

# Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Stimulate the Induction of Th2 Responses by Up-Regulating B7.2 Expression<sup>1</sup>

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Vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase-activating polypeptide (PACAP), two structurally related neuropeptides produced within the lymphoid microenvironment, modulate several immunologic functions. We have recently demonstrated that VIP and PACAP enhance the macrophage costimulatory activity for naive CD4<sup>+</sup> T cells exposed to allogeneic or anti-CD3 stimuli through the differential regulation of the B7 costimulatory molecules. In this study, we report on the role of VIP and PACAP on macrophage B7 expression and costimulatory function for Ag-primed CD4<sup>+</sup> T cells, and on the macrophage-induced regulation of Th1/Th2 differentiation in vitro and in vivo. VIP and PACAP up-regulate the costimulatory activity of macrophages for Ag-primed CD4<sup>+</sup> T cells. VIP-/PACAP-treated macrophages gain the ability to induce Th2-type cytokines such as IL-4 and IL-5 and reduce Th1-type cytokines such as IFN- $\gamma$  and IL-2. In vivo administration of VIP or PACAP in Ag-immunized mice reduce the numbers of IFN- $\gamma$ -secreting cells and enhance the numbers of IL-4-secreting cells. One of the consequences of the VIP-/PACAP-induced shift in cytokine profile is a change in the Ag-specific Ig isotype, increasing IgG1 and decreasing IgG2a levels. Finally, the preferential differentiation into Th2 effector cells after Ag stimulation induced by VIP-/PACAP-treated macrophages is mediated through the up-regulation of B7.2 expression. *The Journal of Immunology*, 1999, 163: 3629–3635.

Upon antigenic stimulation, CD4<sup>+</sup> Th cells can differentiate into two distinct types of effector cells, each producing its own set of cytokines and mediating separate functions (reviewed in Ref. 1). Th1 cells secrete cytokines (IL-2, IFN- $\gamma$ , and TNF- $\beta$ ) critical for the generation of a cellular immune response, thereby activating macrophages, inducing delayed-type hypersensitivity responses, and stimulating IgG2a Ab production in mice. Th2 cells produce IL-4, IL-5, and IL-10, which are critical for IgG1 and IgE Ab production and immunity against helminthic parasites and inhibit macrophage activation and Ag presentation, thereby down-regulating the cellular immune responses. Because their respective cytokines act antagonistically, these two cell subpopulations regulate each other's function (1). The molecular basis for the differentiation of Th cells is just beginning to be delineated. Activation of precursor Th cells (Th0) results in the production of IL-2 with relatively little IFN- $\gamma$  or IL-4. Subsequent events appear to bias the cells toward differentiation into the Th1 or Th2 phenotype. Determining factors include the nature of the APC, the nature and amount of Ag, and the genetic background of the host, with the cytokine microenvironment as the dominant factor. Increasing evidence demonstrates that IL-12 and, to a lesser extent IFN- $\gamma$ , direct CD4<sup>+</sup> T cells to differentiate into Th1. In contrast, IL-4 is necessary to induce the development of Th2. When both IL-4 and IL-12

are added to in vitro cultures, IL-4 dominates over IL-12, driving naive CD4<sup>+</sup> T cells toward the Th2 phenotype (1).

The activation of naive CD4<sup>+</sup> T lymphocytes requires two signals delivered by APCs, leading to enhanced cytokine production and proliferation. The first signal, which confers specificity, is provided by the interaction of the antigenic peptide/MHC complex with the T cell receptor. The second signal is provided by costimulatory molecules expressed on APCs. Among the accessory molecules, the B7 family appears to be the most potent. The B7 costimulatory pathway involves at least two molecules, B7.1 (CD80) and B7.2 (CD86), both of which can interact with their counter-receptors, CD28 and CTLA-4, on T cells (2). B7.1 and B7.2 are expressed on a variety of APCs, including B cells, dendritic cells, Langerhans' cells, and monocytes (reviewed in Ref. 2) and are regulated separately as demonstrated by the kinetics of their expression on APCs upon activation (3), as well as their regulation by cytokines and inflammatory mediators (4). The biologic significance of the differential expression of B7.1 and B7.2 is not clear. Although signals provided through B7/CD28 interactions appear to be essential for the development of Th2-type cytokine responses (5–8), the question whether B7.1 and B7.2 play different roles in the differentiation of Th cells is still under debate. Signals or agents that trigger the B7 accessory molecules are likely to be essential for the generation of an immune response, and their dysregulation may be responsible for certain autoimmune diseases.

Vasoactive intestinal peptide (VIP)<sup>3</sup> and the structurally related peptide, pituitary adenylate cyclase-activating polypeptide (PACAP), are two neuropeptides present in the immune microenvironment (9, 10), with a broad spectrum of biologic functions, including actions on natural and acquired immunity (reviewed in

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<sup>3</sup> Abbreviations used in this paper: VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; KLH, keyhole limpet hemocyanin; ELISPOT, enzyme-linked immunospot.

Refs. 10–12). VIP and PACAP bind to specific receptors expressed in immune cells (13–15) and regulate T cell cytokine production and proliferation, and several macrophage functions, expressing predominantly an anti-inflammatory activity (reviewed in Refs. 11 and 16). In a recent study, we reported that VIP and PACAP differentially regulate B7.1 and B7.2 expression in resting murine macrophages (36). Moreover, both neuropeptides enhance the costimulatory activity of macrophages for anti-CD3 and allogeneic stimulated naive T cells, by up-regulating B7.2 expression. In the present study, we investigate the role of VIP and PACAP on macrophage B7 expression and costimulatory function for Ag-primed CD4<sup>+</sup> T cells, and on the macrophage-induced regulation of Th1/Th2 differentiation in vitro and in vivo.

