In Vitro Induction of Mucosa-Type Dendritic Cells by All-Trans Retinoic Acid

Leslie Saurer, Kenneth C. McCullough and Artur Summerfield

*J Immunol* 2007; 179:3504-3514; doi: 10.4049/jimmunol.179.6.3504

http://www.jimmunol.org/content/179/6/3504

---

**References**  This article cites 37 articles, 16 of which you can access for free at: http://www.jimmunol.org/content/179/6/3504.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2007 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
In Vitro Induction of Mucosa-Type Dendritic Cells by All-Trans Retinoic Acid

Leslie Saurer, Kenneth C. McCullough, and Artur Summerfield

Efficient induction of mucosal immunity usually employs nasal or oral vaccination while parenteral immunization generally is ineffective at generating mucosal immune responses. This relates to the unique ability of resident mucosal dendritic cells (DC) to induce IgA switching and to imprint mucosa-specific homing receptors on lymphocytes. Based on the well-established plasticity of the DC system, this study sought to investigate whether peripheral DC could be modulated toward “mucosa-type” DC by treatment with immunomodulatory, and therefore potentially adjuvant-like, factors. In this study, we show that monocyte-derived DCs pretreated with the vitamin A derivative all-trans retinoic acid (RA) indeed acquired several attributes characteristic of mucosal DC: secretion of TGF-β and IL-6 and the capacity to augment mucosal homing receptor expression and IgA responses in cocultured lymphocytes. Addition of a TGF-β-neutralizing Ab to cocultures significantly inhibited αβ integrin, but not CCR9 mRNA expression by the lymphocytes. Both αβ integrin and CCR9 mRNA expression, but not IgA production, were suppressed in the presence of a RA receptor antagonist. None of the observed effects on the lymphocytes were influenced by citral, a retinal dehydrogenase inhibitor, arguing against a role for de novo-synthesized RA. Collectively, our findings identified a novel role for RA as a mucosal immune modulator targeting DC. Our results further demonstrate that DC can act as efficient carriers of RA at least in vitro. Consequently, RA targeting of DC shows potential for promoting vaccine-induced mucosal immune responses via a parenteral route of immunization. The Journal of Immunology, 2007, 179: 3504–3514.

With most infectious agents invading the host via mucosal surfaces, there is a strong demand for vaccines with the potential to induce mucosal immunity. The development of such vaccines has been impeded by the fact that the well-characterized route of parenteral immunization is generally ineffective at eliciting mucosal immune responses. Moreover, oral or nasal application of vaccines requires higher Ag payloads and novel adjuvants, which need to be both effective and safe to use (1, 2).

Accumulating evidence suggests that the long-observed compartmentalization of mucosal and systemic immune responses is mainly based on the selective expression of homing receptors by lymphocytes. These receptors target effector and memory cells to specific ligands expressed in the corresponding extralymphoid site of original Ag encounter (3). Accordingly, lymphocytes primed in Peyer’s patches or mesenteric lymph nodes (MLN) will acquire expression of the integrin αβ, and the chemokine receptor CCR9. They will localize to the small intestinal mucosa where their appropriate ligands—mucosal addressin cell adhesion molecule-1 and CCL25 (TECK)—are expressed by postcapillary venules and intestinal epithelial cells, respectively. In contrast, effector T cells trafficking to inflamed skin are devoid of αβ and CCR9. Instead, they express ligands for vascular P- and E- selectins and the chemokine receptors CCR4 and/or CCR10. A number of studies have demonstrated an important role for resident dendritic cells (DC) in instructing naïve lymphocytes to express the appropriate homing receptor profile (4–6). DC isolated from Peyer’s patches or MLN were necessary and sufficient to induce αβ and CCR9 expression in cocultured T or B cells in vitro. In contrast, DC from peripheral lymph nodes only promoted expression of P- and E-selectin ligands. The ability of intestinal DC to promote gut-tropic lymphocytes was assigned to their unique expression of retinoid hydroxylase enzymes converting dietary vitamin A to retinoic acid (RA), which was shown to directly up-regulate αβ and CCR9 expression on T cells (7). Importantly, in addition to this modulation of the homing properties of lymphocytes to mucosal sites, gut DC-derived RA—in combination with IL-6 or IL-5 and other yet unknown factors—was also identified as an important mediator of IgA secretion in B cells (8). Furthermore, a recent report associated the presence of the integrin αE chain (CD103) on lamina propria (LP) DC and a subset of MLN DC with their capacity to up-regulate CCR9 in T cells (9).

