (LightCycler-FastStartDNA SYBR Green 1 (Roche), 2 mM MgCl₂, 0.5 µg/ml proteinase K, 50 mM Tris–HCl, pH 8.3) was added, and the sample was amplified for 30 cycles (45 s at 94°C, 45 s at 56°C, and 40 s at 72°C) using primers specific for the IFN-γ transcript. The ratio of 398 nm (Indo-1 bound to Ca²⁺) to 482 nm (unbound Ca²⁺) was determined in CD4⁺ T cells over a period of 3.5 min.

Western blot

Five × 10⁶ naïve CD4⁺ T cells were activated as described above. For total cellular protein, cells were collected, washed three times with PBS, lysed with 1% Triton X-100 lysis buffer, and frozen at −80°C until analysis. Protein samples (5–10 µg) were run on a denaturing 7.5% polyacrylamide gel and transferred to Immunoblot-P PVDF membranes (Millipore). Membranes were blocked with TBST plus 5% dried milk, probed with primary Abs diluted in TBST plus 5% dried milk, and washed. Membranes were then probed with HRP-labeled secondary Abs diluted in TBST plus 5% dried milk, washed, and HRP-labeled Abs were detected using the LumiGlo Detection Kit (Cell Signaling Technology) and specific bands were visualized on Kodak Biomax MS film using an imaging screen-enabled film cassette. Abs used were anti-T-bet (C-15) (Santa Cruz Biotechnology), anti-actin (C-11), and anti-phosphorylated tyrosine (clone PY20) (Upstate Biotechnology).

Chromatin immunoprecipitation (ChIP)

ChIP analysis was conducted essentially as described previously (20). Naïve T cells (10 × 10⁶ cells) activated as described above were collected on day 3, fixed with 1.1% formaldehyde solution, and cross-linking was stopped by the addition of glycine at a final concentration of 0.125 M for 5 min. Cells were rinsed with cold PBS and resuspended in 0.25% Triton X-100 lysis buffer. Nuclei were pelleted, resuspended, and sonicated ten times for 30 s, with a 1 min cooling period on ice in-between. Debris was removed and samples were precleared with protein A/protein G agarose beads that had been blocked with sonicated salmon sperm DNA and BSA. The beads were removed and chromatin samples were incubated at 4°C with the chromatin immunoprecipitation Abs (Millipore) during the final 1 h as described previously (19). Membranes were blocked with TBST plus 5% dried milk, probed with primary Abs diluted in TBST plus 5% dried milk, and washed. Membranes were then probed with HRP-labeled secondary Abs diluted in TBST plus 5% dried milk, washed, and HRP-labeled Abs were detected using the LumiGlo Detection Kit (Cell Signaling Technology) and specific bands were visualized on Kodak Biomax MS film using an imaging screen-enabled film cassette. Abs used were anti-T-bet (C-15) (Santa Cruz Biotechnology), anti-actin (C-11), and anti-phosphorylated tyrosine (clone PY20) (Upstate Biotechnology).

Statistical analyses

Data were analyzed by a one-way ANOVA to determine whether an overall all statistically significant change existed before using the two-tailed unpaired Student’s t test. Statistically significant differences were reported when the p value was <0.05.

