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Viral Macrophage-Inflammatory Protein-II: A Viral Chemokine That Differentially Affects Adaptive Mucosal Immunity Compared with Its Mammalian Counterparts

Udai P. Singh, Shailesh Singh, Palaniappan Ravichandran, Dennis D. Taub, and James W. Lillard, Jr.

Chemokines play a profound role in leukocyte trafficking and the development of adaptive immune responses. Perhaps due to their importance in host defense, viruses have adopted many of the hallmarks displayed by chemokines. In particular, viral MIP-II (vMIP-II) is a human chemokine homologue that is encoded by human herpes virus 8. vMIP-II is angiogenic, selectively chemotactic for Th2 lymphocytes, and a homologue of human I-309 and mouse TCA-3, which also differentially attracts Th2 cells. To better understand the effect of viral chemokines on mucosal immunity, we compared the affects of vMIP-II, I-309, and TCA-3 on cellular and humoral immune responses after nasal immunization with OVA. These CCR8 ligands significantly enhanced Ag-specific serum and mucosal Abs through increasing Th2 cytokine secretion by CD4+ T cells. These alterations in adaptive humoral and cellular responses were preceded (12 h after immunization) by an increase in CD4+ T and B cells in nasal tracts with decreases of these leukocyte populations in the lung. Interestingly, vMIP-II increased neutrophil infiltration in the lung and Ag-specific IL-10-secreting CD4+ T cells after immunization. Although I-309 increased the number of CD28-, CD40L-, and CD30-positive, Ag-stimulated naive CD4+ T cells, vMIP-II and TCA-3 decreased the number of CD28-, CD40L-, and CD30-positive, resting naive CD4+ T cells. Taken together, these studies suggest that CCR8 ligands direct host Th2 responses, and vMIP-II up-regulates IL-10 responses and limits costimulatory molecule expression to mitigate host immunity.


While the mucosa serves as the first natural defense against mucosal pathogens, another major function of mucosal epithelial cells is the transport of polymeric IgA and the production of numerous effector molecules (1), namely defensins (2), chemokines (3–5), and cytokines (6), to initiate mucosal immune responses. Numerous studies have demonstrated the ability of chemokines to regulate the migration of lymphocytes to sites of disease. We have shown, under certain conditions, that chemokines can also modulate adaptive immunity. Besides chemotaxis, our data suggest that chemokines, such as lymphotactin (3), RANTES (4), MIP-1α as well as MIP-1β (5), and others (7), can serve as potent and effective adjuvants for adaptive mucosal immunity (humoral and cell mediated).

Perhaps due to the importance of chemokines in host immunity, a number of viruses have evolved endogenous chemokine homologues and binding proteins that presumably interfere with immune cell function so that these microbes can evade immune detection (8–18). Recently, viral MIP-II (vMIP-II), a viral chemokine homologue encoded by human herpes virus 8 (19), has been shown to compete with native chemokines by binding to human chemokine receptors (20–22). Specifically, vMIP-II was shown to bind CCR3 and CCR8 to block the action of host chemokines on human monocytes (20), selectively attract eosinophils (23), and recruit Th2 lymphocytes that express CCR8, which could have implications in the immunopathogenesis of human herpes virus 8 (HHV-8) (24, 25).

HHV-8 was originally identified in Kaposi’s sarcoma (KS) lesions, and is considered to be the etiologic agent responsible for KS patients with or without HIV infection (26). HHV-8 has also been found in patients with primary effusion lymphoma and multicentric Castleman’s disease, suggesting that it may play an important role in these lymphoproliferative diseases (27). This viral chemokine may also play an important role in the immunopathogenesis of HHV-8. Perhaps vMIP-II is used by HHV-8 to alter the recruitment of leukocytes as part of a viral defense mechanism against the mucosal immune system. However, the selective advantage, if any, that these virally encoded chemokines confer to pathogenesis is not known. However, it has been demonstrated recently that vMIP-II inhibits virus-specific delayed-type hypersensitivity responses in vivo, suggesting that vMIP-II is an inhibitor of type 1 T cell-mediated inflammation (28). Hence, this viral chemokine may direct host immune responses that would not effectively clear viral infections (i.e., Th1→Th2).

I-309 is a human CC chemokine that was first identified during a search of genes expressed by activated T cell lines (29). I-309 induces the chemotaxis of lymphocytes expressing CCR8 (30) and protects murine thymic lymphoma cell lines from dexamethasone-induced apoptosis (31). The murine homologue of I-309 is TCA-3, which induces calcium mobilization in murine CCR8 (mCCR8) transfectants (32). It has been reported that mCCR8-binding affinity of
TCA-3 and I-309 is relatively high ($K_d = 3–4$ and 5 nM, respectively) (33). Hence, I-309 and TCA-3 can be used to study important mCCR8-mediated events.

In this study, we analyzed the immunoregulatory properties of vMIP-II to determine how this viral chemokine modulates mucosal immune responses, relative to its closest or most similar mammalian homologue (i.e., I-309 at 57% homology). Specifically, this study tests the hypothesis that vMIP-II negatively affects adaptive immune responses compared with I-309, but not TCA-3, which is <20% homologous to vMIP-II. Although 10 nM I-309 or TCA-3 is able to provoke calcium mobilization in mCCR8 transfectants, human CCR8 transfectants only respond to I-309 (32). Another point that supports this comparative study is that vMIP-II, but not TCA-3, competitively inhibits the binding of I-309 (34). Hence, the comparison of I-309/TCA-3 and vMIP-II is appropriate due to their high homology, binding affinities to mCCR8, and functional activity when binding mCCR8.