Nonetheless, several studies suggest that the ability of intestinal DC to confer gut tropism to T cells must not necessarily be the attribute of a specialized, distinct, and tissue-restricted DC subset (5, 6, 10–12). Indeed, injection of in vitro bone marrow-derived “neutral” DC into mice induced skin-homing and gut-homing receptors, which was dependent on the route of administration: intracutaneous or i.p., respectively (10). Moreover, i.p. injection of Ag-pulsed DC preferentially up-regulated αβ expression on adoptively transferred T cells, irrespective of the DC origin: MLN or peripheral lymph nodes (11). These experiments indicate a dominant role for the local tissue environment in shaping the mucosal imprinting capacity of DC. Such a flexibility is certainly known for...
DC, being reminiscent of the Th1, Th2, or T regulatory cell polarizing ability they can adopt following exposure to particular cytokine and microbial signals (13). Based on this well-recognized plasticity of the DC system, the present study sought to investigate whether peripheral DC could be modulated toward “mucosa-type” DC by targeting with immunomodulatory and therefore potentially adjuvant-like factors.

Materials and Methods

Animals

Specific pathogen-free Swiss White Landrace pigs were kept at the institute and used as blood donors. For Ag-specific (re)stimulation assays, some of the pigs had received repeated vaccinations with inactivated foot-and-mouth disease virus (FMDV) type O1 Lausanne Ag (provided by Dr. P. Barnett, Institute for Animal Health, Pirbright, U.K.) formulated in Montanide 20 (donated by Seppic, Paris, France). Vaccinations and regular bleeding were performed in compliance with the regulations approved by the local committee for animal experimentation.

Generation and differential treatment of monocyte-derived DCs (MoDC)

PBMC were isolated from citrated blood of pigs by Ficoll-Paque (1.077 g/L, Amersham Pharmacia Biotech) density centrifugation. CD172a-H11001 monocytes were isolated using the mAb 74-22-15 (American Type Culture Collection) plus MACS separation system (Miltenyi Biotec), and cultured in DMEM (Invitrogen Life Technologies) supplemented with 10% v/v porcine serum (Sigma-Aldrich), 100 U/ml recombinant porcine GM-CSF (provided by Dr. S. Inumaru, Institute for Animal Health, Ibaraki, Japan) and 100 U/ml recombinant porcine IL-4 (prepared in our laboratory) as described previously (14). MoDC were fed on day 3 of culture by addition of fresh medium containing GM-CSF and IL-4. On day 4 of culture, when nonadherent cells with the morphology of immature DC dominated, the MoDC were treated for an additional 48 h with the immunomodulatory factors including: porcine TNF-H9251 (prepared from TNF-H9251-expressing L929 cells provided by Dr. G. Bertoni, University of Bern, Switzerland (15)), heat-labile enterotoxin (LT; provided by Berna Biotech, Bern, Switzerland), Pam3Cys (Pam3Cys; provided by Dr. K. H. Wiesmüller, EMC Microcollections, Tübingen, Germany), Escherichia coli-derived LPS, polyinosine-polycytidolic acid (pIC), 1α,25-dihydroxyvitamin D3 (VitD3), all-trans RA, retinol (Ret) (all reagents obtained from Sigma-Aldrich, unless specified.

FIGURE 1. β1 integrin and CCR9 mRNA expression by lymphocytes after coculture with MoDC. The MoDC were either untreated or pretreated with immunomodulatory factors TNF-α (20 ng/ml), LPS (1 μg/ml), LT (0.1, 1, or 10 μg/ml), VitD3 (10 or 1000 nM), RA (10 or 1000 nM), Ret (10 or 1000 nM). After 48 h, the MoDC were loaded with SEB, washed four times, and cocultured with CFSE-labeled lymphocytes for another 4 days. A, Increase of β1 integrin expression by lymphocytes cocultured with treated MoDC relative to β1 integrin expression induced by untreated MoDC. B, Increase in CCR9 mRNA levels in lymphocytes cocultured with treated MoDC relative to the levels induced by untreated MoDC. C, Increase of CFSElow cells in cocultures with treated MoDC relative to increase in cocultures with untreated MoDC. The box plots in A–C represent median values of independent experiments (n = 1–5) and the bars represent mean values with error bars indicating the range. D, Gating strategy and representative dot plots for three of the cocultures: the numbers indicate MFI for β1 integrin (top left corner) and percentage of CFSElow lymphocytes (bottom left corner) defined within the gated region. The dotted lines indicate the position of the isotype controls. **, p < 0.01.
On day 6 of culture, treated MoDC were used for phenotyping or coculture experiments, as described below.