Results

Cross-linking CD80 induces an increase in IFN-γ produced

Our previous findings indicated that addition of an intact anti-CD80 mAb may send a signal to the CD4⁺ T cell resulting in increased IFN-γ production (11). Alternatively, the addition of anti-CD80 mAb may block a negative costimulatory molecule that interacts with CD80, such as CTLA-4 (2) or PD-L1 (21). PD-L1 is hypothesized to act as a negative regulatory molecule for effector CD4⁺ T cell function. Therefore, we wished to determine whether the addition of anti-CD80 mAb was indeed sending a direct signal as opposed to blocking a negative regulatory signal. Because the addition of the non-cross-linking anti-CD80 Fab does not induce an increase in the level of IFN-γ produced, the later possibility appeared to be unlikely (11). However, we wished to test further whether the addition of anti-CD80 mAb was indeed sending a direct signal as opposed to blocking a negative regulatory signal. Because the addition of the non-cross-linking anti-CD80 Fab does not induce an increase in the level of IFN-γ produced, the later possibility appeared to be unlikely (11). However, we wished to test further whether the two aforementioned possibilities may play a role in the anti-CD80 mAb-induced increase in IFN-γ production. Naïve CD4⁺ T cells were activated in the presence of Th1 cell-promoting conditions and the level of CD80 and CTLA-4 was determined over a 48 h time course. As shown in Fig. 1a, CD80 is expressed by the 38.7% of the CD4⁺ T cells by 12 h.
and increases to 63.3% by 24 h. CD80 expression appears to peak at 48 h, and a small percentage of CD80/CTLA-4 double positive cells (10.5%) are observed by 48 h. Furthermore, we found that both CD28 (22, 23) and PD-L1 (21) were constitutively expressed by the cultured CD4\(^+\) T cells (data not shown).

We next determined whether the addition of a natural ligand, CTLA-4, for CD80 was able to induce an increase in the level of IFN-\(\gamma\) produced. As shown in Fig. 1b, the addition of CTLA-4 Ig induced an increase in the level of IFN-\(\gamma\) produced by naive CD4\(^+\) T cells activated in Th1-promoting conditions. Furthermore, both

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Anti-CD80 mAb induces an increase in IFN-\(\gamma\) in the absence of PD-L1. CD4\(^+\) T cells were isolated from naive wild-type SJL mice and cultured (10\(^6\) cells) with anti-CD3 plus anti-CD28 coated beads plus IL-2 and IL-12 (4 ng/ml) plus anti-IL-4 (10 \(\mu\)g/ml) (Th1-promoting conditions). Cells were collected over a 48-h time course and the percent of CD4\(^+\)/CD28\(^+\) T cells expressing CD80 vs CTLA-4 (A) was determined by flow cytometry. B, Naive CD4\(^+\) T cells were activated in the presence of Th1-promoting conditions in the presence of Control Ig, intact anti-CD80 mAb or CTLA-4 Ig (1 \(\mu\)g/ml) plus increasing concentrations of anti-CD80 Fab (0–100 \(\mu\)g/ml) and culture supernatants were collected on day 3 to assay the amount of IFN-\(\gamma\) produced via ELISA. C, Likewise, naive CD4\(^+\) T cells from wild-type and PD-L1\(^{-/-}\) C57BL/6 mice were activated in the presence of Th1-promoting conditions plus Control Ig, anti-CD80 mAb, anti-CD80 Fab, CTLA-4 Ig, anti-CTLA-4, or soluble anti-CD28 in the absence or presence of anti-CD80 mAb (1 \(\mu\)g/ml). Data are presented as the mean units of IFN-\(\gamma\)/ml produced from three replicate wells. * Statistically significant increase in the level of total IFN-\(\gamma\) produced in comparison to control Ig-treated cells, \(p < 0.05\). One representative of two independent experiments is shown.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Anti-CD80 mAb treatment-induced increase in T-bet expression and tyrosine phosphorylation. Naive CD4\(^+\) T cells were activated in culture as described in Fig. 1, plus the addition of cells cultured in the presence of anti-CD3 plus anti-CD28 (1 \(\mu\)g/ml) coated beads plus IL-2 (200 U/ml) (Th0-promoting conditions); or anti-CD3 plus anti-CD28 coated beads plus IL-2 plus IL-4 (11.1 ng/ml) plus anti-IL-12 and anti-IFN-\(\gamma\) (10 \(\mu\)g/ml) (Th2-promoting conditions). After 24 h in culture, either a species and isotype-matched Control Ig, or anti-CD80 mAb (1 \(\mu\)g/ml) was added to the cultures and total cellular protein isolated 3 h later. A, The level of T-bet expression was determined by Western blot, and the level of tyrosine phosphorylated T-bet was determined by first immunoprecipitating total T-bet, and probing the blots with either anti-T-bet or anti-phosphotyrosine Abs. B, The fold increase in the level of total T-bet expression and the level of tyrosine phosphorylated T-bet following the addition of anti-CD80 mAb as determined by densitometry compared with cells that received Control Ig treatment from three individual experiments is shown. * Statistically significant increase in the level of total T-bet and phosphorylated T-bet in comparison to control Ig treated cells, \(p < 0.05\).
the anti-CD80 mAb- and CTLA-4 Ig-induced increases in IFN-γ production were blocked by the addition of anti-CD80 Fab. To determine whether the addition of anti-CD80 mAb potentially blocks a negative regulatory signal resulting from CD80 interacting with PD-L1 during T cell-T cell interactions, naive CD4⁺ T cells from wild-type and PD-L1−/− mice were activated in Th1-promoting conditions. As shown in Fig. 1c, the addition of anti-CD80 mAb, CTLA-4 Ig, or anti-CD28 induces an increase in the level of IFN-γ produced in both the presence or absence of PD-L1 expression. Furthermore, the anti-CD80 mAb-induced increase in IFN-γ is blocked by the addition of anti-CD80 Fab. Collectively, these findings suggest that the anti-CD80 mAb-induced increase in the level of IFN-γ is due to cross-linking CD80 on the surface of CD4⁺ T cells, not due to blockade of a negative regulatory signal.