Unfortunately, the multiple biological activities related to the immunopathogenesis of viral chemokines are poorly understood. Indeed, the receptor-binding properties related to vMIP-II are unique among all known chemokines. The present study is the first of its kind to determine some of the cellular and molecular mechanisms that vMIP-II may use to modulate adaptive immunity, which could possibly favor HHV-8 pathogenesis. We report that vMIP-II, I-309, and TCA-3 differentially modulate costimulatory molecule expression and Ag-specific responses to suppress or direct host immunity, which provides new insights into the function of CCR8 ligands in immune regulation.

**Materials and Methods**

**Immunogens**

I-309 and vMIP-II were purchased from PeproTech (Rocky Hill, NJ). The mouse TCA-3 was purchased from R&D Systems (Minneapolis, MN). The potential level of endotoxin contamination was quantified by the chromogenic Limulus amebocyte lysate assay (Associates of Cape Cod, Falmouth, MS) to be <5 EU/ml. Chicken egg albumin (OVA) and BSA were purchased from Sigma-Aldrich (St. Louis, MO).

**Mice and immunizations**

Female C57BL/6, BALB/c, and DO11.10 mice, aged 6–8 wk, were procured from The Jackson Laboratory (Bar Harbor, MN). All mice were housed in horizontal laminar flow barrier cabinets free of microbial pathogens. Routine Ab screening for a large panel of pathogens and histological analysis of organs and tissues were performed to ensure that mice were pathogen free. Following anesthesia, mice were nasally immunized on days 0, 7, and 14 with 75 μg of OVA alone or OVA plus 1 μg of vMIP-II, I-309, or TCA-3 in 15 μl of PBS (7.5 μl per nare). BALB/c mice of similar age were also immunized to confirm the results obtained using C57BL/6 mice. Experimental groups consisted of five mice, and studies were repeated three times. The guidelines proposed by the committee for the Care of Laboratory Animal Resources Commission of Life Sciences-National Research Council were followed to minimize animal pain and distress.

**Sample and tissue collection**

Fecal samples were weighed and dissolved in 1 ml of PBS containing 0.1% sodium azide per 100 mg of fecal pellet. Following suspension by vortexing for 10 min, fecal samples were centrifuged and supernatants were collected for analysis. Vaginal cavities were rinsed twice with 100 μl of PBS to collect reproductive tract secretions. Blood samples were collected by supraorbital capillary puncture, and serum was obtained following centrifugation. Serum and mucosal secretions were collected 1 wk after the last immunization and analyzed for OVA-specific Ab responses by ELISA. Mice were sacrificed by CO2 inhalation 1 wk after the last immunization to quantify the OVA-specific T cell responses present in immune compartments.

**Cytokine and OVA-specific Ab detection by ELISA**

For the assessment of cytokine production by spleen, lungs, nasal tract, and cervical lymph nodes, culture supernatants were harvested after 3 days of incubation. Th cytokines, IL-4, IL-10, GM-CSF, IL-2, IFN-γ, and TNF-α, in cell culture supernatants were determined by ELISA, following manufacturer’s instructions (E-Biosciences, San Diego, CA). Fecal, vaginal, and serum sample levels of OVA-specific Abs were measured by ELISA, as previously described (3). Briefly, 96-well Falcon 3912 flexible ELISA plates (Fisher Scientific, Pittsburgh, PA) were coated with 100 μl of 1 mg/ml OVA in PBS overnight at 4°C and blocked with 10% FCS (Atlanta Biologicals, Norcross, GA) in PBS (FCS-PBS) for 3 h at room temperature. Individual samples (100 μl) were added and serially diluted in FCS-PBS. After overnight incubation at 4°C, ELISA plates were washed three times. The plates were read at 450 nm after 10 min.

**Cell isolation**

Seven days after three (weekly) nasal immunizations with PBS and/or 75 μg of OVA alone or OVA plus 1 μg of vMIP-II, I-309, or TCA-3, single cell suspensions of spleen, lungs, cervical lymph nodes, and nasal tract were passed through a sterile wire screen (Sigma-Aldrich). The lower respiratory tract (lungs and mediastinal lymph nodes) was injected with 1 ml of cold PBS to remove blood, dissected into small pieces, and digested in collagenase type IV (Sigma-Aldrich) in RPMI 1640 (collagenase solution) for 45 min with stirring at 37°C (2–4). Nasal tract mucosal tissue was removed by scraping, and single cell suspensions of spleen, lungs, nasal tract, and cervical lymph node cells were passed through a sterile wire screen (Sigma-Aldrich). Cell suspensions were washed twice in RPMI 1640. Lung and nasal tract lymphocytes were further purified using a discontinuous Percoll (Pharmacia Biotech, Uppsala, Sweden) gradient, collecting at the 40–75% interface. Lymphocytes were maintained in complete medium, which consisted of RPMI 1640 supplemented with 10 ml/L nonessential amino acids (Mediatech, Washington, DC), 1 mM sodium pyruvate (Sigma-Aldrich), 10 nM HEPES (Mediatech), 100 U/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamicin (Elkins-Sinn, Cherry Hill, NJ), 50 μM 2-ME (Sigma-Aldrich), and 10% FCS (Atlanta Biologics).