## Materials and Methods

### Animals

Female C57BL/6 (H-2<sup>b</sup>) and DBA/2 (H-2<sup>d</sup>) mice obtained from The Jackson Laboratory (Bar Harbor, ME) were kept in pathogen-free conditions. All mice used were between 7 and 12 wk of age.

### Reagents and Abs

Synthetic VIP and PACAP38 were purchased from Novabiochem (Laufelfingen, Switzerland). Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (San Diego, CA). Monoclonal Abs to Mac-1, CD24, Thy-1, CD8, IgM  $\mu$ -chain (6B2), B7.1 (IG10, rat IgG2a) and B7.2 (GL1, rat IgG2a), and capture and biotinylated monoclonal anti-murine IL-4, IL-2, IL-5, and IFN- $\gamma$  Abs were purchased from PharMingen (San Diego, CA). OVA, 5-bromo-4-chloro-3-indolyl-phosphate, nitroblue tetrazolium, and avidin-peroxidase were purchased from Sigma (St. Louis, MO).

### Cell cultures

All cells were cultured in DMEM (HyClone Laboratories, Logan, UT) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 10  $\mu$ g/ml streptomycin, and 2% FCS (Life Technologies, Grand Island, NY).

### In vivo followed by in vitro Ag-stimulation of T cells

To generate KLH-specific CD4<sup>+</sup> T cells, DBA/2 mice were immunized with 150  $\mu$ g KLH in CFA in the footpads as previously described by DeKruyff et al. (17). Two weeks later, the draining lymph nodes were used as a source for the KLH-primed CD4<sup>+</sup> T cells. Single-cell suspensions were depleted of B cells by adherence to goat anti-mouse Ig-coated dishes (5  $\mu$ g/ml; 100  $\mu$ l per well) for 1 h at 4°C. The remaining cells were depleted of CD8<sup>+</sup> T cells, residual B cells, and other accessory cells by treating the cells with a mixture of anti-CD8 (clone 53-6.7), anti-class II (clone 34-2-12), and anti-CD24 (clone J11d) mAbs on ice for 10 min, followed by complement-mediated lysis (rabbit complement from Pel Freez, Rogers, AR) at 37°C for 45 min. The purified T cells were >98% CD4<sup>+</sup> by FACS analysis.

Nonimmune DBA/2 mice were used as a source for peritoneal macrophages. Mice were injected i.p. with 2 ml of 3% thioglycollate broth (Difco, Detroit, MI), and 4 days later, the animals were killed, injected i.p. with 5 ml of cold DMEM, and the peritoneal fluid was harvested. The peritoneal exudate cells were washed and treated with anti-Thy-1 and anti-B220 mAbs followed by complement-mediated lysis to remove contaminating B and T cells. The surviving cells were >96% macrophages as judged by Mac-1<sup>+</sup> expression (FACS analysis) and by morphologic and phagocytic criteria.

Purified peritoneal macrophages ( $1 \times 10^6$  cells/ml) were allowed to adhere for 24 h, followed by removal of possible nonadherent contaminants by washing, and cultured in the presence or absence of different concentrations of VIP or PACAP, for 22 h at 37°C. After washing, the VIP-/PACAP-treated macrophages ( $1 \times 10^5$  cells) were added to the Ag-primed CD4<sup>+</sup> T cells ( $4 \times 10^5$  cells/well) in the presence of soluble KLH (10  $\mu$ g/ml). T cell proliferation was determined as described below.

### In vivo Ag stimulation of T cells

C57BL/6 mice were immunized i.p. with 50  $\mu$ g of KLH in monophosphoryl lipid A + synthetic trehalose dicorynomycolate adjuvant (RIBI Immunochemical Research, Hamilton, MT) as previously described (18, 19). Two weeks later, the mice were challenged i.p. with 100  $\mu$ g of KLH, in PBS. One group of mice was bled immediately before Ag challenge for

serum Ig measurements. VIP or PACAP (5 nmol/mouse, 30  $\mu$ g neuropeptide/kg) and/or anti-B7.1, anti-B7.2, or isotype-matched control Abs (100  $\mu$ g Ab/mouse) were injected i.p. simultaneously with the Ag challenge. Two weeks later, serum samples were obtained and assayed for anti-KLH Abs. In some experiments, splenocytes and peritoneal cells were obtained 2 weeks after the Ag challenge, stimulated in vitro with soluble Ag (50  $\mu$ g KLH), and subjected to enzyme-linked immunospot (ELISPOT) analysis.

### FACS analysis

DBA/2 mice were injected i.p. with medium alone or with VIP or PACAP (5 nmol/mouse, 30  $\mu$ g neuropeptide/kg). After 8 h, macrophages were purified from the peritoneal exudate as indicated above. Cells were washed twice with PBS containing 0.1% sodium azide plus 2% heat-inactivated FCS and incubated with anti-B7.1 or anti-B7.2 mAbs (2.5  $\mu$ g/ml) at 4°C for 1 h. Isotype-matched Abs were used as controls, and IgG block (purchased from Sigma) was used to block the nonspecific binding to Fc receptors. The cells were washed and further stained with FITC-conjugated goat F(ab')<sub>2</sub> anti-rat IgG (Sigma) (2.5  $\mu$ g/ml) for 30 min at 4°C. After extensive washing, the cells were fixed in 1% buffered paraformaldehyde. The stained macrophages, gated according to scatter characteristics, were analyzed on a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA). Fluorescence data are expressed as mean channel fluorescence and as percentage of positive cells after subtraction of background isotype-matched values.