**Cross-linking CD80 induces T-bet phosphorylation**

Naive CD4⁺ T cells activated in Th1-promoting conditions demonstrate a time-dependent increase in the levels of CD80 expression (Fig. 1a) (11, 15, 24), and cross-linking CD80 by the addition of an anti-CD80 mAb increases the level of IFN-γ message and protein, and T-bet message expressed (11, 25). To determine whether the CD80-induced increase in the level of T-bet message is translated into the functional protein, naive CD4⁺ T cells were activated in neutral (anti-CD3/28 plus IL-2), Th1-promoting (added IL-12 and anti-IL-4 mAb), or Th2-promoting (added IL-4, anti-IL-12 mAb, and anti-IFN-γ mAb) conditions in the presence of either a species and isotype matched control Ig or anti-CD80 mAb. As shown in Fig. 2a the level of T-bet protein present within

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**FIGURE 3.** Cross-linking CD80 increases the rate of IFN-γ transcription. Naive CD4⁺ T cells were activated in culture as described in Fig. 1. A. After 3 days in culture, nuclei were isolated from 20 × 10⁶ cells and nuclear run-on was performed in the presence of biotin-16-UTP. Biotin-labeled RNA was incubated with streptavidin-labeled magnetic beads, and isolated by magnetic separation. The level of IFN-γ transcript was analyzed by RT-PCR. The level of IFN-γ transcript produced was determined for cultures that received Control Ig (●), anti-CD80 mAb (1 μg/ml) (▲), anti-CD80 mAb (1 μg/ml) plus anti-CD80 Fab (1 μg/ml) (○), and anti-CD80 mAb (10 μg/ml) (●). Data are presented as the normalized OD of IFN-γ transcript from one representative of two independent experiments. *, Statistically significant increase in the level of total IFN-γ in comparison to control Ig treated cells, p < 0.05. B. To determine the stability of the IFN-γ transcript, 10⁶ cells were collected (t = 0), and then actinomycin-D (20 μg/ml) was added to the remaining cells in culture. Cells with actinomycin-D were collected over a 4 h time course. Cell viability was analyzed by trypan blue exclusion and total cellular RNA was collected for quantitation of IFN-γ transcript by real-time PCR. Linear regression was done to determine the rate of IFN-γ degradation. One representative experiment of three independent experiments is shown. C. After 3 days, 10 × 10⁶ cells were fixed, nuclei isolated, lysed, and DNA was sheared into 200–500 bp fragments by sonication. T-bet bound to the DNA was then immunoprecipitated by the use of an anti-T-bet Ab. The amount of T-bet bound to the IFN-γ promoter and third intronic enhancer was analyzed by PCR. The amount of T-bet-specific PCR product (T) for each sample was determined by subtracting out the nonspecific PCR product from samples that received a species and isotype matched control Ab (C) during the immunoprecipitation and normalized to the amount of input DNA (I). One representative experiment of two is shown.
CD4⁺ T cells activated in Th1-promoting conditions in the presence of anti-CD80 mAb was increased as determined by Western blot. However, when naive CD4⁺ T cells were activated in Th2-promoting conditions, T-bet expression was not induced in either the presence or absence of anti-CD80 mAb.