**DO11.10 primary lymphocyte stimulation**

Splenocytes from DO11.10 mice were isolated and added at a density of 5 × 10^6 cells/ml in complete medium containing 0, 1, 10, 100, or 1000 ng/ml vMIP-II, I-309, or TCA-3. A class II-restricted OVA peptide conjugated to mouse I-309 or TCA-3, or vMIP-II, I-309, or TCA-3 in 15 μl of PBS (7.5 μl per nare). BALB/c mice of similar age were also immunized to confirm the results obtained using C57BL/6 mice. Experimental groups consisted of five mice, and studies were repeated three times. The guidelines proposed by the committee for the Care of Laboratory Animal Resources Commission of Life Sciences-National Research Council were followed to minimize animal pain and distress.

**In vitro cell proliferation assay**

Lymphocyte proliferation was measured by a BrdU absorption-detection kit (Roche Diagnostics, Dusseldorf, Germany). In brief, after 2 days of cultures, at the density of 10^6 cells/ml, cells were transferred to polystyrene 96-well plates (Corning Glass, Corning, NY). A total of 10 μl of BrdU labeling solution (10 μM final concentration per well) was added and incubated for 18 h at 37°C with 5% CO2. The cells were then fixed and incubated with 100 μl of nuclease in each well for 30 min at 37°C. The cells were washed with complete medium and again incubated with BrdU-peroxidase solution for 30 min at 37°C. The incorporation was developed with an ABTS solution, and the change in OD was read at 450 nm (OD450).

**Flow cytometry analysis**

Twelve hours after nasal administration of PBS, OVA alone, or OVA plus I-309, TCA-3, or vMIP-II, nasal tract, cervical lymph node, lung, and spleen tissue were isolated for flow cytometry analysis. Cell suspensions
were washed twice in RPMI 1640. Lungs and nasal tract lymphocytes were further purified using a discontinuous Percoll (Pharmacia Biotech) gradient, collecting at the 40–75% interface. Similar to the methods described above, leukocytes were stained with PE-Cy5-, FITC-, or PE-conjugated anti-CD3, anti-CD4, anti-CD8, anti-B220, anti-CD11b, anti-CD11c, anti-NK1.1, and/or anti-Ly-6G (BD Pharmingen) for 30 min with shaking. Lymphocytes were then washed with FACS staining buffer (PBS with 1% BSA) and fixed in 2% paraformaldehyde in PBS and analyzed by flow cytometry (BD Biosciences).

**Statistics**

The data are expressed as the mean ± SEM and compared using a two-tailed Student’s t test or an unpaired Mann-Whitney U test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Berkeley, CA) for Macintosh computers and were considered statistically significant if p values were <0.05. When cytokine levels were below the detection limit (below detection), they were recorded as one-half the lower detection limit (e.g., 15 pg/ml for IL-10) for statistical analysis.

**Results**

**vMIP-II, I-309, and TCA-3 stimulate OVA-specific systemic Ab responses**

We have previously used the nasal route of immunization to study how chemokines modulate acquired mucosal immunity (2–5). Therefore, we have chosen a similar model to study the effects of vMIP-II, TCA-3, and I-309 on humoral and cellular immune responses. The optimal dose of these chemokines was established after nasally administering, three times at weekly intervals, 75 μg of OVA alone or in the presence of increasing concentrations of vMIP-II, I-309, or TCA-3 (e.g., 0.0, 0.01, 0.1, 1.0, and 5.0 μg). Accordingly, we analyzed OVA-specific Ab titers in serum and mucosal secretions. Significant titers of OVA-specific Ab responses were elicited when mice were given a 1.0 μg dose of vMIP-II, I-309, or TCA-3. Ab responses were not significantly increased when doses >5 μg were used (data not shown); therefore, a 1 μg dose of vMIP-II, I-309, or TCA-3 was used for subsequent experiments.

Mice nasally immunized three times with 75 μg of OVA plus 1 μg of vMIP-II, I-309, or TCA-3 displayed significant, yet different, increases in Ag-specific serum Ab levels, compared with mice receiving OVA alone (Fig. 1). Serum IgM Ab responses were not significantly increased after immunization. I-309 induced significant increases in anti-OVA IgG1 Ab responses, compared with vMIP-II and TCA-3. Neither chemokine increased Ag-specific IgG2a, IgG2b, or IgG3 Ab levels, compared with control groups that received OVA alone.

**Mucosal adjuvant effects of TCA-3, I-309, and vMIP-II**

We next asked whether the adjuvant activity of nasally coadministered vMIP-II, I-309, or TCA-3 could promote mucosal secretory (S)-IgA Ab responses. Analysis of OVA-specific Ab responses in mucosal secretions revealed higher Ag-specific S-IgA and IgG Ab titers in fecal extracts as well as nasal and vaginal wash samples from mice immunized with OVA plus vMIP-II, I-309, or TCA-3 (Fig. 1). I-309 significantly increased the levels of Ag-specific S-IgA and IgG Ab titers in fecal samples, compared with groups receiving vMIP-II or TCA-3 as adjuvants. Moreover, vMIP-II-mediated Ag-specific IgG Ab effects on vaginal and nasal secretions were significantly less compared with mice receiving I-309 or TCA-3 plus OVA. These findings highlight the similarities and differences between I-309/TCA-3- and vMIP-II-mediated mucosal humoral responses.

