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CD80 Costimulation Is Required for Th2 Cell Cytokine Production But Not for Antigen-Specific Accumulation and Migration into the Lung[1]

Nicola L. Harris,* Melanie Prout,* Robert J. Peach,[†] Barbara Fazekas de St. Groth,[‡] and Franca Ronchese[2]*

The CD28 ligands CD80 and CD86 are expressed on APC, and both provide costimulatory function. However, the reason for the expression of two separate CD28 ligands remains unclear. We have previously shown that blockade of CD80 costimulation by Y100F-Ig, a CTL-associated Ag-4 (CTLA4)-Ig mutant that does not bind CD86, inhibits the development of lung inflammatory immune responses, but does not affect blood eosinophilia or Ab production. Each of those responses was inhibited by treatment with CTLA4-Ig, which binds both CD80 and CD86. To clarify the mechanism underlying these observations we have developed a model of lung inflammation using adoptively transferred CD4$^+$ T cells expressing a Vα11$^+$Vβ3$^+$ transgenic TCR specific for I-E^k and moth cytochrome c. Treatment with Y100F-Ig inhibited the induction of lung eosinophilia in adoptively transferred mice. However, Y100F-Ig did not detectably affect the accumulation of Ag-specific T cells at the site of peptide deposit or in the draining lymphoid tissues. Acquisition of an activated phenotype and expression of adhesion molecules required for migration into the lung were modestly affected. Importantly, treatment with Y100F-Ig diminished the ability of T cells to produce the cytokines IL-4 and IL-5 following intranasal challenge with Ag. All the responses examined were severely inhibited by treatment with CTLA4-Ig. We conclude that T cells require CD80 costimulation for the optimal production of IL-5 following intranasal administration of Ag. Decreased IL-5 production is the most likely explanation for the diminished airway eosinophilia observed. *The Journal of Immunology,* **2001, 166: 4908–4914.**

T cell activation requires a signal delivered via interaction of the TCR with specific Ag on MHC molecules and a costimulatory signal. The most fully characterized costimulatory signal is mediated by the binding of CD80 and CD86 on APC to their receptor CD28 on T cells (1). Although CD80 and CD86 are only distantly related (2), they show similar equilibrium binding to CD28 (3). Both molecules also serve as ligands for a second receptor on T cells, CTLA4, which appears to deliver inhibitory signals to T cells (4). CD80 and CD86 do not appear to be functionally redundant and may have distinct roles in the regulation of T cell immunity (5). However, the exact mechanisms by which CD80 and CD86 could differentially affect T cell activation are not known.

We have previously investigated the role of CD80 costimulation during in vivo T cell responses using a mutant form of CTLA4-Ig, Y100F-Ig. Y100F-Ig and CTLA4-Ig bind equally well to CD80, but the binding of Y100F-Ig to CD86 is undetectable (6). Consequently, Y100F-Ig can be used to selectively block CD80 costimulation. In a model of chicken OVA-induced airway eo-

sinophilia, we have shown that treatment with Y100F-Ig suppressed eosinophil and, to a lesser extent, lymphocyte infiltration into the lung of OVA-challenged mice, but had no effect on systemic blood eosinophilia or Ab production (6, 7).

There are several possible explanations for the ability of Y100F-Ig to suppress OVA-induced lung inflammation. Y100F-Ig treatment may limit the activation and/or clonal expansion of Ag-specific T cells in secondary lymphoid tissues, such that responses in the lung can no longer be induced. Alternatively, CD80 blockade may result in altered regulation of the adhesion marker expression or the chemokine production necessary for the migration of CD4$^+$ Th2 cells into the lungs of airway-challenged mice. Lastly, CD4$^+$ Th2 cells may be able to migrate into the lungs of airway-challenged mice, but CD80 blockade may prevent T cell activation at this site, resulting in decreased local IL-5 and cytokine production.

We have developed a new model of Ag-induced lung eosinophilia mediated by peptide-specific T cells expressing a transgenic TCR. Using this model we can identify the T cells responsible for mediating airway inflammation by specific mAbs and investigate the effect of Y100F-Ig treatment on peptide-induced T cell activation and migration in vivo. This has enabled us to clarify the mechanism by which Y100F-Ig suppresses lung inflammatory responses while leaving systemic eosinophilia and Ag-specific Ab production intact.

Materials and Methods

Mice

All mice were bred and maintained at the animal facility of the Wellington School of Medicine. B10.A mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained by brother × sister mating. The −I line 5C.C7 transgenic mice (8, 9) were backcrossed to B10.A mice and maintained by breeding transgenic males to B10.A females. The

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TCR used to generate the 5C.C7 transgenic strain was derived from the cytochrome *c*-specific T cell clone 5C.C7. These cells are specific for pigeon cytochrome *c* fragment 81–104 and I-E^k, but proliferate more vigorously when stimulated with peptide fragment 81–103 from the tobacco horn worm moth cytochrome *c* (MCC)[3] (10, 11). All animal experimental procedures used in this study were approved by the Wellington School of Medicine animal ethics committee and were conducted in accordance with the guidelines of the University of Otago (Dunedin, New Zealand).

Purification of lung lymphocytes, peritoneal cells, and PBLs

Mice used for the preparation of lung lymphocytes were injected i.p. with 150 U of heparin (Leo Pharmaceutical Products, Denmark) and sacrificed. The lower vena cava was severed, and lungs were perfused via the heart right ventricle with ~5 ml of PBS to remove circulating blood. Minced lung tissue was incubated for 30 min in complete IMDM containing 2.4 μg/ml collagenase type II (Life Technologies, Auckland, New Zealand) and 0.1% DNase I (Sigma, St. Louis, MO). Complete IMDM consisted of IMDM (Sigma) with 5% FCS (Life Technologies), 2 mM glutamine (Sigma), 1% penicillin-streptomycin (Sigma), and 5×10^{-5} M 2-ME (Sigma). Remaining tissue was then broken down by passage through an 18-gauge needle, and mononuclear cells were purified by gradient centrifugation over Lympholyte-M (Cedarlane Laboratories, Ontario, Canada) according to the manufacturer's instructions. Macrophages were depleted by culturing cells on plastic dishes in complete IMDM for 1–2 h at 37°C.