Isolation and CFSE labeling of lymphocytes

Lymphocytes were isolated from freshly prepared PBMC by depletion of CD172a/H11001 cells using the MACS system and LD columns (for additional depletion of CD172alow APC). For some experiments, lymphocytes were labeled with CFSE (Molecular Probes). Briefly, CD172/H11002 cells were resuspended at 1/10^7 cells/ml in PBS and incubated with 5/10^M CFSE for 10 min at 39°C. Lymphocytes were washed three times in DMEM 10% v/v FBS (Invitrogen Life Technologies) and used in cocultures with autologous MoDC which had received the different treatments mentioned above.

MoDC-lymphocyte cocultures

For superantigen-dependent T cell activation, MoDC were loaded with 1/10^g/ml staphylococcal enterotoxin B (SEB; Toxin Technology) for 1 h at 39°C, washed four times and cultured at 2/10^4 cells/well with CFSE-labeled lymphocytes (2 × 10^6/well) in DMEM 10% v/v FBS in 96-well U-bottom plates (Costar) in replicates of three to five wells. On day 4 of culture, cells were harvested and stained for the β7 integrin with the anti-mouse (anti-porcine cross-reactive) mAb FIB27 (BD Biosciences) and an anti-rat IgG PE-conjugated goat F(ab’)2 Ig (Southern Biotechnology Associates). In some experiments, cells were additionally stained for α4 integrin chain expression with the mAb L25 (BD Biosciences). Proliferation (as based on CFSE profile) and integrin expression was assessed by flow cytometry (FCM) using a FACSCalibur with the CellQuest pro software (both BD Biosciences). In some of the experiments, MoDC were fixed with 1% w/v paraformaldehyde (PFA) for 15 min after SEB loading.

For FMDV-specific T cell activation, differentially treated 6-day-old MoDC were washed four times and cocultured with lymphocytes (5 × 10^6/well) at a 1:10 ratio of MoDC to CD172/H11002 lymphocytes in 24-well plates (Costar) with inactivated FMDV as Ag. To this end, FMDV O1 Lausanne was propagated in BHK-21 cells and inactivated by exposure to UV light, as described previously (16, 17). β7 integrin expression was assessed on day 5 of culture using the mAb FIB27 and mAbs against porcine CD4 (74-12-4) and CD8 (11-295-33) (hybridomas provided by Dr. A. Saalmüller, Veterinary University, Vienna, Austria) to allow for gating on the FMDV-responsive CD4+/CD8+ porcine memory Th population (18, 19). In some experiments, MoDC were separated from lymphocytes by a 0.4 μM pore-size cell culture insert (BD Biosciences).

Addition/neutralization of cytokines and inhibition of RA pathways in cocultures

For neutralization of TGF-β in cocultures, a pan-TGF-β (1, 2, 3) mAb (mAb 1835; R&D Systems) was used and purified mouse IgG1 (Sigma-Aldrich) was used as an isotype-matched control. Neutralization of IL-6...
used an anti-porcine polyclonal Ab (AF686; R&D Systems). Recombinant porcine IL-6 was also obtained from R&D Systems. The RA receptor α (RARα) antagonist Ro-41-5253 and the retinal dehydrogenase inhibitor citral were obtained from Biomol and Sigma-Aldrich, respectively.

**Phenotyping of MoDC**

MoDC cell surface expression of MHC class II and CD80/86 was assessed by FC using the mAb MSA3 (donated by A. Saalmüller) and a human CTLA4-mouse-Ig fusion protein (Alexis), respectively, followed by anti-mouse isotype-specific PE-conjugated goat F(ab′)2, Igs (Southern Biotechnology Associates). Endocytic activity (primarily macrophagocytosis) of MoDC was evaluated by incubation of the cells with 10 μg/ml OVA-dQ (Molecular Probes) in serum-free medium for 1 h at 39°C. OVA-dQ acquires fluorescence after dequenching through proteolytic enzyme cleavage, permitting the analyses of both Ag uptake and processing by FC. As control, the uptake at 4°C was measured and mean fluorescence intensity (MFI) values were subtracted from the 39°C values.