**CD4+ T cell proliferative and cytokine responses induced by vMIP-II, TCA-3, and I-309**

Because vMIP-II and I-309/TCA-3 differentially regulated mucosal and systemic Ab responses, we next examined whether the differences in their activities also extended to the pattern of Th cell subsets and cytokine responses that they promoted in vivo. CD4+ T cells isolated from the nasal tract, cervical lymph nodes, lungs, or mesenteric lymph nodes, but not from Peyer’s patches of mice immunized with OVA plus vMIP-II, I-309, or TCA-3 exhibited marked increases in OVA-specific proliferative responses, when compared with CD4+ T cells from mice immunized with OVA alone (Fig. 2). However, CD4+ T cells from the spleen of mice immunized with vMIP-II plus OVA exhibited significantly higher increases in Ag-specific proliferative responses than mice immunized with I-309 or TCA-3 plus OVA. IL-2 and TNF-α, but not IFN-γ secretion patterns of OVA-restimulated CD4+ T cells from vMIP-II, I-309, or TCA-3 plus OVA-immunized mice were significantly higher than those isolated from mice immunized with OVA alone. Interestingly, IL-2, TNF-α, and IFN-γ secretion patterns of OVA-restimulated CD4+ T cells from vMIP-II, I-309, or TCA-3 plus OVA-immunized mice were not statistically different from controls.

vMIP-II, I-309, and TCA-3, as adjuvants, lead to robust increases in Th2 responses (Fig. 3). Most notably, IL-4 secretion by ex vivo OVA-restimulated CD4+ T cells from vMIP-II, I-309, or TCA-3 plus OVA-immunized mice was dramatically elevated compared with mice immunized with OVA alone. Another finding that highlighted the striking differences between vMIP-II vs I-309/TCA-3-mediated immunity was observed among IL-10 and GM-CSF expression by splenic and mesenteric lymph node-derived CD4+ T cells restimulated with OVA. For example, vMIP-II, as adjuvant, significantly increased IL-10 responses to Ag, compared with I-309 and TCA-3. However, I-309 plus OVA immunization was more effective at increasing GM-CSF responses by Ag-restimulated splenic and mesenteric lymph node T cells, compared with similar T lymphocytes isolated from vMIP-II or TCA-3 plus OVA-immunized mice. Taken together, these results show that vMIP-II, I-309, and TCA-3 enhanced IL-4 responses;
and IFN-γ-presented are the mean OD450 for proliferative responses or IL-2, TNF-α repeated twice. Proliferation was measured by BrdU incorporation. The data are statistically significant differences, i.e., p < 0.05, between OVA plus I-309-, TCA-3-, and vMIP-II-immunized mice compared with mice immunized with OVA alone (∗) or significant differences between I-309, TCA-3, and vMIP-II plus OVA-immunized groups (∗∗).

### FIGURE 2. Proliferation response and Th1 cytokine secretion by ex vivo Ag-stimulated T cells, previously immunized with OVA plus vMIP-II, TCA-3, or I-309. Groups of five C57BL/6 mice were nasally immunized on days 0, 7, and 14 with 75 µg of OVA alone (□) or OVA plus 1.0 µg of vMIP-II (Ⅲ), I-309 (Ⅰ), or TCA-3 (Ⅱ). One week after the last immunization, lung-, spleen (SP)-, nasal tract (NT)-, cervical lymph node (CLN)-, Peyer’s patch (PP)-, and mesenteric lymph node (MLN)-derived CD4+ T cells were purified and cultured at a density of 5 × 10^6 cells/ml to 10^7 cells/ml γ-irradiated syngeneic feeder cells with 1 mg/ml OVA for 3 days. Experimental groups consisted of five mice, and studies were repeated twice. Proliferation was measured by BrdU incorporation. The data presented are the mean OD_{450} for proliferative responses or IL-2, TNF-α, and IFN-γ levels ± SEM of quadruplicate cultures. Asterisk(s) indicates statistically significant differences, i.e., p < 0.05, between OVA plus I-309-, TCA-3-, and vMIP-II-immunized mice compared with mice immunized with OVA alone (∗) or significant differences between I-309, TCA-3, and vMIP-II plus OVA-immunized groups (∗∗).

### FIGURE 3. Th2 cytokine secretion by ex vivo Ag-stimulated T cells immunized with OVA plus vMIP-II, TCA-3, or I-309. Groups of five C57BL/6 mice were intranasally immunized on days 0, 7, and 14 with 75 µg of OVA alone (□) or OVA plus 1.0 µg of vMIP-II (Ⅲ), I-309 (Ⅰ), or TCA-3 (Ⅱ). One week after the last immunization, spleen (SP)-, lung-, cervical lymph node (CLN)-, Peyer’s patch (PP)-, mesenteric lymph node (MLN)-, and nasal tract (NT)-derived lymphocytes were purified and cultured at a density of 5 × 10^6 cells/ml with 10^6 cells/ml γ-irradiated syngeneic feeder cells with 1 mg/ml OVA for 3 days in complete medium. Experimental groups consisted of five mice, and studies were repeated three times. IL-4, IL-10, and GM-CSF production of cultured supernatants was determined by ELISA and presented as the mean cytokine level (pg/ml) ± SEM of duplicate cultures from each group. Asterisk(s) indicates statistically significant differences, i.e., p < 0.05, between OVA plus I-309-, TCA-3-, and vMIP-II-immunized mice compared with mice immunized with OVA alone (∗) or significant differences between I-309, TCA-3, and vMIP-II plus OVA-immunized groups (∗∗).