Peritoneal lymphocytes were obtained by sacrificing mice and flushing ~10 ml of PBS into the peritoneal cavity three times. PBLs were purified by collecting 100 μl of tail blood into 1 ml of Alsevers' solution (2% dextrose, 0.4% NaCl, and 0.8% sodium citrate) and lysing RBC by incubation in 0.14 M NH$_4$Cl and 17 mM Tris-HCl for 10–20 min at 37°C.

FACS staining

FACS analysis of lymphocytes was conducted by staining in 96-well round-bottom plates at 10^5–10^6 cells/well for 10–15 min on ice using the appropriate mAbs diluted in 100 μl of FACS buffer (PBS plus 2% FCS and 0.01% sodium azide). 2.4G2 (10 μg/ml) was used to inhibit FcγRII-mediated uptake. Flow cytometric analysis was performed on a FACSort (Becton Dickinson, Mountain View, CA) using CellQuest software.

Reagents and mAbs

CTLA4-Ig, Y100F-Ig, and L6-Ig were purified from culture medium of stably transfected Chinese hamster ovary cells as previously described (6). Anti-CD4 (GK1.5) was grown from hybridoma culture supernatant and conjugated to FITC or biotin. Anti-Vα11-FITC, anti-Vβ3-PE, anti-Vβ3-biotin, anti-CD49d-PE, anti-CD44-PE, anti-CD62L-PE, anti-CD80-FITC, anti-CD86-PE, and streptavidin-FITC, -PE, and -Cy-Chrome were all obtained from PharMingen (San Diego, CA).

Ag-induced airway inflammation

The protocol for induction of OVA-dependent airway inflammation has been described previously (6). For the adoptive transfer model, cell suspensions were prepared from lymph nodes of 5C.C7 mice, and the percentage of Vα11, Vβ3 TCR-expressing lymphocytes was determined by FACS analysis. A total of 3×10^6 Vα11$^+$Vβ3$^+$ T cells were injected into the tail vein of sex-matched B10.A mice in a total volume of 500 μl of IMDM. Two days later recipient mice were immunized i.p. with 250 μg of MCC$_{87–103}$ in 200 μl of alum adjuvant (SERVA, Heidelberg, Germany) on days 0 and 20 after adoptive transfer. Six days after the last i.p. immunization, mice were anesthetized by injection of a mixture of ketamine and xylazine (Phoenix, Auckland, New Zealand) and challenged with 100 μg of MCC$_{87–103}$ in 50 μl of PBS by intranasal (i.n.) inoculation. At the corresponding times, nonimmunized mice received alum adjuvant i.p. and PBS i.n. Mice were sacrificed at the indicated time points after i.n. challenge, and cellular infiltration into the airways was determined by bronchoalveolar lavage (BAL) and differential cell counting as previously described (6).

Enzyme-linked immunospot (ELISPOT)

Cells were cultured at various dilutions for 6 h at 37°C in 96-well plates (Nunc, Copenhagen, Denmark) coated with 10 μg/ml anti-CD3 mAb (2C11) and 20 μg/ml anti-IL-5 mAb (TRFK5), anti-IL-4 mAb (11B11), or anti-IFN-γ mAb (R4-6A2) in the presence of IL-2 (10 U/ml). As a control,

cells were also incubated on plates coated with the capture Ab only. After the initial 6-h incubation plates were washed thoroughly with PBS-Tween, then 5 μg/ml biotinylated detection Abs (anti-IL-5 mAb, TRFK4; anti-IL-4 mAb, BVD6-24G2; anti-IFN-γ mAb, xMG-D6) were added, and plates were incubated overnight at 4°C. Washed plates were then incubated with avidin-alkaline phosphatase conjugate (Sigma) for 1 h at room temperature. Reactions were developed using 5-bromo-4-chloro-3-indoyl-phosphate (Sigma) and were stopped by addition of distilled water. The number of spots was determined by counting under an inverted microscope.

RNA isolation and cDNA preparation.

RNA was isolated from mediastinal lymph node (MLN) and lung using TRIzol (Life Technologies). The RNA was quantified using GeneQuant, and 1 μg of total RNA was used in the cDNA synthesis reaction. The cDNA reaction was conducted for 60 min at 37°C using 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies) and 0.5 μg of oligo-(dT)$_{12–18}$ primer (Life Technologies).

Polymerase chain reaction

Primers and probes for IL-4, IL-5, IFN-γ, and β_2-microglobulin were designed and synthesized as described previously (12). Probes were modified to incorporate a reporter dye at the 5' end (6-carboxy-fluorescein or tetracholoro-6-carboxy-fluorescein) and a quencher at the 3' end (6-carboxytetramethyl-rhodimine). The sequences of the oligonucleotides and PCR conditions are detailed in the report by Hook et al. (12). PCRs were conducted in an ABI Prism 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA). Reactions were set up using the TaqMan core reagents (PE Applied Biosystems) according to the manufacturer's instructions. MgCl$_2$ (5 mmol/L) was used in the IL-4, IL-5, IFN-γ, and β_2-microglobulin reactions. Cycling conditions consisted of 50°C for 2 min, 94°C for 10 min, and then 35 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s. DNA standards were made for each of the cytokines and serially diluted and included with each PCR. Data were analyzed using ABI Prism Sequence Detection System version 1.63 software (PE Applied Biosystems). All amplifications were performed in duplicate, and the amount of DNA in the samples was calculated from the standard curve. All results were normalized to β_2-microglobulin to compensate for differences in the amount of cDNA in the samples.