**RNA extraction and quantitative TaqMan RT-PCR for porcine CCR9**

SEB-stimulated MoDC/lymphocyte cocultures (pool of five replicate wells for each coculture) or FMDV-stimulated cocultures were harvested on days 4 and 5 of culture, respectively. RNA was extracted using the Nucleospin RNA II Extraction kit (Macherey Nagel) according to manufacturers’ instructions. Four microliters of RNA was reversed transcribed in a final reaction volume of 40 μl with RNasin (32 U), dNTPs (2.5 μM each; both Promega), buffer A, MgCl2 (25 mM final), Multiscribe RT (50 U) (all Applied Biosystems), and random hexamers (25 μM final; Pharmacia). The reaction was allowed to proceed for 30 min at 48°C and stopped by incubation for 10 min at 95°C. Four microliters of cDNA in a final reaction volume of 25 μl with buffer A, MgCl2 (25 mM final), dNTP (2.5 mM final each), AmpliTaq-Gold (0.5 U), and specific primers (0.5 μM each final) and probe (0.1 μM final) were subsequently used for TaqMan RT-PCR with the ABI PRISM 7700 Sequence Detector System (Applied Biosystems). Specific primers and probes for porcine GAPDH and CCR9 were designed based on sequences obtained from GenBank and synthesized by Microsynth. Sequences were as follows: pGAPDH-forward (F): catctacg caccgcagga, pGAPDG-reverse (R): tgcacacatgactccac, pCCR9-F: ggtgagacctggatggaa, pCCR9-R: tcagactcacccagaaga, pGAPDH-reverse (R): atgatgttctgggcagccc, pGAPDH-P: tgt

**Expression and functional readout**

The expression and the functional readout of the CCR9 mRNA expression were analyzed at day 4 of culture by FACS and by TaqMan RT-PCR as described above.

**ELISA for TGF-β1, IL-6, and FMDV-specific IgG and IgA**

IL-6 in MoDC culture supernatants was assessed by the porcine IL-6 Duo-Set ELISA Development System from R&D Systems. For analysis of TGF-β1, day 4 MoDC were transferred to serum-free medium before treatment with immunomodulatory factors for 24 and 48 h to avoid interference from serum-derived TGF-β1 in the ELISA. Cell-free supernatants were acidified with 10 μl/100 μl supernatant 1 M HCl for 30 min at 4°C, neutralized with equal amounts of 1 M NaOH, and used at a 1/2 dilution in the ELISA. This was performed using anti-TGF-β mAb 1835 (R&D Systems) for capture and biotinylated anti-TGF-β1 mAb 240 (R&D Systems) for detection, following the manufacturers’ guidelines. Recombinant human TGF-β1 (PeproTech) was used as standard. The ELISA for FMDV-specific IgG was based on the method described by Ridgen et al. (20), with modifications. Briefly, MaxiSorp plates (Nunc) were coated overnight at 4°C with a rabbit anti-swine IgG F(ab′)2 (Jackson ImmunoResearch Laboratories) diluted in the same buffer was added, followed by a 1-h incubation at 37°C. O-phenylenediamine (Sigma-Aldrich) H2O2 was used as substrate. Ab titers are expressed as absorbance (OD50). FMDV-specific IgA was assessed analogously but using an anti-swine IgA mAb (CVL.SwIgA.27.9.1; Cedi Diagnostics) and a rabbit anti-mouse Ig-HRP conjugate (DakoCytomation) for detection.

**Statistical analysis**

All statistical analyses were performed using the Wilcoxon pairwise rank sum test.

**Results**

RA pretreatment of MoDC leads to up-regulated β3 integrin and CCR9 mRNA expression by cocultured T cells

The initial analyses focused on screening a number of immunoregulatory factors for their potential to modulate peripheral DC toward mucosa-type DC. An in vitro coculture model of MoDC and lymphocytes was used, with analysis of lymphocyte expression of the β3 integrin and CCR9 mRNA as functional readout. The factors used for pretreatment of MoDC were chosen based on their already well-established association with the induction and functioning of mucosal immune responses (LT, Di-VitD3, Ret, RA) or on account of their known overall stimulating capacity for DC (TNF-α, LPS, Pam3Cys, pIC).
When MoDC were pretreated with 1000 nM RA before coculture with autologous lymphocytes, significantly higher β7 integrin expression was noted on proliferating (CFSElow) lymphocytes, compared with untreated MoDC (Fig. 1A). This was not observed when the MoDC were treated with the other factors or the lower concentration of RA (10 nM). CCR9 mRNA expression by the lymphocytes was also modulated in the cocultures containing the RA-pretreated MoDC, in a similar fashion to the β7 integrin expression—CCR9 mRNA levels were increased up to 43-fold in cocultures with MoDC pretreated with 1000 nM RA (Fig. 1B). Interestingly, a mean 4-fold increase of CCR9 mRNA was also observed in cocultures with LT-pretreated MoDC for 4 days in the absence or presence of the RARα antagonist Ro-41-5253. After 4 days of culture, β7 integrin expression was analyzed in proliferating cells. F, β7 integrin expression by CFSElow lymphocytes cocultured with untreated or RA-pretreated MoDC for 4 days in the absence or presence of the RALDH inhibitor citral. All experiments in A–F were performed in replicates of three wells. Mean values are shown with error bars indicating the range. Experiments were repeated twice with similar results.