Tyrosine phosphorylation of T-bet has been shown to regulate the Th2 genetic program through the physical interaction with GATA-3 (26). To further determine whether the role of the anti-CD80 mAb-induced increase in T-bet protein is functionally relevant, we evaluated the level of T-bet phosphorylation. T-bet was immunoprecipitated and membranes probed with either an anti-T-bet mAb or a species and isotype-matched control Ig or various concentrations of anti-CD80 mAb. As shown in Fig. 3a, as the concentration of anti-CD80 mAb added to the cultures was increased, the rate of IFN-γ transcription increased as determined by nuclear run-on. Furthermore, the anti-CD80 mAb-induced increase in the rate IFN-γ transcription was blocked by the addition of a non-cross-linking anti-CD80 Fab.

To determine whether the stability of IFN-γ transcript was affected by cross-linking CD80, actinomycin D was added to the in vitro cultures on day 3 after initial activation. Cells were collected before, and at various times after, the addition of actinomycin D. Cell viability was assessed at each of these time points by trypan blue exclusion, and the overall viability of the cells did not change significantly until 4–16 h after the addition of actinomycin D. Because a two-fold decrease in cell viability was seen at the 6 h time point, these cells were eliminated from analysis. The level of IFN-γ transcript present at the remaining time points was quantified by real-time PCR. Linear regression was done for each of the treatment groups, so that the stability of the mature transcript could be determined following cross-linking CD80 in our in vitro model system. As shown in Fig. 3b, stimulation of CD80 does not affect the stability of IFN-γ transcript as compared with the species- and isotype-matched control Ab group. These results indicate that cross-linking CD80 on a CD4⁺ T cell activated in Th1-promoting conditions increases the rate of IFN-γ transcription, while not affecting the stability of the transcript produced.

We next determined whether anti-CD80 mAb-induced increase in T-bet expression and phosphorylation is relevant to the increase in the rate of IFN-γ transcription by assessing the level of T-bet bound to the IFN-γ regulatory regions, i.e., the IFN-γ promoter and the third intronic enhancer, using ChIP. PCR primer sets were designed to amplify regions of DNA that are known to contain the IFN-γ promoter and the third intronic enhancer, and of the regions containing T-bet binding sequences (16, 28). The data show that the addition of anti-CD80 mAb induces an increase in the level of T-bet bound to the third intronic enhancer (Fig. 3c). In contrast, the level of T-bet bound to the IFN-γ promoter was not altered by the addition of anti-CD80 mAb. Collectively, cross-linking CD80 on the surface of CD4⁺ T cells activated in Th1-promoting conditions increases the expression, phosphorylation, and binding of T-bet to the IFN-γ regulatory regions.

**FIGURE 4.** Inhibition of anti-CD80 mAb-induced T-bet phosphorylation and IFN-γ. Naive CD4⁺ T cells were activated in culture as described in Fig. 1. After 24 h, either a vehicle control or specific inhibitors (Wortmannin (50 nM), Go6967 (2.3–20 nM), p38 MAPK inhibitor (35 nM), GF109203X (20 nM), SB202190 (30 nM), or U73122 (1.5 μM)) were added, followed 30 min later by the addition of either a species and isotype matched Control Ig or anti-CD80 mAb (1 μg/ml). After 3 h in culture, total cellular protein was isolated and the level of tyrosine phosphorylated T-bet was determined by first immunoprecipitating T-bet and probing the blots with either anti-T-bet or anti-phosphotyrosine Abs. B. After 48 h, culture supernatants were collected and the level of IFN-γ produced was determined by ELISA. One representative experiment of three is shown. *, Statistically significant decrease in the level of total IFN-γ in comparison to anti-CD80 mAb treated cells in the vehicle, p < 0.05.