Results

Model of MCC$_{87–103}$-induced airway eosinophilia

A model of peptide-induced lung eosinophilia was developed using the adoptive transfer of T cells from 5C.C7 mice (8, 9), which are transgenic for a Vα11$^+$Vβ3$^+$ TCR specific for I-E^k and MCC$_{87–103}$ (10, 11).

Initial experiments were conducted to determine the optimal immunization conditions for the induction of lung eosinophilia. The variables examined included the number of adoptively transferred T cells, the peptide dose, and the number and timing of i.p. immunizations. It was found that a large number of adoptively transferred TCR transgenic T cells induced a good response, but was associated with occasional lethality at the time of i.n. challenge. Moreover, i.p. immunizations with larger amounts of peptide and at longer intervals appeared to yield more substantial and more reproducible eosinophil infiltration (data not shown). Fig. 1 outlines the optimal protocol as determined in those experiments. Briefly, 3×10^6 5C.C7 T cells expressing the transgenic Vα11$^+$Vβ3$^+$ TCR were adoptively transferred into normal syngeneic B10.A mice 2 days before the first immunization. Recipient mice were immunized i.p. with 250 μg of MCC$_{87–103}$ in alum adjuvant on days 0 and 20, and then given an i.n. challenge with 100 μg of MCC$_{87–103}$ in PBS on day 26. Cellular infiltration into the airways and lung was determined by BAL. Importantly, no airway eosinophilia was observed in B10.A mice that were immunized and airway challenged with MCC$_{87–103}$, but had not received T cells expressing the transgenic Vα11$^+$Vβ3$^+$ TCR, using these immunizing conditions.

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[3] Abbreviations used in this paper: MCC, moth cytochrome *c*; i.n., intranasal(ly); MLN, mediastinal lymph node; BAL, bronchoalveolar lavage; ELISPOT, enzyme-linked immunospot; VLA-4, very late Ag 4.
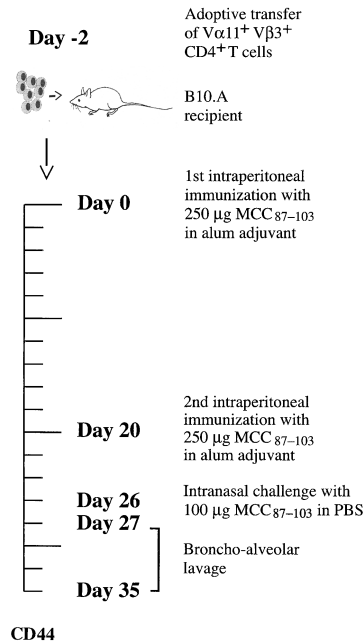
FIGURE 1. Protocol of immunization for the induction of lung eosinophilia by MCC_{87-103}. Normal B10.A mice were injected with 3×10^6 $V\alpha 11^+ V\beta 3^+$ T cells from 5C.C7 mice and immunized as outlined. Cellular infiltration into the lung was determined by collecting BAL fluid and performing differential cell counts on stained cytospins.

FIGURE 2. Treatment with Y100F-Ig inhibits lung eosinophilia in MCC_{87-103}-immunized and airway-challenged mice. Normal B10.A mice were injected with T cells from 5C.C7 TCR transgenics, then immunized and airway challenged as detailed in Fig. 1, or were left nonimmunized (×). Mice were treated throughout the experiment with CTLA4-Ig (○), Y100F-Ig (◇), or the control molecule L6-Ig (□) every 48 h, beginning on the day of the first i.p. immunization. Mice were sacrificed, and differential cell counts were made on BAL fluid collected at the indicated time points after the i.n. challenge. Values represent the mean number of cells per milliliter of BAL fluid ± SE of five individual mice. Values for the nonimmunized group are given for day 3 only. The data shown are from one representative experiment. Similar results were obtained in three separate experiments using four to six mice per group.

Treatments with Y100F-Ig and CTLA4-Ig suppress MCC_{87-103}-induced lung eosinophilia

To determine the effect of Y100F-Ig treatment on MCC_{87-103}-induced airway eosinophilia B10.A mice were adoptively transferred with $V\alpha 11^+ V\beta 3^+$ T cells from 5C.C7 donors, then immunized with MCC_{87-103}/alum and airway challenged as detailed in Fig. 1. Mice were treated with i.p. injections of 400 μg of human or 200 μg of murine CTLA4-Ig, Y100F-Ig, or the control molecule L6-Ig every 48 h, beginning on the day of the first MCC_{87-103} i.p. immunization.

The BAL fluid obtained from mice treated with the isotype control L6-Ig contained high numbers of eosinophils and lymphocytes. Treatment with CTLA4-Ig completely abrogated eosinophil and lymphocyte infiltration into the BAL fluid of MCC_{87-103}-challenged mice (Fig. 2). Treatment with Y100F-Ig decreased the number of eosinophils in the BAL 3- to 5-fold on days 1, 3, and 5 after i.n. challenge (Fig. 2), but did not alter MCC_{87-103}-induced lymphocyte infiltration into the BAL (Fig. 2). Although there was some variability in the MCC_{87-103}-induced eosinophilia, the suppressive effect of Y100F-Ig treatment was statistically significant over all time points using ANOVA of log data ($p = 0.0001$).

These data indicate that CD80 costimulation is required for the development of MCC_{87-103}-induced lung eosinophilia. Therefore, the inhibitory effect of Y100F-Ig on lung eosinophilia is not limited to the OVA system in C57BL/6 mice (6), but applies also to lung eosinophilia mediated by TCR transgenic T cells.