### Induction of cytokine synthesis in CD4<sup>+</sup> T cells

Purified KLH-primed CD4<sup>+</sup> T cells were incubated in 96-well plates (final volume, 150  $\mu$ l of complete DMEM) at a concentration of  $4 \times 10^5$  cells/well with various numbers of syngeneic macrophages preincubated with VIP or PACAP for 18 h; soluble KLH (10  $\mu$ g/ml) was added to the cultures. Samples were performed in quadruplicate. OVA (10  $\mu$ g/ml) was used as an irrelevant Ag. Culture supernatants were harvested on day 2 or 4 as indicated and assayed for levels of IL-2, IL-4, IL-5, and IFN- $\gamma$  by ELISA. In some experiments, the T cells were recovered after 4 days of culture and restimulated at a concentration of  $4 \times 10^5$  cells/well with fresh normal peritoneal macrophages ( $1 \times 10^5$  cells/well) and Ag (KLH, 10  $\mu$ g/ml). Supernatants were harvested 24 h after restimulation.

### Cytokine ELISA

The content of cytokines in the supernatants was determined by specific sandwich ELISAs as previously described. Briefly, 96-well plates were coated overnight with primary anti-cytokine capture Ab. The plates were washed and blocked, and dilutions of supernatants or standards were added. Dilutions of culture supernatants were incubated overnight at 4°C, and after washing, the wells were incubated with biotin-conjugated anti-cytokine detecting mAbs. After a 2-h incubation, the plates were washed and a horseradish peroxidase-streptavidin conjugate was added. The plates were incubated for an additional hour, and after washing, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate was added. After developing, the OD was determined at 405 nm. The cytokine amount in each supernatant was extrapolated from the standard curve. The Ab pairs used were as follows, listed by capture/biotinylated detection Abs: IL-4, BVD4-1D11/BVD6-24G2; IFN- $\gamma$ , R4-6A2/XMG1.2; IL-5, TRFK5/TRFK4; IL-2, and JES6-1A12/JES6-5H4.

### Assay of the macrophage costimulatory activity

The proliferation of the KLH-specific T cells was determined by incubating the KLH-primed CD4<sup>+</sup> T cells in flat-bottom 96-well plates ( $4 \times 10^5$  cells/well) with KLH (10  $\mu$ g/ml) and varying numbers of macrophages, in 150  $\mu$ l of complete DMEM. Samples were assayed in quadruplicate. OVA (10  $\mu$ g/ml) was used as an irrelevant Ag. The cultures were pulsed with 0.5  $\mu$ Ci [<sup>3</sup>H]TdR (specific activity, 97 Ci/mmol; Amersham, Arlington Heights, IL) for the last 16 to 18 h of a 4-day culture, and the proliferation was determined by measuring [<sup>3</sup>H]TdR incorporation in a  $\beta$ -scintillation counter (Beckman, Palo Alto, CA). Results are expressed as the mean cpm  $\pm$  SD of triplicate assays.

### ELISPOT assay

The frequency of Ag-specific T cells producing IFN- $\gamma$  or IL-4 was determined by the ELISPOT technique according to the supplier's protocol (PharMingen). Groups of four C57BL/6 mice were immunized and challenged with KLH as described above. Fourteen days after the Ag challenge,  $5 \times 10^7$ /ml splenocytes or  $1 \times 10^7$ /ml peritoneal cells were stimulated ex vivo with 50  $\mu$ g/ml KLH in complete DMEM for 24 h. Viable cells were recovered by passage through a Histopaque 1007 density gradient (Sigma).

The viable cells were washed, serially diluted, and seeded in nitrocellulose-bottomed 96-well Milititer HA plates (Millipore, Bedford, MA) precoated with anti-IL-4 (BVD4-1D11) or anti-IFN- $\gamma$  (R4-6A2) capture mAbs (5  $\mu$ g/ml in 0.1 M bicarbonate buffer, pH 8.2, for 24 h at 4°C). The cells were cultured for 20 h at 37°C in 5% CO<sub>2</sub>. After washing with PBS (three times), followed by 0.05% (v/v) Tween-20/PBS (three times), the cytokines captured on the cellulose ester membranes were detected with biotinylated anti-IL-4 (BVD6-24G2) or anti-IFN- $\gamma$  (XMG1.2) mAbs. Spots, representing single IL-4- or IFN- $\gamma$ -producing cells were visualized using avidin-peroxidase and 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium and were counted using a dissecting microscope. Data represent total cytokine-producing cells/10<sup>5</sup> splenocytes or peritoneal cells, calculated from the serially diluted samples. No spots were detected in unstimulated cultures without KLH or in cultures stimulated with an irrelevant Ag (OVA, 100  $\mu$ g/ml).

#### Determination of Ab responses

Specific anti-KLH Ab responses in the KLH-immunized C57BL/6 mice were determined by ELISA. Briefly, Maxisorb plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100  $\mu$ l of soluble KLH (10  $\mu$ g/ml) in 0.1 M bicarbonate buffer, pH 9.6. After washing with PBS containing 0.05% v/v Tween-20, the plates were blocked with 3% BSA and incubated with serial dilutions of serum for 2 h at 37°C. After washing, biotinylated anti-IgG1 (2.5  $\mu$ g/ml) and anti-IgG2a (2.5  $\mu$ g/ml) (Serotec, Oxford, U.K.) were added for 1 h at 37°C. The plates were washed, followed by incubation with streptavidin-horseradish peroxidase. The bound enzyme was detected with the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate, and quantitated at OD<sub>405</sub> in an ELISA reader (Bio-Tek Instruments, Winooski, VT). A standard curve was constructed for each assay by coating wells with an isotype-specific anti-mouse Ig followed by addition of known concentrations of the mouse Ig isotype as previously described (20). The remaining steps for the ELISA were identical with those used for the KLH-coated wells. The values for the experimental samples were interpolated using the standard curve.