However, these viral and mammalian chemokines differentially modulate IL-10 and GM-CSF responses in both mucosal and systemic lymphoid compartments.

**Alteration of primary T cell cytokine and proliferative responses by vMIP-II, I-309, and TCA-3**

We next examined the ability of vMIP-II, TCA-3, and I-309 to promote differential Ag-specific cytokine and proliferation responses by primary T cell lymphocytes isolated from DO11.10 mice that express a transgenic TCR to the class II-restricted peptide of OVA (Fig. 4). IFN-γ and TNF-α secretion levels were significantly increased by I-309, while TCA-3 costimulation had no effect on cytokine secretion by OVA-stimulated DO11.10 T cells. DO11.10 lymphocytes stimulated with OVA expressed significantly less TNF-α and IFN-γ when cocultured with vMIP-II. Neither vMIP-II, I-309, nor TCA-3 significantly modulated IL-4 or GM-CSF secretion by OVA-stimulated DO11.10 T cells; however, IL-10 levels expressed by vMIP-II-treated cultures were significantly higher than those incubated with I-309 or TCA-3 plus OVA or OVA alone (Fig. 4). These results are also in correlation with the observed in vivo effects when these chemokines were used as adjuvants. Together, the data suggest that vMIP-II, I-309, and TCA-3 act differently to influence Ag-specific Th responses, while vMIP-II mediates significant increases in IL-10 with decreases in TNF-α and IFN-γ secretion, when compared with I-309- or TCA-3-treated Ag-stimulated T cells.

**vMIP-II, I-309, or TCA-3 effects on surface costimulatory molecule expression**

Earlier studies with chemokines (4, 5, 36) and viral proteins (37–39) have shown that they can differentially modulate costimulatory molecule expression and proliferative responses by lymphocytes. To better elucidate the effects of vMIP-II, I-309, and TCA-3 on T cell responses, we assessed their potential to modulate costimulatory molecule expression by resting and OVA-stimulated primary T cells from naive DO11.10 mice. In parallel with the ability of I-309 to produce greater levels of Ag-specific IgG1 and mucosal Ab responses, I-309, but not vMIP-II or TCA-3, increased the expression of CD28 and CD30 by Ag-stimulated CD4+ T cells (Fig. 5). vMIP-II modestly down-regulated CD28, CD40L, and CD30 expression by resting CD4+ T cells in a dose-dependent fashion. Although I-309 was involved in the differential regulation of costimulatory molecule expression by resting and Ag-stimulated DO11.10 T lymphocytes, the data indicate that vMIP-II mediates the down-regulation of important costimulatory molecules for T cell activation and differentiation. TCA-3 did not alter the expression of CD28 by either resting or Ag-stimulated T lymphocytes (Fig. 5). However, we noticed that TCA-3 dose dependently declined CD40L expression by resting T lymphocytes and CD30 expression by both resting and Ag-stimulated T lymphocytes.
from DO11.10 mice were cultured at density of 5 × 10^6 cells/ml to 10^6 cells/ml γ-irradiated syngeneic feeder cells with 1 mg/ml OVA plus 0 ( ), 1, 10, 100, or 1000 ng/ml vMIP-II ( ), I-309 ( ), or TCA-3 ( ). Cytokine levels in cultured supernatants were determined by ELISA. The data presented are IL-2, TNF-α, IFN-γ, IL-4, IL-10, and GM-CSF levels (pg/ml) ± SEM of quadruplicate cultures. Asterisk(s) indicates statistically significant differences, i.e., *p < 0.05, between OVA plus I-309-, TCA-3-, and vMIP-II-immunized mice compared with mice immunized with OVA alone or significant differences between I-309, TCA-3, and vMIP-II plus OVA-immunized groups ( ).

**FIGURE 4.** Th cytokine secretion by OVA-stimulated CD4^+ T cells from DO11.10 mice. CD4^+ T cells from DO11.10 mice were incubated with 0, 1, 10, 100, 1000 ng/ml vMIP-II ( ), I-309 ( ), or TCA-3 ( ). The percentage of increase (or decrease) in the IL-2, IL-4, IL-10, TNF-α, IFN-γ, and GM-CSF responses, augments neutrophil recruitment to lungs, and lowers mucosal macrophage infiltration. All of these mechanisms combined could contribute to altering mucosal immunity.