Treatment with Y100F-Ig does not alter the accumulation of $V\alpha 11^+ V\beta 3^+$ T cells in the secondary lymphoid tissues or at the site of Ag deposit

A possible explanation for the observation that Y100F-Ig can suppress airway eosinophilia is that CD80 costimulation is required for optimal activation and/or clonal expansion of Ag-specific T
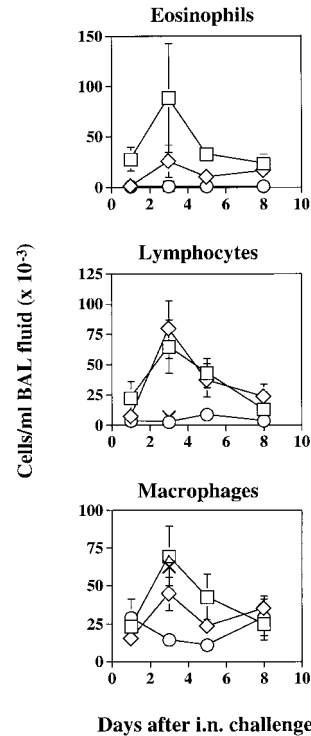
cells following i.p. immunization. Alternatively, CD80 blockade may suppress lung eosinophilia by altering T cell responses following the i.n. challenge of sensitized mice. To distinguish between these possibilities, the activation and accumulation of T cells expressing $V\alpha 11^+ V\beta 3^+$ TCR were determined at different times after the second i.p. immunization and after the i.n. challenge.

After the second i.p. immunization with MCC_{87-103} in alum, a considerable accumulation of $V\alpha 11^+ V\beta 3^+$ cells was observed in the spleen and peritoneal cavity of immunized mice compared with nonimmunized controls (Fig. 3A). A detectable, but smaller, accumulation of $V\alpha 11^+ V\beta 3^+$ T cells was also observed in the lymph nodes of immunized mice (data not shown). Treatment with Y100F-Ig had no effect on the number of $V\alpha 11^+ V\beta 3^+$ T cells that accumulated in the spleen or peritoneal cavity (Fig. 3A). In contrast, treatment with CTLA4-Ig abrogated MCC_{87-103}-induced accumulation of $V\alpha 11^+ V\beta 3^+$ cells at both sites (Fig. 3A). Therefore, these data do not support the possibility that Y100F-Ig treatment alters the clonal expansion or site-specific accumulation of Ag-reactive T cells following i.p. immunization.

To investigate the alternative possibility that Y100F-Ig treatment may affect the T cell response after i.n. challenge with MCC_{87-103}, we examined the accumulation of $V\alpha 11^+ V\beta 3^+$ T cells in the MLN, lung, and BAL fluid following i.n. challenge with MCC_{87-103} peptide. As shown in Fig. 3B, treatment with

A)

Spleen **Peritoneum**

Number of Vα11+Vβ3+ CD4+ T cells (x 10^{-3})

B)

Mediastinal lymph node

Lung

BAL fluid

Number of Vα11+Vβ3+ CD4+ T cells (x 10^{-3})
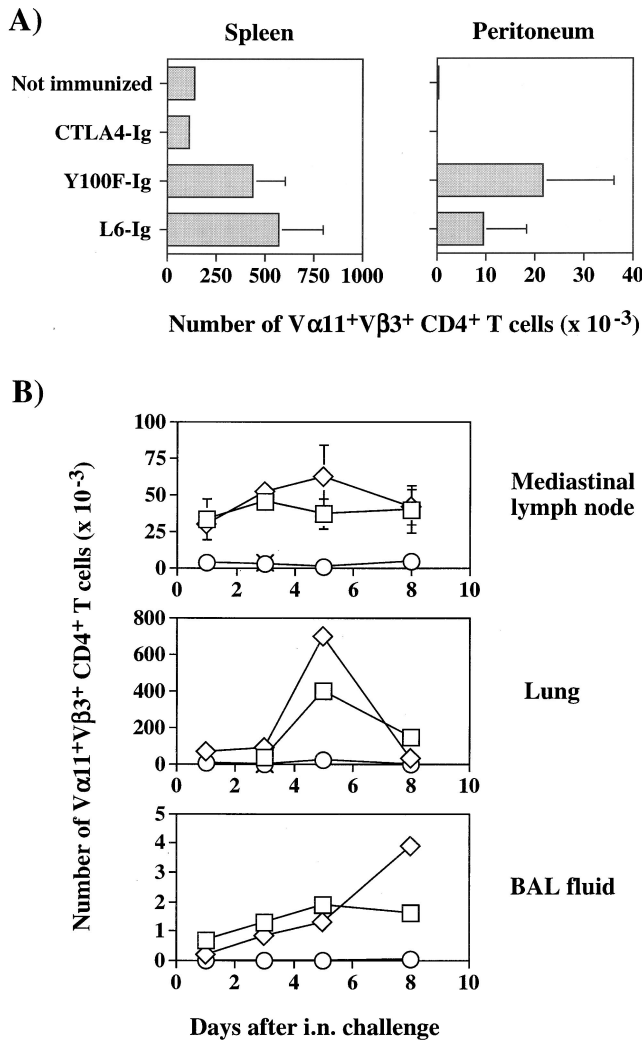
Days after i.n. challenge

FIGURE 3. Treatment with Y100F-Ig does not alter the accumulation of Vα11+Vβ3+ T cells in lymphoid organs or peripheral tissues. Normal B10.A mice were injected with T cells from 5C.C7 TCR transgenics and then immunized and airway challenged as detailed in Fig. 1 or were left nonimmunized (×). Mice were treated throughout the experiment with CTLA4-Ig (○), Y100F-Ig (◇), or L6-Ig (□) every 48 h, beginning on the day of the first i.p. immunization. Total numbers of Vα11+Vβ3+ T cells were determined for spleen and peritoneal washing on day 3 after the second i.p. immunization (A) and for MLN, lung, and BAL fluid at the indicated times after i.n. challenge (B). Values represent the mean number of cells ± SE of five individual mice, except for lung and BAL fluid, where the results from pooled samples are shown. Data from nonimmunized mice are shown for day 3 only. The data shown are from one representative experiment. Similar results were obtained in three separate experiments using four to six mice per group.