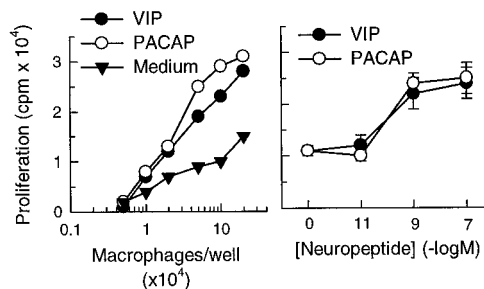
## Results

#### VIP and PACAP stimulate the macrophage costimulatory activity for Ag-primed CD4<sup>+</sup> T cells

We have recently reported that VIP and PACAP enhance the costimulatory activity of macrophages for naive T cells stimulated with anti-CD3 or in allogeneic reactions (36). In this study, we investigate whether VIP and PACAP regulate the costimulatory capacity of macrophages for the proliferation of Ag-primed T cells. DBA/2 peritoneal macrophages were treated with VIP or PACAP for 24 h, washed, and added to the responder T cells in the presence of soluble KLH. The responder T cells were obtained from the draining lymph nodes of DBA/2 mice injected with KLH in CFA 2 weeks previously. The proliferation of the responder T cells after exposure to macrophages and soluble Ag was measured through [<sup>3</sup>H]TdR incorporation. Macrophages cultured with medium alone had a low stimulatory activity for the proliferation of CD4<sup>+</sup> T cells. However, VIP and PACAP significantly up-regulated the costimulatory capacity of macrophages (Fig. 1). The rate of proliferation increased with the number of VIP/PACAP-treated macrophages. The ability of VIP and PACAP to induce the costimulatory activity of macrophages was dose dependent, with maximal effects in the 10<sup>-7</sup> to 10<sup>-9</sup> M concentration range (Fig. 1).

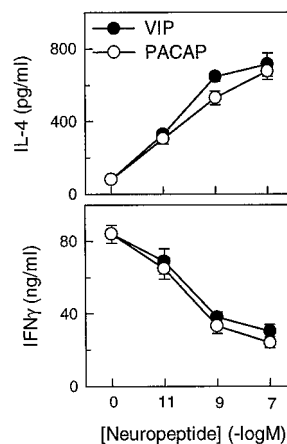
#### Pretreatment of macrophages with VIP or PACAP enhances their capacity to induce Th2 cytokine synthesis

In a previous study, we showed that VIP and PACAP up-regulate expression of B7.2 but not B7.1 on macrophages over an extended time period (from 12 to 72 h) (36). Recent studies indicated that expression of B7, and especially of B7.2, is essential for the development of Th2 effector cells. Therefore, we asked whether the cytokine profile of the Ag-primed CD4<sup>+</sup> T cells cultured in the presence of VIP- or PACAP-pretreated macrophages would be altered. Peritoneal macrophages obtained from DBA/2 mice were



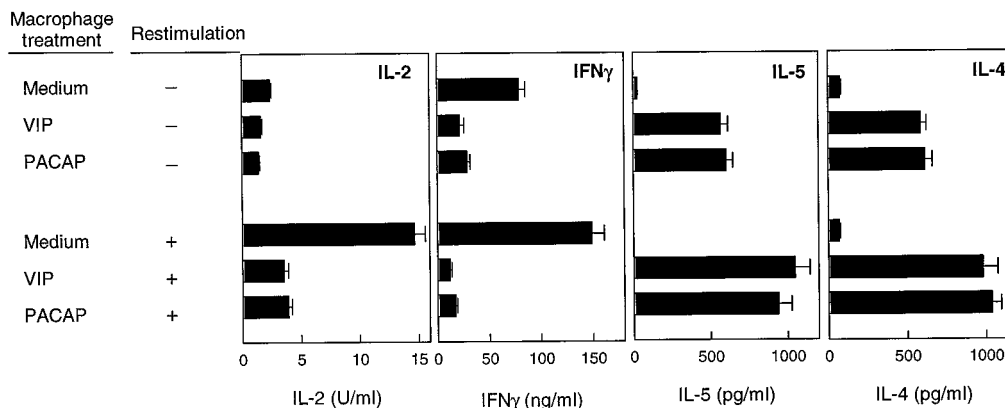
**FIGURE 1.** VIP and PACAP stimulate the macrophage costimulatory activity for Ag-primed CD4<sup>+</sup> T cells. DBA/2 peritoneal macrophages ( $1 \times 10^6$  cells/ml) were treated for 24 h with medium alone or with different concentrations of VIP or PACAP ( $10^{-8}$  M, left panel). After washing with medium and PBS, the macrophages were added in various numbers ( $10^5$  cells/well, right panel) to KLH-primed CD4<sup>+</sup> T cells ( $4 \times 10^5$  cells/well) in the presence of KLH (10  $\mu$ g/ml). [<sup>3</sup>H]TdR was added for the last 18 h of a 4-day culture. Cells cultured with an irrelevant Ag (OVA, 10  $\mu$ g/ml) did not induce any proliferative response:  $576 \pm 32$  cpm for controls and  $662 \pm 24$  cpm for  $10^{-8}$  M VIP. Results are expressed as cpm [<sup>3</sup>H]TdR incorporation; each value is the mean of three determinations.

cultured in vitro with VIP or PACAP for 18 h, washed, and added to syngeneic CD4<sup>+</sup> T cells purified from lymph nodes of KLH-primed mice in the presence of soluble Ag. Stimulation with macrophages pretreated with medium alone resulted in the development of effector T cells producing high levels of IFN- $\gamma$  and low levels of IL-4 (Fig. 2). However, pretreatment of macrophages with either VIP or PACAP led to the inhibition of IFN- $\gamma$  and to significant increase in the IL-4 production (Fig. 2). In addition to the effect on IL-4 and IFN- $\gamma$  production, macrophages pretreated with VIP or PACAP developed an increased capacity to induce another Th2 cytokine, IL-5, and a reduced capacity to induce IL-2 (Fig. 3). No cytokine production by T cells was detectable in the absence of macrophages (data not shown) or in the presence of an