FIGURE 4. Role for carryover of RA. A and B, PBMC isolated from several individual pigs were incubated with SEB and the indicated RA concentrations for 4 days. A, RA-induced increase in β7 integrin expression by proliferating cells relative to β7 integrin expression in cells cultured in the absence of RA. B, Relative RA-induced increase in CCR9 mRNA expression. Lines represent responses from individual pigs. C and D, Untreated and 1000 nM RA-treated, SEB-loaded MoDC were cocultured with CFSE-labeled lymphocytes for 4 days in the absence or presence of the RARα antagonist Ro-41-5253. C, β7 integrin expression by CFSElow cells. D, CCR9 mRNA expression in cocultures. E, PBMC were stimulated with SEB in the absence or presence of different concentrations of RA and the RARα antagonist Ro-41-5253. After 4 days of culture, β7 integrin expression was analyzed in proliferating cells. F, β7 integrin expression by CFSElow lymphocytes cocultured with untreated or RA-pretreated MoDC for 4 days in the absence or presence of the RALDH inhibitor citral. All experiments in A–F were performed in replicates of three wells. Mean values are shown with error bars indicating the range. Experiments were repeated twice with similar results.

The above results were obtained in an SEB-dependent stimulation assay. Although DC do present SEB in a costimulation-dependent manner (21), Ag processing is not required. To ascertain whether the above observations would also be operative during Ag presentation, as would occur during vaccination, a second model system was used. Therein, an identical set of experiments was performed using FMDV as Ag together with lymphocytes isolated from FMDV-immune pigs. Selective gating on FMDV-specific blasting receptors.

Influence of RA treatment of MoDC on β7 integrin and CCR9 mRNA expression during an Ag-specific response

The above results were obtained in an SEB-dependent stimulation assay. Although DC do present SEB in a costimulation-dependent manner (21), Ag processing is not required. To ascertain whether the above observations would also be operative during Ag presentation, as would occur during vaccination, a second model system was used. Therein, an identical set of experiments was performed using FMDV as Ag together with lymphocytes isolated from FMDV-immune pigs. Selective gating on FMDV-specific blasting receptors.

The αβδ-integrin expression was seen to be cell cycle dependent (Fig. 1D) (4). Thus, it was possible that the results shown in Fig. 1, A and B, were due to an increased stimulatory capacity of the RA pretreated MoDC. Consequently, the relative increase in lymphocyte proliferation, determined as the frequency of CFSElow cells, was quantified in cocultures of lymphocytes with the pretreated MoDC, compared with cultures using untreated MoDC. The RA-pretreated MoDC were indeed more efficient than untreated MoDC at inducing T cell proliferation (Fig. 1, C and D). However, their stimulatory capacity was exceeded by LT-treated MoDC, which induced significantly more lymphocyte proliferation compared with untreated MoDC (Fig. 1, C and D). In fact, there was no precise relationship between the induction of lymphocyte proliferation and up-regulation of mucosal homing receptors.
CD4+CD8+ memory/activated cells, but not on any other lymphocyte subset (data not shown), revealed a significantly up-regulated expression of the β7 integrin by these cells after coculture with 1000 nM RA-treated or 1 μg/ml LT-treated MoDC (Fig. 2, A and D), although the latter were less effective than the RA-treated MoDC. Despite the low frequency of FMDV-responsive lymphocytes, an increase in CCR9 mRNA expression similar to the SEB-dependent system was also detected in these cocultures (Fig. 2B). Again, analysis of the percentage of proliferating lymphocytes (frequency of CD4+CD8+ blasts) failed to demonstrate a clear-cut correlation between the stimulatory potential and homing receptor imprinting capacity of the treated MoDC (Fig. 1C). Thus, despite variations between individual experiments in both the SEB and FMDV systems, the presence of RA-treated (and to a lesser extent of LT-treated) MoDC consistently led to an increased mucosal homing receptor expression by cocultured lymphocytes. Interestingly, this imprinting of mucosal homing receptors through the presence of RA-treated MoDC was seen with both naïve and Ag-experienced lymphocytes (Figs. 1 and 2).