Y100F-Ig did not notably alter the MCC$_{87-103}$-induced accumulation of Vα11+Vβ3+ T cells at any of those sites. In contrast, treatment with CTLA4-Ig inhibited the accumulation of Vα11+Vβ3+ T cells at all sites investigated (Fig. 3B). Thus, these data do not support the alternative possibility that Y100F-Ig treatment may prevent the specific accumulation of Ag-reactive T cells following i.n. administration of Ag.

The expression of activation markers was examined on Ag-specific T cells in the MLN after i.n. challenge with MCC$_{87-103}$. In immunized mice, Vα11+Vβ3+ T cells expressed significantly decreased levels of CD62L (L-selectin) compared with the same cells in naive 5C.C7 mice, while their expression of CD44 (PgP-1) was

increased (Fig. 4). This phenotype is characteristic of Ag-activated T cells. Treatment with CTLA4-Ig inhibited almost completely the progression of Vα11+Vβ3+ T cells from the naive to the activated phenotype (Fig. 4). In contrast, Vα11+Vβ3+ T cells in mice treated with Y100F-Ig clearly showed an activated phenotype in terms of CD62L and CD44 expression. However, on close examination the ratio of activated, CD62L^{low} CD44^{high}, to naive CD62L^{high} CD44^{low} Vα11+Vβ3+ T cells was slightly lower in the Y100F-Ig group compared with that in the control, L6-Ig-treated group.

In summary, these data indicate that treatment with Y100F-Ig does not prevent the activation of Vα11+Vβ3+ T cells following MCC$_{87-103}$ immunization and airway challenge, but does result in a slightly less activated phenotype compared with that of control, L6-Ig-treated mice. The likely outcome of a small change in activation marker expression in terms of T cell effector function is unknown.
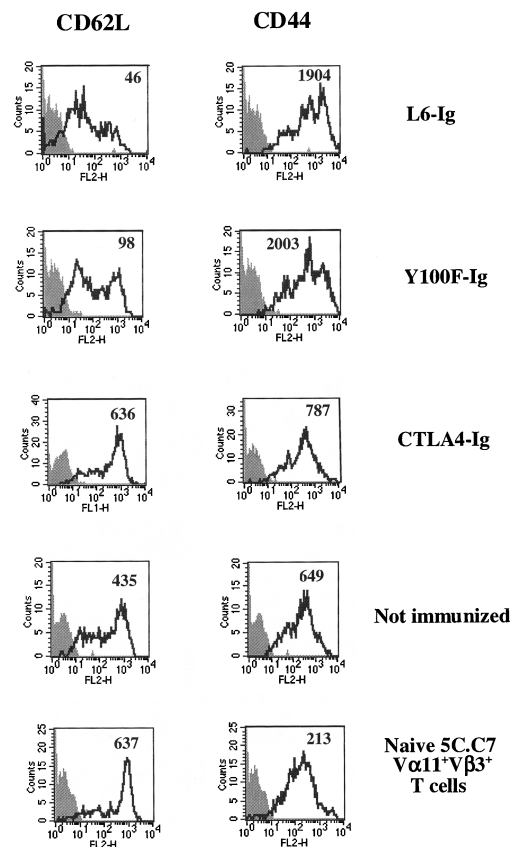

FIGURE 4. Treatment with Y100F-Ig has a modest effect on the activation phenotype of Vα11+Vβ3+ T cells in MLN. Normal B10.A mice were injected with T cells from 5C.C7 TCR transgenics, then MCC$_{87-103}$ immunized and airway challenged as detailed in Fig. 1. Mice were treated with CTLA4-Ig, Y100F-Ig, or L6-Ig every 48 h, beginning at the time of the first immunization. Four days after the i.n. challenge mice were sacrificed, and MLN cells were analyzed for the expression of CD62L and CD44 using three-color fluorescent staining and FACS analysis. Histogram plots show Vα11+Vβ3+ gated T cells. ■, nonstained samples; □, samples stained with anti-CD62L-PE or anti-CD44-PE mAbs as indicated. The mean fluorescence intensities for each staining are shown in the *top right* corner of each histogram. The data shown are from the pooled cells of five mice in one representative experiment. Similar results were obtained in three separate experiments using four to six mice per group.

Treatment with Y100F-Ig does not prevent the depletion of $V\alpha11^+V\beta3^+$ T cells from peripheral blood following i.n. challenge with MCC_{87-103} or expression of integrins involved in migration into inflammatory sites

The data presented in the previous section demonstrate that treatment with Y100F-Ig did not affect the accumulation of $V\alpha11^+V\beta3^+$ T cells in lymphoid organs and lung after i.p. immunization and i.n. challenge with MCC_{87-103}. The expression of the activation markers CD62L and CD44 was moderately affected.

T cell migration and recruitment can also be examined by determining number of $V\alpha11^+V\beta3^+$ T cells in peripheral blood at various times throughout the MCC_{87-103} immunization and airway challenge protocol. Over a large number of experiments we have observed that i.p. immunization with MCC_{87-103} in alum results in an increase in the percentage of $V\alpha11^+V\beta3^+$ T cells in peripheral blood. $V\alpha11^+V\beta3^+$ T cells rapidly disappear from the blood following i.n. challenge with MCC_{87-103} (Fig. 5*A*), presumably reflecting migration of activated T cells into the lung and airways. As shown in Fig. 5*A*, treatment with Y100F-Ig did not affect the accumulation of $V\alpha11^+V\beta3^+$ T cells in peripheral blood following i.p. immunization. Y100F-Ig treatment also did not affect the depletion of $V\alpha11^+V\beta3^+$ T cells from peripheral blood following i.n. challenge with MCC_{87-103} (Fig. 5*A*). In contrast, accumulation of $V\alpha11^+V\beta3^+$ T cells in the blood was completely inhibited by treatment with CTLA4-Ig, preventing further analysis of their migration.