**FIGURE 2.** VIP and PACAP enhance the capacity of macrophages to induce Th2 cytokine production in CD4<sup>+</sup> T lymphocytes. Macrophages ( $1 \times 10^5$  cells/well) pretreated for 24 h with different concentrations of VIP or PACAP were incubated with Ag (KLH, 10  $\mu$ g/ml) and Ag-primed CD4<sup>+</sup> T cells ( $5 \times 10^5$  cells/well). Supernatants were harvested after 4 days and assayed for IL-4 and IFN- $\gamma$  by ELISA. Cells cultured with an irrelevant Ag (OVA, 10  $\mu$ g/ml) did not induce IL-4 and IFN- $\gamma$ :  $<0.2$  ng IFN- $\gamma$ /ml and  $<30$  pg IL-4/ml for both controls and  $10^{-8}$  M VIP. Results are the mean  $\pm$  SD of three independent experiments performed in duplicate.





**FIGURE 3.** VIP-/PACAP-treated macrophages induce CD4<sup>+</sup> T cells to a Th2 pattern of cytokine synthesis. Purified peritoneal macrophages ( $1 \times 10^5$  cells/well) were pretreated with medium alone, VIP, or PACAP ( $10^{-8}$  M). After 24 h, the macrophages were washed and incubated with Ag (KLH, 10  $\mu$ g/ml) and Ag-primed CD4<sup>+</sup> T cells ( $5 \times 10^5$  cells/well). Culture supernatants were harvested after 24 h (for IL-2), 96 h (for IL-4 and IFN- $\gamma$ ), and 120 h (for IL-5). T cells were recovered from some cultures after 96 h and restimulated at  $4 \times 10^5$  cells/well with fresh peritoneal macrophages ( $1 \times 10^5$  cells/well) and Ag (KLH, 10  $\mu$ g/ml). Supernatants were harvested 24 h after restimulation. Cytokine content in the supernatants was assayed by ELISA. Each result is the mean  $\pm$  SD of three independent experiments performed in duplicate.

unrelated Ag (see Fig. 2 legend). Thus, pretreatment of macrophages with VIP or PACAP enhances their capacity to preferentially induce Th2-cytokine synthesis and inhibits Th1-cytokine synthesis. These results suggest that Ag-specific T cells stimulated with VIP- or PACAP-treated macrophages preferentially differentiate into Th2 effector cells. The change in cytokine profile induced by VIP and PACAP was even more pronounced when the T cells were restimulated after 4 days with normal APCs and KLH (Fig. 3, lower panels). This was to be expected whether costimulation with VIP-/PACAP-treated macrophages led to an increased number of Th2 effector cells.

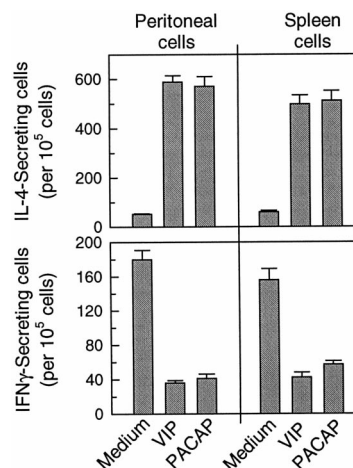
#### VIP and PACAP regulate APC-dependent Th1 and Th2 development in vivo

After activation by Ag, naive CD4<sup>+</sup> T cells differentiate into Th1 and/or Th2 effector cells. To identify the differentiation pathway that is predominantly activated by VIP and PACAP after immunization, we analyzed the number of cells that release Th1 vs Th2 cytokines. To raise a sufficient number of Ag-reactive T cells, C57BL/6 mice were immunized with KLH emulsified in adjuvant and challenged with soluble KLH 2 weeks later. VIP and PACAP were injected simultaneously with the Ag challenge. Two weeks after the Ag challenge, splenocytes and peritoneal cells were harvested and restimulated in vitro for 24 h with KLH, presented by spleen or peritoneal APCs. The live cells were recovered, and the frequency of Ag-reactive Th1 and Th2 cells was assessed on the basis of IFN- $\gamma$ - and IL-4-secreting cells, respectively, using ELISPOT assays. Mice injected with the Ag in the absence of VIP/PACAP developed high numbers of IFN- $\gamma$ -producing Th1 cells and very low numbers of IL-4-producing Th2 cells (Fig. 4). In contrast, mice injected with VIP or PACAP generated few IFN- $\gamma$ -specific spots, whereas they developed high numbers of IL-4-secreting Th2 cells (Fig. 4). The results obtained by this approach mirror the in vitro changes seen by the analysis of the Th1 and Th2 cytokine profile and confirm that VIP and PACAP shift the T cell response to Th2 differentiation and/or proliferation, in vivo as well as in vitro.