**Inhibition of anti-CD80 mAb-induced T-bet phosphorylation and IFN-γ**

Because cross-linking CD80 on CD4⁺ T cells activated in Th1-promoting conditions increases the level of T-bet phosphorylation, a putative starting point for the identification of the CD80 signaling pathway has been identified. Published data show that T-bet is phosphorylated on tyrosine residues through the activation of Ca²⁺–dependent pathways (26, 29, 30). Therefore, we determined whether the inhibition of various signaling pathways that involve tyrosine phosphorylation was able to inhibit the anti-CD80 mAb-induced increase in T-bet phosphorylation. Following initial activation for 24 h in Th1-promoting conditions, cells were treated with inhibitors of various signaling pathways or vehicle for 30 min, at which time anti-CD80 mAb was added and total cellular protein isolated after an additional 3 h. As shown in Fig. 4a, blockade of the Ca²⁺–dependent pathways was able to inhibit the phosphorylation of T-bet. To determine whether the inhibition of T-bet...
was functionally relevant, the level of IFN-γ protein secreted was determined via ELISA. The data show that blockade of the Ca\(^{2+}\)-dependent pathways also inhibits the anti-CD80 mAb-induced increase in the level of secreted IFN-γ (Fig. 4B). These findings indicate that cross-linking CD80 on CD4\(^+\) T cells activated in Th1-promoting conditions induces a putative Ca\(^{2+}\)-dependent pathway.

**Cross-linking CD80 induces an intracellular Ca\(^{2+}\) flux**

Because the above findings suggest that cross-linking CD80 on the surface of CD4\(^+\) T cells activated in the Th1-promoting conditions activates a Ca\(^{2+}\)-dependent signaling pathway, we sought to determine whether cross-linking CD80 on naive CD4\(^+\) T cells activated in Th1-promoting conditions induces a Ca\(^{2+}\)-flux within CD4\(^+\) T cells. Naïve CD4\(^+\) T cells activated in Th1-promoting conditions for 24 h induces the surface expression of CD80 (11). CD4\(^+\) T cells were then treated with a vehicle control (DMSO), specific signaling pathway inhibitors, or anti-CD80 Fab for 30 min before stimulation with anti-CD80 mAb or anti-CD3 mAb as a control for the induction of a Ca\(^{2+}\)-flux. As shown in Fig. 5, the addition of anti-CD3 mAb or anti-CD80 mAb to CD4\(^+\) T cells activated in the Th1-cell-promoting conditions and DMSO induces a Ca\(^{2+}\)-flux. Pretreatment of naive CD4\(^+\) T cell activated in Th1-promoting conditions cells with anti-CD80 Fab was able to inhibit the anti-CD80 mAb-induced Ca\(^{2+}\)-flux demonstrating the specificity of the induction. In addition, pretreatment with the inhibitors, wortmannin and U73122, blocked the induction of the anti-CD80 mAb-induced Ca\(^{2+}\)-flux. In contrast, when cells are pre-treated with the PKCa inhibot inhibitor Go6976, which is down stream of the Ca\(^{2+}\) flux, had no effect on the anti-CD80 mAb-induced Ca\(^{2+}\)-flux. Collectively, the present findings suggest that cross-linking CD80 on CD4\(^+\) T cells activated in Th1-promoting conditions induces a Ca\(^{2+}\) flux.