The integrin very late Ag-4 (VLA-4) is involved in the migration of activated T cells to inflammatory sites, and anti-VLA-4 Ab treatment has been shown to block the migration of T cells into the lungs of allergen-challenged animals (13). Thus, we examined expression of the VLA-4 α subunit, CD49d, on $V\alpha11^+V\beta3^+$ T cells from i.p. immunized mice. No CD49d-expressing $V\alpha11^+V\beta3^+$ T cells could be demonstrated in the lymph nodes or spleen of immunized mice (not shown). In control L6-Ig-treated mice peripheral blood $V\alpha11^+V\beta3^+$ T cells expressed increased levels of CD49d compared with $V\alpha11^+V\beta3^+$ T cells from naive 5C.C7 transgenic mice (Fig. 5*B*). Cells expressing high levels of CD49d were preferentially depleted from blood after i.n. challenge with MCC_{87-103} (data not shown). Treatment with Y100F-Ig did not alter the MCC_{87-103}-induced increase in CD49d expression on blood $V\alpha11^+V\beta3^+$ T cells (Fig. 5*B*). In contrast, $V\alpha11^+V\beta3^+$ T cells in CTLA4-Ig treated mice expressed only low levels of CD49d (Fig. 5*B*), comparable to the levels observed in naive 5C.C7 mice.

Taken together, the data in Fig. 5 indicate that Y100F-Ig treatment does not prevent the emigration of $V\alpha11^+V\beta3^+$ T cells from the blood upon i.n. Ag challenge, nor does it alter their expression of the integrins required for entry into the lung and airways. These data support the conclusion in the previous section that Y100F-Ig treatment does not prevent the homing of $V\alpha11^+V\beta3^+$ T cells to the lungs after i.n. administration of Ag.

Treatment with Y100F-Ig inhibits T cell cytokine production

Local IL-5 production is critical to the development of lung eosinophilia (7, 14). Thus, Y100F-Ig may lower airway eosinophilia by preventing T cell activation and local IL-5 production following re-encounter with specific Ag in the lung and airways. We investigated the effects of L6-Ig, Y100F-Ig, and CTLA4-Ig treatment on the ability of T cells to produce cytokines following Ag immunization and airway challenge. However, despite extensive analysis at different time points after i.n. challenge with MCC_{87-103} peptide, little cytokine production by $V\alpha11^+V\beta3^+$ T cells could be demonstrated in the lung or lymph node by analysis of ELISPOT
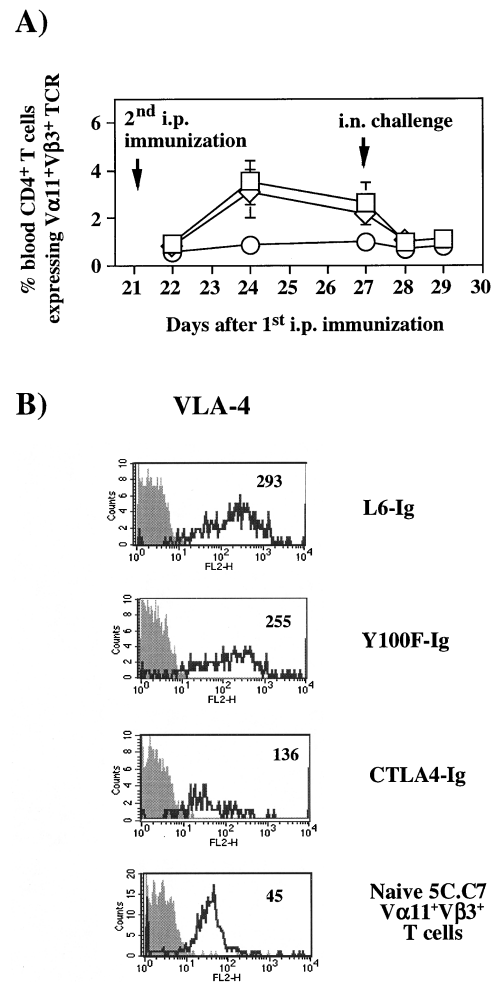

FIGURE 5. Treatment with Y100F-Ig does not affect depletion of $V\alpha11^+V\beta3^+$ T cells from the blood or their expression of CD49d. Normal B10.A mice were injected with T cells from 5C.C7 TCR transgenics, then MCC_{87-103} immunized and airway challenged as detailed in Fig. 1. Mice were treated with CTLA4-Ig (○), Y100F-Ig (◇), or L6-Ig (□) every 48 h, beginning at the time of the first i.p. immunization. *A*, Depletion of Ag-specific T cells from the blood following i.n. challenge was examined by determining the percentage of CD4$^+$ peripheral blood T cells that are $V\alpha11^+V\beta3^+$ using fluorescent staining and three-color FACS analysis. *B*, Three days after the second i.p. immunization peripheral blood $V\alpha11^+V\beta3^+$ T cells were analyzed for expression of the VLA-4 subunit CD49d. Histogram plots show $V\alpha11^+V\beta3^+$ gated T cells. ■, nonstained samples; □, samples stained with anti-CD49d-PE mAb. The mean fluorescence intensities are shown in the *top right* corner of each histogram. The data shown are from the pooled cells of five mice in one representative experiment. Similar results were obtained in three separate experiments using four to six mice per group.

or bulk culture supernatants. We next attempted to determine the effects of L6-Ig, Y100F-Ig, and CTLA4-Ig treatment on T cell cytokine production in mice immunized and i.n. challenged with the whole protein Ag OVA. These experiments were conducted using B10.A mice that had been immunized and i.n. challenged with OVA as previously described (6), as this immunization protocol induces a T cell response in which ex vivo cytokine production can be more easily measured. As seen using the MCC_{87-103}-induced airway eosinophilia model, Y100F-Ig treatment of OVA-immunized B10.A mice resulted in a reduction of airway eosinophilia. In contrast, BAL lymphocyte numbers were similar in the L6-Ig- and Y100F-Ig-treated groups. CTLA4-Ig treatment

inhibited infiltration of either eosinophils or lymphocytes into the airways (data not shown).