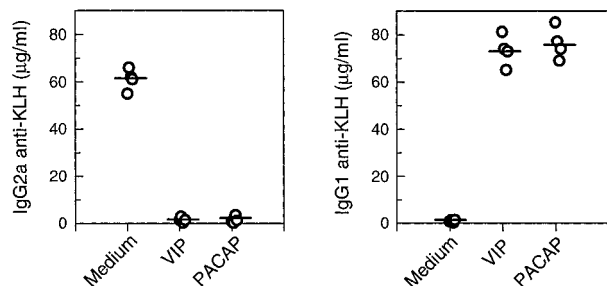
#### VIP and PACAP influence the Ag-specific Ab responses

Th1 and Th2 lymphocytes differentially affect the shift from the T cell-independent IgM isotype to IgG isotypes in activated B lymphocytes, inducing a switch to IgG2a/b and IgG1, respectively

(21). Because VIP/PACAP favor the differentiation into Th2 effector cells, we expected a switch in anti-KLH Abs toward the IgG1 isotype. C57BL/6 mice were immunized with KLH emulsified in adjuvant, followed by a challenge with free KLH, and the isotype of the serum anti-KLH Abs was determined 2 weeks later. The mice injected with KLH in the absence of VIP/PACAP produced IgG2a, but not IgG1 anti-KLH Abs (Fig. 5). In contrast, mice injected with VIP or PACAP simultaneously with the second Ag challenge produced IgG1 (but not IgG2a) anti-KLH Abs (Fig. 5). This remarkable IgG isotype composition indicates a nearly



**FIGURE 4.** VIP and PACAP regulate the ratio of IFN- $\gamma$ /IL-4-secreting cells. Groups of C57BL/6 mice (four per group) were Ag primed with 50  $\mu$ g of KLH emulsified in adjuvant i.p. After 2 wk, mice were challenged with 100  $\mu$ g KLH i.p., in the presence or absence of VIP or PACAP (5 nmol/mouse, 30  $\mu$ g neuropeptide/kg). Fourteen days after Ag challenge,  $5 \times 10^7$  splenocytes and  $1 \times 10^7$  peritoneal cells were stimulated ex vivo with 50  $\mu$ g/ml KLH in complete DMEM for 24 h, after which the numbers of IFN- $\gamma$ - and IL-4-producing T cells per  $10^5$  viable cells were determined by ELISPOT as described in *Materials and Methods*. Peritoneal cells from mice injected with medium generated 15 spots and 1 spot for IFN- $\gamma$  and IL-4, respectively, after stimulation with KLH. These numbers were subtracted from the counts shown. No spots were detected in unstimulated cultures or in cultures stimulated with an irrelevant Ag (OVA, 50  $\mu$ g/ml). Results are the mean  $\pm$  SD of four independent experiments performed in duplicate.



**FIGURE 5.** VIP and PACAP stimulate IgG1 and inhibit IgG2a anti-KLH Abs. Groups of C57BL/6 mice (four per group) were Ag primed with 50  $\mu$ g KLH emulsified in adjuvant. After 2 wk, mice were challenged with 100  $\mu$ g KLH, in the presence or absence of VIP or PACAP (5 nmol/mouse, 30  $\mu$ g neuropeptide/kg). Fourteen days after Ag challenge, serum levels of IgG1 and IgG2a anti-KLH were determined by isotype-specific ELISA. For controls, the average IgG1 and IgG2a levels, determined in sera obtained from four mice before immunization, were  $1.2 \pm 0.2$   $\mu$ g/ml and  $0.9 \pm 0.2$   $\mu$ g/ml, respectively.

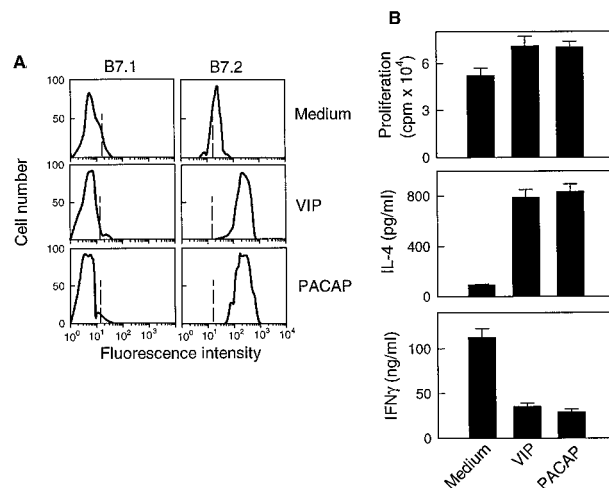
exclusive activation of Th2 cells, in agreement with the results obtained by ELISPOT.

*The Th2 response induced by VIP and PACAP is mediated by the up-regulation of B7.2 expression on macrophages*

As a result of LPS and/or IFN- $\gamma$  activation, B7.2 expression on macrophages occurs earlier and at higher levels than B7.1. In a previous study, we determined that VIP and PACAP induce B7.2, but not B7.1, expression in macrophages, at least during an initial period of up to 72 h (36). Here, we determined whether VIP and PACAP up-regulate B7.2 expression in vivo and whether the expression of B7.2 is responsible for the preferential development of a Th2 response. DBA/2 mice were injected i.p. with 5 nmol VIP or PACAP (30  $\mu$ g neuropeptide/kg), and B7 expression was analyzed on purified peritoneal macrophages 8 h later by flow cytometry. As expected, VIP and PACAP increased in vivo B7.2, but not B7.1, expression on macrophages (Fig. 6A). In addition, the in vivo treatment with VIP or PACAP enhanced the macrophage costimulatory activity for Ag-primed CD4 $^{+}$  T cells (Fig. 6B, top panel). Moreover, macrophages from VIP- and PACAP-treated mice preferentially induced a Th2 cytokine profile in the Ag-primed CD4 $^{+}$  T cells (Fig. 6B, middle and bottom panels).

To investigate the relationship between the increase in B7.2 expression on macrophages and the preferential development of a Th2 response, two different assays were performed. First, KLH-immunized C57BL/6 mice were challenged 2 weeks later with KLH and VIP or PACAP (5 nmol, 30  $\mu$ g neuropeptide/kg), with or without anti-B7.1 or anti-B7.2 mAbs (100  $\mu$ g/animal). After 8 h, the peritoneal macrophages were harvested and cultured in vitro with the Ag-primed CD4 $^{+}$  T cells in the presence of soluble KLH. IL-4 and IFN- $\gamma$  production were assayed by ELISPOT. Anti-B7.2, but not anti-B7.1 Abs, reversed the VIP effect, increasing IFN- $\gamma$  and reducing IL-4 production (Fig. 7A).