**Discussion**

Although limited data are available to support a functional role of CD80 expression by CD4\(^+\) T cells during an immune response (31, 32), the current results support the hypothesis that stimulation via CD80 on CD4\(^+\) T cells positively regulates effector Th1 function. Cross-linking CD80 on T cells was previously shown to induce an increase in the tyrosine phosphorylation of multiple intracellular proteins (33), and increases the level of IFN-γ and T-bet transcription (11). To our knowledge, this is the first report that begins to identify a putative signaling pathway induced following cross-linking of CD80 on CD4\(^+\) T cells or any other cell type. Our findings indicate that cross-linking CD80 on CD4\(^+\) T cells activated in Th1-promoting conditions increases the level of IFN-γ produced by increasing the rate of IFN-γ transcription via the activation of a Ca\(^{2+}\)-dependent signaling pathway.

Over the past several years there has been an increase in the number of findings that suggest the ability of two B7 family members, i.e., CD80 and CD86, to induce intracellular signals directly to the cell that the molecule is expressed on. For example, cross-linking CD80 on the surface of B cells activated in the presence of IFN-γ increases the level of IgG2a produced, as well as increases proapoptotic genes (12). Likewise, dendritic cells activated in the presence of soluble CD80/86 increases the expression of express IL-6 and IFN-γ in a CD80-, CD86-, and p38 MAPK-dependent manner (13). Considerable progress has been made toward determining the signaling pathway activated following cross-linking CD86. Cross-linking CD86 on the surface of B cells activated in the presence of IL-4 increases the rate of mature IgG1 transcript produced via PI3K-dependent activation of the NF-kB subunits p65 and p50. Likewise, activation of these same NF-kB subunits is induced in macrophage cell lines following the cross-linking of CD80 and CD86 (34). The present findings suggest that the CD80-induced increase in IFN-γ is similarly PI3K-dependent. Taken together, it is tempting to conclude that the same intracellular signaling pathway is activated following both CD80 and CD86 cross-linking. However, cross-linking CD80 on B cells induces proapoptotic gene expression, while cross-linking CD86 induces anti-apoptotic gene expression (12). Therefore, although there may be common intracellular signaling intermediates activated by both CD80 and CD86, presumably receptor-specific intracellular signaling intermediates must also be activated.

The high affinity interaction between CD4\(^+\) T cell-expressed CD80 and CD4\(^+\) T cell-expressed CTLA-4 is hypothesized to play a regulatory role during T cell-T cell interactions. It has been observed that Th2 cells express higher levels of CTLA-4 than Th1
cells. This finding was correlated with data showing that Th2 cells, but not Th1 cells, show variations in the organization of the immunological synapse dependent on B7 expression by the APC (35). The current data show that CD80 and CTLA-4 are co-expressed by a small percentage of the CD4+ T cells by 48 h after activation allowing for CD80-CTLA-4 interaction. Cross-linking CTLA-4 inhibits CD4+ T cell activity by decreasing tyrosine phosphorylation of Fyn and ZAP70 and Ca2+ mobilization following TCR stimulation (36–39). Our results show that cross-linking CD80 induces a Ca2+ influx. Because studies in human T cells line suggest that an increase in intracellular Ca2+ would increase the level of CTLA-4 expressed (40, 41), we are currently investigating whether cross-linking CD80 also alters the level of CTLA-4 expressed. These data may point to a putative T cell-T cell regulatory mechanism by which CD80 interaction with its ligand CTLA-4 would induce a temporal increase in cytokine production and survival signals for CD4+ T cells expressing CD80 during the early stages of an immune response. This hypothesis is supported by the finding that transfer of CD80/CD86−/− T cells resulted in a significantly increased severity of GVHD as compared with transferred wild-type T cells, while transfer of T cells over-expressing CD86 decreased GVHD as compared with wild-type T cells (15). This finding suggests that CD80/CD86 expression on T cells down-regulated allogeneic responses through CTLA-4 ligation. In contrast, CD4+ T cell-expressed CD80 has been shown to play a critical role in CD4+ T cell-mediated antitumor activity suggesting that inflammatory CD4+ T cell responses may be increased by CD80 expressed on tumor-infiltrating CD4+ T cells (14, 42). However, because cross-linking CD80 may then also increase the level of CTLA-4 expressed by the activated CD4+ T cells, this would eventually result in down-regulation of the response.