**FIGURE 5.** I-309, TCA-3, and vMIP-II regulation of CD28-, CD40L-, and CD30-positive CD4^+ T cells. Resting or Ag-stimulated splenic T cells from DO11.10 mice were incubated with 0, 1, 10, 100, 1000 ng/ml vMIP-II ( ), I-309 ( ), or TCA-3 ( ). In 96-well culture plates containing OVA peptide containing aa 323–339 ( ), lymphocytes was calculated as the percentage of double-positive CD3^+ , CD4^+ and CD28^− , CD30^− , and CD40L^− cells in cultures containing vMIP-II, TCA-3, or I-309 minus the percentage gated of double-positive cells in cultures without these chemokines, divided by the latter. Studies were repeated three times, and the data presented are the mean percentage of change ± SEM of these experiments.

**vMIP-II-, I-309-, and TCA-3-mediated in vivo migration of leukocyte subpopulation**

To further establish the effect of vMIP-II, I-309, and TCA-3 in the modulation of cellular and humoral immunity, mice were nasally immunized as before with OVA alone or chemokines plus OVA. Although nasal immunization (with or without chemokines) did not significantly change the percentage of leukocyte subpopulations in the spleen or cervical lymph nodes, all of the chemokines studied significantly increased the number of CD4^+ T and B cells in the nasal tract 12 h after immunization (Table I). However, the percentage of CD4^+ T and B cells in the lungs was significantly less than mice that received intranasal PBS (negative control) or OVA alone, which suggests that the increase in T and B cell lymphocytes in the nasal tract may have arisen from mucosal homing of these cells from the lower respiratory tract (lung). Modest, yet statistically significant decreases in the percentage of nasal tract CD11b^+ macrophages were also observed 12 h after OVA plus vMIP-II immunization.

Although neutrophils do not express substantial levels of CCR3 or CCR8, these cells are among the first leukocytes to be recruited to the site of an infection or immunization. We therefore characterized the changes in Ly-6G^+ leukocytes in the spleen, nasal tract, cervical lymph nodes, and lungs 12 h after nasal administration of PBS, OVA alone, or OVA plus vMIP-II, I-309, or TCA-3. Surprisingly, vMIP-II was the only chemokine tested that increased the percentage of neutrophils present in the lung. Taken together, these data indicate that vMIP-II and I-309/TCA-3 differentially mediate leukocyte recruitment to and from the nasal tract and lungs.

**Discussion**

The unique features of vMIP-II (as well as TCA-3 and I-309) provided the rationale(s) to test our hypothesis that this viral chemokine differentially affects adaptive mucosal and systemic immunity to favor viral pathogenesis. For the first time, we have shown that vMIP-II, I-309, and TCA-3 modulate adaptive immunity that is fostered by the differential regulation of Th2 cytokine and costimulatory molecule expression for the support (I-309/TCA-3) or misdirection (vMIP-II) of humoral and cellular immunity. Previous studies have shown that the classical mucosal adjuvant, cholera toxin, can induce serum Ag-specific IgE and IgG1 Ab titers supported by Th2 cytokine help (40). However, RANTES, when used as a mucosal adjuvant, induces predominantly Th1-driven Ag-specific IgG2a, followed by IgG2b, IgG3, and IgG1 Abs (4). Similarly, MIP-1α functions as a Th1 inducer to propagate cytotoxic T cell responses, serum IgG2a, and mucosal IgA Ab responses (5). Lymphotactin also acts as an innate mucosal adjuvant to induce Th2 > Th1 responses, and dramatically increases serum IgG subclasses and robust mucosal IgA responses (3). It has been shown previously that vMIP-II inhibits virus-specific type 1 T cell-mediated inflammation in vivo (28). In this study, we show that vMIP-II induces host immunity, enhances Th2 immune responses (with predominant serum IgG1 Ab responses), and, in contrast with I-309 and TCA-3, promotes differential IL-10 and GM-CSF responses, augments neutrophil recruitment to lungs, and lowers mucosal macrophage infiltration. All of these mechanisms combined could contribute to altering mucosal immunity.
It is now well established that IL-4 supports IgG1 Ab generation (41–43), and the levels of anti-OVA IgG1 Abs by vMIP-II, I-309, or TCA-3 plus OVA-immunized mice were consistent with cytokine secretion patterns of IL-4 and GM-CSF by OVA-restimulated CD4+ T cells. I-309, as adjuvant, was effective at increasing IFN-γ- and TNF-α-responsive CD4+ T cells. vMIP-II, as an adjuvant, was effective at increasing Ag-specific IL-10 responses, compared with mice immunized with I-309 or TCA-3 plus OVA. Similar trends were observed during primary T cell responses to Ag stimulation and vMIP-II, I-309, or TCA-3 coculture. For example, while I-309 coculture with OVA-stimulated DO11.10 lymphocytes enhanced TNF-α and IFN-γ secretion, vMIP-II dramatically decreased TNF-α production and increased IL-10 generation.

The precise cytokine signals required for S-IgA production, and mucosal immunity in general, are not completely understood. Studies have supported that both Th1 and Th2 cell-derived cytokines are important for S-IgA (44–46). We have shown previously that lymphotactin, MIP-1α, MIP-1β, and RANTES also induce S-IgA (3–5). Clearly, mucosal Ag-specific Ab responses were enhanced by vMIP-II, TCA-3, and I-309. However, I-309 yielded higher increases in S-IgA responses in fecal secretions, while both I-309 and TCA-3 increased nasal and vaginal IgG levels when compared with those induced by vMIP-II. The heightened mucosal Ab responses generated by I-309 also correlated with the predominant GM-CSF response displayed by Ag-restimulated CD4+ T cells from I-309 plus OVA-immunized mice. Perhaps the reduced levels of Ag-specific fecal IgA Abs, by vMIP-II plus OVA-immunized mice, were the result of higher levels of IL-10 expression by Ag-restimulated mesenteric lymph node T cells. In fact, IL-10 can profoundly inhibit T cell immunity required for viral clearance (47). To this end, IL-10 has been reported to serve as an autocrine growth factor for AIDS-related B cell lymphoma, in which HHV-8 can play a role (48).