In a second experiment, DBA/2 peritoneal macrophages were treated in vitro with VIP in the presence or absence of anti-B7.1 or anti-B7.2 Abs. After washing, the macrophages were added to KLH-primed CD4 $^{+}$  T cells in the presence of soluble KLH, and the amounts of IFN- $\gamma$  and IL-4 were determined by ELISA in the culture supernatants. Again, anti-B7.2, but not anti-B7.1 Abs, reversed the effects of VIP, by increasing IFN- $\gamma$  and decreasing IL-4 production (Fig. 7B).

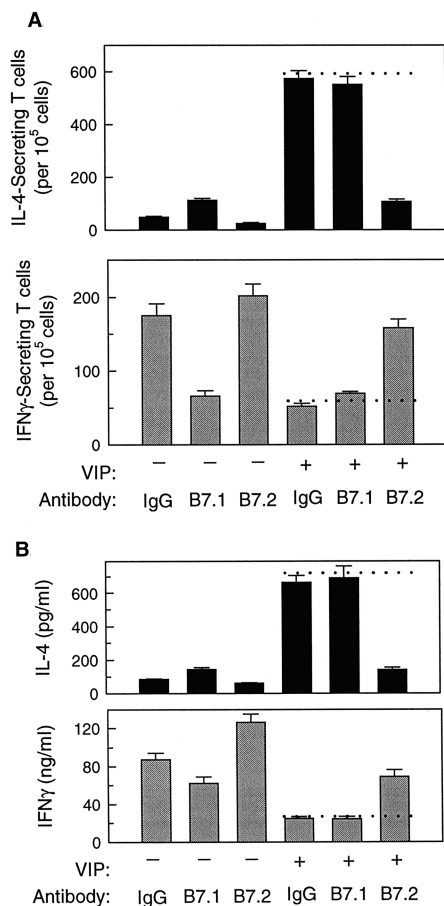


**FIGURE 6.** In vivo effect of VIP and PACAP on B7.1/B7.2 expression and macrophage costimulatory activity. A, VIP and PACAP differentially regulate the expression of B7.1 and B7.2 in macrophages. DBA/2 mice (three per group) were injected i.p. with medium, VIP, or PACAP (5 nmol/mouse, 30  $\mu$ g neuropeptide/kg). After 8 h, macrophages were purified from peritoneal exudate, and B7.1 and B7.2 expression was analyzed by flow cytometry. The gates were set based on staining with unrelated isotype control Abs. Data are representative of three similar experiments. B, DBA/2 mice (three per group) were injected i.p. with medium, VIP, or PACAP (5 nmol/mouse, 30  $\mu$ g peptide/kg). After 8 h, macrophages were purified from peritoneal exudate and incubated ( $1 \times 10^5$  cells/well) with Ag (KLH, 10  $\mu$ g/ml) and Ag-primed CD4 $^{+}$  T cells ( $5 \times 10^5$  cells/well). [ $^3$ H]TdR was added to the wells for the last 18 h of a 4-day culture. Results are expressed as cpm [ $^3$ H]TdR incorporation. In addition, supernatants were harvested after 4 days and assayed for IL-4 and IFN- $\gamma$  by ELISA. Each result is the mean  $\pm$  SD of three independent experiments performed in duplicate.

## Discussion

VIP and PACAP are two immunomodulatory neuropeptides that regulate cytokine production and proliferation in T cells and modulate several macrophage functions (reviewed in Refs. 10–12). We have recently demonstrated that VIP and PACAP enhance the macrophage costimulatory activity for naive CD4 $^{+}$  T cells exposed to allogeneic or anti-CD3 stimuli (36). In the present study, we show that VIP and PACAP increase the costimulatory activity of macrophages for Ag-primed CD4 $^{+}$  T cells. In addition, the VIP/PACAP-treated macrophages gain the ability to induce Th2-type cytokines such as IL-4 and IL-5, and reduce Th1-type cytokines such as IFN- $\gamma$  and IL-2.

Previous reports from our laboratory indicated that VIP and PACAP inhibit the production of IL-2, IL-4, and IL-10 from naive T cells stimulated with Con A or anti-CD3 (11, 22–24). The inhibitory effect of VIP on IL-4 production by naive T cells is in apparent contradiction with the results presented in this study, in which VIP and PACAP promote IL-4 production. However, the two experimental systems are different. In the first system we investigated the effect of VIP directly on naive T cells stimulated with Con A or anti-CD3 mAbs. Moreover, we concluded that the effect of VIP on IL-4 production by T cells is indirect, being mediated through the reduction in available IL-2 (23). Here, we investigate the effects of VIP/PACAP on macrophages and indirectly on the differentiation of Ag-primed Th cells. The possibility that VIP/PACAP could be carried over from the macrophage to the T cell cultures is extremely unlikely, because the pretreated macrophages were washed extensively, and the neuropeptides have a very short life span in culture.