The numbers of cytokine-producing cells were evaluated using an ELISPOT assay. Given the short restimulation period required, we considered that the cytokine production measured by this technique is likely to reflect cytokine production in vivo. Significant numbers of lymphocytes producing IL-4 and IL-5 were found in the lung, airways, and MLN of OVA-immunized and airway-challenged B10.A mice on day 4 following i.n. challenge (Fig. 6, L6-Ig-treated group). The numbers of IL-4- and IL-5-producing cells were reduced in the airways, lung, and MLN of Y100F-Ig-treated mice and were virtually absent from these tissues in CTLA4-Ig-treated mice (Fig. 6). The number of IFN-γ-producing cells in the lung and MLN of OVA-immunized and challenged mice did not differ dramatically from that in nonimmunized mice (Fig. 6). The numbers of IFN-γ-secreting cells found in the BAL fluid were increased in immunized, L6-Ig-treated mice, and this increase was reduced by treatment with either Y100F-Ig or CTLA4-Ig (Fig. 6).
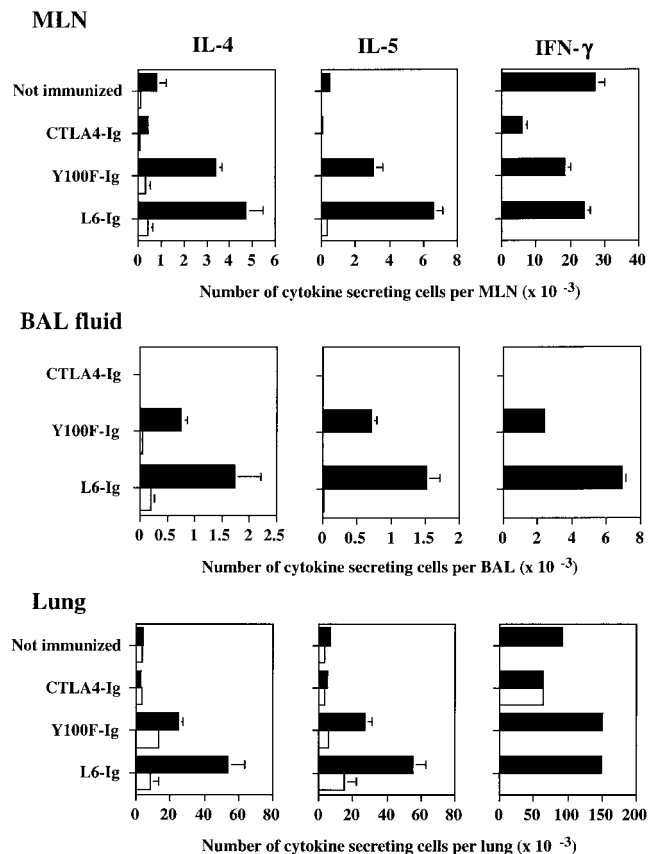
However, it is not clear whether the IFN-γ produced was derived from CD4^{+} or CD8^{+} T cells.

To further evaluate cytokine production in Y100F-Ig- and CTLA4-Ig-treated mice, a quantitative RT-PCR assay was used. IL-4 mRNA copy number was increased in the MLN and lungs of immunized L6-Ig-treated mice compared with that in nonimmunized mice (Fig. 7). In parallel with the ELISPOT data, treatment with either Y100F-Ig or CTLA4-Ig led to a reduction in the production of IL-4 mRNA in these tissues (Fig. 7). No IL-5 or IFN-γ could be detected using this assay.

Together, these data suggest that the inhibition of lung eosinophilia by Y100F-Ig treatment is due to decreased IL-5 production by specific T cells. Interestingly, this defective T cell cytokine production in Y100F-Ig-treated mice is not limited to the lung and airways, but also occurs in the MLN.

Discussion

We have previously shown that CD80 costimulation is necessary for the induction of airway eosinophilia in immunized mice (6). In the present study we also show that this finding is not a result of any defect in the accumulation of Ag-specific T cells at inflamed sites or in the draining lymphoid tissue. Instead, CD80 blockade decreased the production of the cytokines IL-4 and IL-5 by specific T cells following exposure to soluble Ag delivered i.n. This decreased cytokine production is likely to result in decreased eosinophilia, as IL-5 production has been shown previously to be critical for the development of airway eosinophilia (7, 14).