The importance of CD80-mediated costimulation in autoimmune disease progression, such as in R-EAE, is supported by studies analyzing the temporal expression of B7 costimulatory molecules. Active immunization of SJL mice with PLP139−151 in CFA, resulted in a temporal up-regulation of surface CD80 expression, relative to CD86, on B cells, T cells, and macrophages in the spleen (43). Furthermore, a CD80-dominant expression pattern is seen on all peripheral inflammatory cell types in the CNS-infiltrating population (44). The relevance of CD80 expression by CD4+ T cells is not limited to the mouse system. CD80 is also expressed by human peripheral blood CD4+ T cells, and the ability of CD80 to initiate an intracellular signal has also been shown to exist in human immune cells (12, 33). Analysis of lesions from multiple sclerosis (MS) patients has identified an increased level of CD80 expressed at the lesion site (45). Genetic analyses of MS patients support the hypothesis that CD80 expressed by autoreactive CD4+ T cells may contribute to the disease exacerbation. There is a positive correlation between the expression of allelic variants of CTLA-4 that have an increased affinity for binding to CD80 in MS patients (46). Beside the anti-CD80 mAb-induced increase in the level of IFN-γ, ongoing studies in our laboratory have begun to focus on the effect anti-CD80 mAb treatment has on the level of IL-17 produced by encephalitogenic CD4+ T cells in ex vivo recall responses, and naïve CD4+ T cell differentiation into Th17 cells. In this scenario, the expression of CTLA-4 might have the opposite effect of the “classic” negative regulatory role CTLA-4 has on CD4+ T cell activity (47–49). Instead, CTLA-4, with its increased affinity for CD80, might act as a cross-linker for CD80 sending a positive signal to the autoreactive T cell during T cell-T cell interactions.

Considering the current in vitro data along with the aforementioned in vivo data, a putative mechanism is suggested to explain why the timing of anti-CD80 mAb treatment has differing effects on disease outcome in the R-EAE model where it has been reported that mice treated with anti-CD80 mAb during the onset of disease exhibited decreased disease severity (10) while mice treated during remission with an intact anti-CD80 mAb exhibited increased disease severity (44). In contrast, treatment with an anti-CD80 Fab led to significantly decreased disease severity and relapse frequency when administered during disease remission. Both the in vitro and in vivo data support the hypothesis that the initial steps of activation of naïve CD4+ T cells are inhibited by anti-CD80 due to a lack of costimulation through CD28 interacting with CD80 expressed on APCs. For example, the addition of either an intact anti-CD80 mAb, anti-CD80 Fab, or CTLA-4-Ig at the time of naïve CD4+ T cell activation in the presence of antigenic peptide and APCs blocks T cell activation (50, 51). These culture conditions represent the treatment regimen in which anti-CD80 mAb is administered to the mice at the time of priming with encephalitogenic CD4+ T cell epitopes. In contrast, the culture conditions used in this report more closely mimic the in vivo treatment regimen wherein anti-CD80 treatment is initiated during ongoing disease. Therefore, these results support the conclusion that cross-linking CD80 on effector CD4+ T cells induces increased production of IFN-γ (and possibly IL-17) leading to enhanced tissue destruction and increased epitope spreading by increasing CD4+ T cell effector function. The present findings also strongly suggest that extreme caution must be taken when designing treatment regimens for autoimmune diseases such as MS based on Ab-mediated blockade of costimulatory molecules. Any treatment that has the potential to cross-link CD80 must be analyzed with the utmost care in that not only can blocking and/or cross-linking of CD80 on an APC affect cellular activity (12), but these same regimens may also directly up-regulate autoreactive CD4+ T cell effector functions, thereby exacerbating disease.

Disclosures

The authors have no financial conflict of interest.

References