The cytokines produced by CD4+ T cells after mucosal administration of vMIP-II, I-309, or TCA-3 only partially explain the observed OVA-specific Ab and T cell responses. Chemokines may affect several immune enhancing factors or mechanisms required for induction of adaptive mucosal immune responses. Uptake by B cells and macrophages and Ag recognition by T cells are important to initiate adaptive immunity. A mucosal adjuvant that facilitates this would potentially enhance Ag presentation and recognition, which are important adjuvant mechanisms. Our results show that vMIP-II, TCA-3, and I-309 may enhance the recognition phase of the adaptive host response, because all three chemokines increased the frequency of CD4+ T and B cells in the nasal tract 12 h after immunization. Interestingly, vMIP-II differed by recruiting significantly fewer macrophages to the nasal tract and more neutrophils to the lung. Hence, the variances observed in both humoral and cellular immunity induced by vMIP-II, TCA-3, and I-309 coincided with their ability to modulate leukocyte migration to and from mucosal effector sites.

Although these chemotactic molecules directly aid in the accumulation of Th2 lymphocytes, lymphocyte recruitment alone does not ensure the initiation of acquired immunity, particularly not a mucosal immune response (2). To address the potential mechanisms that are used by I-309, TCA-3, and vMIP-II to initiate or hinder, respectively, adaptive immune responses, we investigated how these molecules would affect the expression of costimulatory molecules by T cells. CD154 (i.e., gp39 or CD40L) is considered a major determinant in the outcome of T-B cell interactions (49). CD40L stimulation can also drive B cell activation and IgA production (50, 51). The generation and activation of adaptive immunity often depend on the availability of and help from CD4+ T cells and require CD154 interactions (52, 53), which are required for antiviral humoral immunity as well as the establishment and homeostasis of CD8+ T cell memory (54). To this end, we observed significant changes in CD40L expression by activated T cells cocultured with RANTES, MIP-1α, and MIP-1β (4, 5). Although I-309 increased CD40L expression by resting T cells, vMIP-II and TCA-3 had a modest or negative effect.

CD30 interactions have been shown to control Th2 differentiation and lymphocyte proliferation (55). CD30 is predominantly

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**Table I. vMIP II, I-309, and TCA-3 effects of leukocyte migration**

<table>
<thead>
<tr>
<th>Leukocyte Subpopulation</th>
<th>Additions</th>
<th>Spleen</th>
<th>Nasal tract</th>
<th>Cervical lymph nodes</th>
<th>Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ CD4+</td>
<td>PBS alone</td>
<td>12.5 ± 1.9</td>
<td>3.2 ± 0.5</td>
<td>3.4 ± 0.5</td>
<td>24.2 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>OVA alone</td>
<td>17.9 ± 2.7</td>
<td>1.6 ± 0.3</td>
<td>4.4 ± 0.6</td>
<td>25.3 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>vMIP-II + OVA</td>
<td>18.4 ± 2.7</td>
<td>19.7 ± 2.9*</td>
<td>3.7 ± 0.5</td>
<td>1.4 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>I-309 + OVA</td>
<td>17.7 ± 2.6</td>
<td>23.9 ± 3.5*</td>
<td>7.4 ± 1.1</td>
<td>1.7 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>TCA-3 + OVA</td>
<td>15.0 ± 2.2</td>
<td>18.7 ± 2.8*</td>
<td>3.6 ± 0.5</td>
<td>1.9 ± 0.3*</td>
</tr>
<tr>
<td>B220+</td>
<td>PBS alone</td>
<td>64.6 ± 9.9</td>
<td>20.4 ± 3.6</td>
<td>29.6 ± 4.4</td>
<td>64.3 ± 9.6</td>
</tr>
<tr>
<td></td>
<td>OVA alone</td>
<td>68.4 ± 9.7</td>
<td>24.9 ± 3.7</td>
<td>32.2 ± 4.8</td>
<td>61.2 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>vMIP-II + OVA</td>
<td>66.7 ± 10</td>
<td>65.7 ± 9.8*</td>
<td>27.6 ± 4.0</td>
<td>25.1 ± 3.7*</td>
</tr>
<tr>
<td></td>
<td>I-309 + OVA</td>
<td>67.6 ± 11</td>
<td>65.2 ± 9.2*</td>
<td>34.4 ± 5.2</td>
<td>26.3 ± 3.4*</td>
</tr>
<tr>
<td></td>
<td>TCA 3 + OVA</td>
<td>62.6 ± 7.9</td>
<td>57.9 ± 8.7*</td>
<td>21.6 ± 3.2</td>
<td>26.2 ± 3.4*</td>
</tr>
<tr>
<td>CD11b+</td>
<td>PBS alone</td>
<td>2.3 ± 0.3</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>OVA alone</td>
<td>2.3 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>vMIP-II + OVA</td>
<td>1.8 ± 0.2</td>
<td>0.3 ± 0.1*</td>
<td>1.1 ± 0.1</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>I-309 + OVA</td>
<td>1.6 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>TCA-3 + OVA</td>
<td>1.8 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>0.3 ± 0.1</td>
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<tr>
<td>Ly-6G+</td>
<td>PBS alone</td>
<td>3.5 ± 0.5</td>
<td>1.2 ± 0.2</td>
<td>2.2 ± 0.3</td>
<td>0.6 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>OVA alone</td>
<td>2.9 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td>3.3 ± 0.5</td>
<td>0.4 ± 0.08</td>
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<tr>
<td></td>
<td>vMIP-II + OVA</td>
<td>2.8 ± 0.4</td>
<td>1.7 ± 0.3</td>
<td>4.5 ± 0.7</td>
<td>2.2 ± 0.4*</td>
</tr>
<tr>
<td></td>
<td>I-309 + OVA</td>
<td>2.4 ± 0.3</td>
<td>1.5 ± 0.2</td>
<td>7.2 ± 1.1</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>TCA-3 + OVA</td>
<td>2.4 ± 0.3</td>
<td>1.4 ± 0.2</td>
<td>6.1 ± 0.9</td>
<td>0.7 ± 0.02</td>
</tr>
</tbody>
</table>