We do not think that these data are in conflict with our previous findings that Y100F-Ig treatment lowered airway eosinophilia, but not blood eosinophilia or Ab production, in immunized mice (6). Rather, these data may simply reflect differences in the level of T cell cytokine production required for the development of airway eosinophilia as opposed to the development of peripheral blood eosinophilia or the provision of T cell help to B cells. For instance, IL-5 production is reduced, but not lost, in Y100F-Ig treated mice. Therefore, the amount of Ag-specific IL-5 production occurring during the immunization and challenge of Y100F-Ig-treated mice may be adequate for the differentiation and recruitment of eosinophils from the bone marrow, but not for their recruitment from blood into lung. Likewise, perhaps only a small number of IL-4-producing T cells are required to deliver help to the limited pool of Ag-specific B cells responsible for producing OVA-specific IgE. If
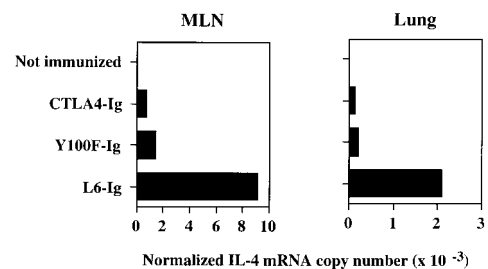

FIGURE 6. Treatment with Y100F-Ig decreases the numbers of IL-4- and IL-5-producing cells in the lung, airways, and MLN of OVA-immunized and airway-challenged mice. Normal B10.A mice were immunized and airway challenged with OVA as previously described (6). Mice were treated with CTLA4-Ig, Y100F-Ig, or L6-Ig every 48 h, beginning at the time of the first immunization. Four days after the i.n. challenge mice were sacrificed, and BAL fluid, lung, and MLN collected. Cell suspensions were plated onto microtiter plates coated with capture mAb with anti-CD3 mAb (■) or without anti-CD3 mAb (□) and cultured for 6 h before revealing bound cytokines using the appropriate detection mAb. The total number of cytokine-producing lymphocytes was calculated by multiplying the fraction of cytokine-producing cells by the total number of cells in that tissue. Data represent the mean ± range of duplicate measurements from the pooled cells of four mice in one representative experiment. Similar results were obtained in three separate experiments using three mice per group.

FIGURE 7. Treatment with Y100F-Ig decreases the amount of IL-4 mRNA found in the MLN and lung of OVA-immunized and airway-challenged mice. Normal B10.A mice were immunized and airway challenged with OVA and treated with L6-Ig, Y100F-Ig, or CTLA4-Ig as described in Fig. 6. RNA was isolated from MLN and lung at 24–48 h after i.n. challenge using TRIzol. IL-4 mRNA copy numbers were then estimated by real-time quantitative RT-PCR using the Applied Biosystems Prism 7700 Sequence Detector as described in *Materials and Methods*. β2-Microglobulin expression was used to normalize samples. Values represent the mean ± range of duplicate samples taken from pooled tissues of three mice.

this were the case one would not expect to see a comparable reduction in IgE production following a reduction in the numbers of IL-4-producing T cells.

It is interesting that cytokine production by T cells was decreased by CD80 blockade while other T cell functions, such as clonal expansion and migration into peripheral tissues, appeared unaffected. This may reflect a unique dependence of Th2 cell differentiation and/or Th2 cytokine production on CD80 costimulation. IL-4 and IL-5 cytokine production are considered to be late events in T cell activation (15), only occurring following a certain number of cell divisions, and may be more dependent on CD80 costimulation due to the slow kinetics of CD80 up-regulation on activated APCs (16). In our system, we detected no evidence of inhibition of T cell division, as similar numbers of $V\alpha11^+V\beta3^+$ transgenic T cells were found to accumulate in the lungs and lymphoid tissues of L6-Ig and Y100F-Ig-treated mice. However, more sensitive techniques may be required to reveal small differences in cell division. It is also possible that CD80-mediated signals are required for the differentiation of T cells into Th2 independently of cell division. This possibility is favored by the observation that expression of adhesion molecules on T cells was modestly, but detectably, decreased in Y100F-Ig-treated compared with L6-Ig-treated mice. Lastly, as further discussed below, Y100F-Ig may simply act by inhibiting the secretion of cytokines by fully differentiated effector T cells. Effector T cells in our model were of the Th2 type; thus, the inhibitory effect was observed on IL-4 and IL-5 secretion. However, we do not rule out the possibility that a similar inhibition may be observed on other types of effector T cells and cytokines. Indeed, we observed that treatment with Y100F-Ig resulted in decreased IFN-γ production by BAL lymphocytes in the present study, and in CD8$^+$ T cells following infection with influenza virus (17).

An alternative explanation for our findings is that CD80 blockade may decrease cytokine production following administration of soluble Ag i.n., but not following administration of Ag in alum adjuvant i.p. In this case Y100F-Ig treatment would not be expected to affect the development of blood eosinophilia or the production of T cell-dependent Abs following Ag/alum immunization, but it would lower cytokine production following the administration of Ag in PBS as for the i.n. challenge. This possibility has not been directly investigated; however, an obvious explanation for such a scenario would be that APCs up-regulate CD86 following challenge with Ag in an adjuvant such as alum, providing adequate costimulation to the responding T cells even in the absence of CD80. Conversely, encounter with Ag in PBS may not result in comparable CD86 up-regulation, deeming the presence of CD80 necessary for adequate costimulation.

Although the lack of IL-5 production by lung T cells is likely to result in defective eosinophil recruitment, we have not ruled out the possibility that this could also occur by altered chemokine production. Many chemokines regulate eosinophil chemotaxis, the most potent being RANTES and eotaxin (18–20). The major cellular sources of eotaxin are thought to be the epithelium, endothelium, and eosinophils themselves rather than T cells (19, 20). However, T cells are known to produce the chemokines RANTES and macrophage inflammatory protein-1α, and this has previously been shown to be CD28 dependent (21). Although we have not formally investigated the possibility, it would not be surprising if T cells that are defective for IL-4 and IL-5 production also fail to produce relevant chemokines.

In conclusion, we have found that the major effect of blocking CD80 costimulation on T cell function appears to be in the production of cytokines following i.n. challenge with Ag. The lack of IL-5 production by these T cells is likely to be responsible for the inhibition of airway eosinophilia seen in Y100F-Ig-treated mice.

Acknowledgments

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