**FIGURE 7.** Up-regulation of B7.2 is involved in the predominant Th2 response induced by VIP and PACAP. **A**, Groups of C57BL/6 mice (four per group) were Ag primed with 50  $\mu$ g KLH emulsified in adjuvant. After 2 wk, mice were challenged with 100  $\mu$ g KLH and injected i.p. with VIP (5 nmol/mouse, 30  $\mu$ g peptide/kg), in the presence or absence of control (IgG), anti-B7.1, or anti-B7.2 Abs (100  $\mu$ g/mouse). Fourteen days after the Ag challenge,  $1 \times 10^7$  peritoneal cells were stimulated ex vivo with 50  $\mu$ g/ml KLH in complete DMEM for 24 h, after which the numbers of IFN- $\gamma$ - and IL-4-producing T cells per  $10^5$  viable cells were determined by ELISPOT as described in *Materials and Methods*. The dotted lines represent values from VIP-treated mice. Each result is the mean  $\pm$  SD of four independent experiments performed in duplicate. **B**, DBA/2 peritoneal macrophages were treated for 24 h with VIP ( $10^{-8}$  M), in the presence or absence of control (IgG), anti-B7.1, or anti-B7.2 Abs (5  $\mu$ g/ml). After washing with medium and PBS, macrophages ( $1 \times 10^5$  cells) were added to KLH-primed CD4<sup>+</sup> T cells ( $5 \times 10^5$  cells/well) in the presence of KLH (10  $\mu$ g/ml). Supernatants were harvested after 4 days and assayed for IL-4 and IFN- $\gamma$  by ELISA. Dotted lines represent values from VIP-pretreated macrophages. Results are the mean  $\pm$  SD of four independent experiments performed in duplicate.

In our experiments we used thioglycollate-injected mice to increase the number of harvested peritoneal macrophages. Although thioglycollate-induced macrophages are not identical with quiescent cells, they express low levels of B7.2 and no B7.1. In contrast, LPS-activated macrophages express B7.1 and up-regulate significantly the expression of B7.2 (reviewed in Ref. 25). In preliminary experiments, we determined that VIP and PACAP affect quiescent macrophages the same way as thioglycollate-induced ones, by up-regulating B7.2, but not B7.1.

Because the Ag-primed T cells exposed to VIP-/PACAP-treated macrophages secrete primarily Th2-type cytokines, we propose that the VIP-/PACAP-treated macrophages preferentially induced

differentiation into Th2 effector cells. This conclusion is supported by the ELISPOT experiments after the in vivo administration of VIP or PACAP in mice immunized and challenged with Ag. Similar to the in vitro experiments, which indicated an increase in IL-4 production and a decrease in IFN- $\gamma$  production, the in vivo inoculation of VIP and PACAP led to significantly reduced numbers of IFN- $\gamma$ -secreting cells and enhanced numbers of IL-4-secreting cells.

One of the consequences of the shift in cytokine profile is the change in the Ig isotype. Cytokines such as IFN- $\gamma$  and IL-4 regulate the Ig isotype switch. In mice, IL-4 promotes IgG1 (26, 27) and inhibits IgG2a (28), whereas IFN- $\gamma$  has the opposite effects (28). Therefore, we expected that the in vivo treatment with VIP or PACAP would induce a shift in the anti-KLH Ab isotype, with an increase in IgG1 and a decrease in IgG2a. This was indeed the case.

One of the most interesting questions is the nature of the mechanism by which VIP and PACAP enable macrophages to induce Th2 differentiation. We have recently shown that VIP and PACAP specifically up-regulate the expression of B7.2 on peritoneal macrophages and that the enhanced B7.2 expression is responsible for the increase in the macrophage stimulatory activity for naive T cells (36). Similar findings are reported in this study. VIP and PACAP were found to up-regulate B7.2 expression on macrophages in vivo, at least in the short term, and to enhance both their costimulatory activity and their subsequent capacity to increase IL-4 and decrease IFN- $\gamma$  production. The direct involvement of B7.2 in the switch in cytokine production is supported by the fact that the in vivo administration of anti-B7.2 Abs reversed the effect of VIP on both cytokine production and number of cytokine-secreting cells. Although the role of B7.1 vs B7.2 in selectively activating the Th1- or the Th2-type response is controversial, several in vivo disease models suggest that B7.2 plays the essential role in the differentiation of Th2 cells (29–32). This is supported by the different cytokine profile exhibited by B7.1<sup>-/-</sup> and B7.2<sup>-/-</sup> deficient mice in a model of murine schistosomiasis. Whereas the B7.1<sup>-/-</sup> mice do not differ significantly from the wild type, the B7.2<sup>-/-</sup> mice express high levels of IFN- $\gamma$  and low levels of IL-4 and IL-10, suggesting the involvement of B7.2 in Th2 differentiation (33). However, the double B7.1/B7.2-deficient mice exhibit even more pronounced changes in the cytokine profile, with very high levels of IFN- $\gamma$  and no IL-4 or IL-10 (33). The most direct interpretation is that, although B7.2 alone induces Th2 differentiation whereas B7.1 does not, together the two isoforms act in a complementary manner.

In addition to the MHC haplotype, the dose and nature of the Ag, the route of Ag administration, and the availability of IFN- $\gamma$  and IL-12, as opposed to IL-4, are decisive for the differentiation to Th1 and Th2, respectively (1, 34). We have recently reported that VIP and PACAP inhibit IL-12 production by activated macrophages, with the subsequent inhibition of IFN- $\gamma$  production by T cells (35). The neuropeptide-induced absence of IL-12 and IFN- $\gamma$ , two cytokines necessary for development of Th1 cells, may further amplify the bias toward Th2 differentiation.

In conclusion, our observations suggest that pretreatment of macrophages with VIP/PACAP enhances their costimulatory capacity for both naive and Ag-primed T cells, through the up-regulation of B7.2 expression. The VIP/PACAP treatment enables macrophages to initiate a predominant Th2 response after Ag stimulation. In addition, the regulatory effect of VIP/PACAP on IL-12 production by macrophages and subsequently on IFN- $\gamma$  production by activated T cells could further promote Th2 differentiation and inhibit the initiation of Th1 responses. Because Th1-dominated responses are associated with inflammatory reactions typical of

cell-mediated immunity, the VIP/PACAP inhibition of Th1 development may represent an additional mechanism for the general anti-inflammatory activity of the two neuropeptides.

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