* BALB/c mice were intranasally challenged with PBS or OVA alone or with OVA and v-MIP-II, I-309, and TCA-3 in a volume of 7.5 μl of PBS. Spleen, nasal tract, cervical lymph nodes, and lung lymphocytes were purified and prepared for FACS analysis after 12 h of induction. SEMs not exceeding 15% are not shown.

b Differences between experimental controls (PBS and OVA) or with chemokine-treated group were considered significant when p < 0.01 (*).
expressed by T cells that secrete Th2-type cytokines and down-regulated by IFN-γ (56). We show a modest, yet dose-dependent, increase in CD30 expression following I-309, but not TCA-3 or vMIP-II coinoculation with OVA-stimulated DO11.10 CD4+ T cells. In fact, vMIP-II and TCA-3 decreased CD30 expression by both resting and Ag-stimulated T cells.

CD28 supplies a coactivation signal for T cell activation (57, 58). Stimulation through CD28 acts in concert with signals provided by Ag recognition to result in IL-2 production and subsequent cell division (59). CD28 is also required for mucosal and T cell-mediated immunity (59, 60), and we have previously shown that RANTES and MIP-1α act as mucosal adjuvants, partly through CD28 up-regulation (4, 5). I-309 was the only chemokine tested that increased CD28 expression by Ag-stimulated CD4+ T cells. In general, our data suggest that I-309 is effective at modulating surface expression of costimulatory molecules necessary for activation, while vMIP-II and TCA-3 suppress or modestly up-regulate, respectively, CD28, CD40L, and CD30 expression by Th cells. In this regard, vMIP-II has been shown to be an efficient immunosuppressive agent for CCR8+ cells (61). vMIP-II potently inhibits MCP-1, -1β, RANTES-, and fractalkine-induced chemotaxis of activated lymphocytes (61). Perhaps viruses encoding viral chemokines, e.g., HHV-8, use similar strategies to circumvent host immunity.

The possible route of HHV-8 transmission by blood components and organ transplant is still debated and raises certain public concerns. However, the mucosal route of entry, following intercourse, appears to be the main route of HHV-8 transmission (62). HHV-8 has also been associated with various forms of malignancies, namely, body cavity-based or high grade B cell lymphoma (63) and primary effusion lymphoma that is a rare form of non-Hodgkin’s lymphoma (64). Castelman’s disease is also caused by HHV-8 infection; this disease results in enlarged hyperplastic lymph nodes with marked vascular proliferation. The presence of HHV-8 in angiosarcomas, in addition to classical KS, is a clear indication that immunosuppression is not a prerequisite for viral infection, but that viruses may mediate immune evasion or modulate adaptive responses to attenuate host immunity (65, 66). Perhaps the unique ability of vMIP-II to enhance both IL-2 and IL-10 Ag-specific Th cell responses contributes to the lymphoproliferative and immunosuppressive diseases caused by HHV-8.

Our results show that Th2-type pathways can be induced by mucosally administered vMIP-II, TCA-3, and I-309. The ability of I-309/TCA-3 to selectively increase Th2 responses may also be shared by other chemokines (e.g., eotaxin-1, -2, and -3) that attract Th2 lymphocytes. We have shown that under certain conditions I-309/TCA-3 can enhance immune cell functions, while vMIP-II suppresses them. Perhaps due to the importance of chemokines in host immunity, a number of pathogens have evolved endogenous chemokine homologues and binding proteins that presumably interfere with host immunity so that these microbes can evade immune detection (8, 13–18). The ability of vMIP-II to only moderately affect mucosal immunity may also contribute to the pathogenesis of HHV-8. The mechanisms demonstrated by vMIP-II may very well be shared by other viral chemokines and chemokine receptors. Although additional studies will be needed to unravel the precise cellular and molecular mechanism of viral and mammalian chemokine-mediated immunity, our studies suggest the vMIP-II as well as I-309/TCA-3 may play an important, yet undefined role in mucosal and systemic adaptive immunity.