Analysis of cell surface molecule expression and cognate interaction relative to the activity associated with the RA-treated MoDC

Microscopic examination revealed that RA-treated MoDC were morphologically distinct to untreated cells, being more similar to the morphology of TNF-α-matured MoDC (data not shown). Accordingly, MoDC phenotyping was used to assess whether the influence of the RA-treated MoDC on the lymphocytes could be related to modified MHC class II or costimulatory molecule expression. RA-treated MoDC did not display modulated MHC class II or CD80/86 expression compared with untreated cells (Fig. 3A). In contrast, RA-treated MoDC had a strongly reduced endocytic and processing capacity, as assessed by the signal intensity obtained with OVA-dQ (Fig. 3A). Thus, RA was influencing the functionality of the MoDC. The relevance of cognate interactions between RA-treated MoDC and T cells, in terms of β7 integrin and CCR9 mRNA expression, was ascertained by separating the treated MoDC from the lymphocytes by a 0.4 μm pore-size cell culture insert. Whole PBMC were stimulated with FMDV to allow for Ag presentation. In the absence of the cell culture insert, the RA-treated MoDC promoted an almost 4-fold increase in β7 integrin expression on the gated CD4+CD8+ Th lymphocytes (Fig. 3B, MoDC + PBMC). The degree of this induction was greater than that obtained with cocultured APC-depleted (CD172a−ly) lymphocytes (Fig. 3B, “MoDC + CD172a−ly”). When the RA-treated MoDC were separated from the PBMC by the 0.4-μm membrane, their capacity to induce β7 integrin expression was unaffected (Fig. 3B, “MoDC + PBMC”). In contrast, when separated from the APC-depleted lymphocytes, the RA-treated MoDC only induced a minor increase in β7 integrin expression (Fig. 3B, “MoDC + CD172a−ly”). This related to the lack of Ag presentation in these cultures (data not shown) and confirmed that separation of the MoDC by the 0.4 μm pore-size membrane was complete (Fig. 3B). These findings suggested that RA-treated MoDC mediated their effect through soluble factors rather than cognate interaction with the lymphocytes.

FIGURE 5. Additional MoDC-derived factors may be involved in the regulation of β7 integrin expression. A, PBMC were stimulated with SEB in the presence of serially diluted supernatants derived from 4-day-old cocultures of CD172a− lymphocytes with untreated and RA-treated MoDC, respectively. β7 integrin expression was analyzed in proliferating PBMC at day 4 of culture. B and C, Comparison of β7 integrin expression and proliferative responses in FMDV-stimulated PBMC cultures supplied with 100 nM RA and in cocultures of CD172a− cells with untreated and RA-treated MoDC, respectively. Mean values of three replicate wells are shown with error bars indicating the range. D–F, Untreated or 48-h RA-treated, SEB-loaded MoDC were fixed with 1% PFA, washed four times, and cocultured with CFSE-labeled CD172a− lymphocytes for 4 days. D, Frequency of proliferating, CFSElow cells. Bars represent values of five replicate wells ± SEM. F, CCR9 mRNA expression in pools of five replicate coculture wells. Each set of experiments A, B and C, and D–F were repeated three times with similar results.
MoDC as carriers of RA

Iwata et al. (7) demonstrated that concentrations of RA as low as the picomolar range can directly induce $\beta_7$ integrin and CCR9 expression by T cells in the absence of DC. In our system, RA-treated MoDC were washed at least four times (with a ~100-fold dilution at each step) before coculture with the lymphocytes. Thus, the original concentration of 1000 nM RA used for MoDC pre-treatment was likely reduced to <0.01 pM. Titrations of RA on SEB-stimulated PBMC isolated from several individual pigs indicated that concentrations of >1 nM RA were required for detectable induction of $\beta_7$ integrin and CCR9 mRNA expression by porcine lymphocytes (Fig 4, A and B). Considering this together with the known instability of RA, a carryover of residual RA into the cocultures seemed unlikely. Nevertheless, additional analyses to rule out a “carry-over” of RA were pursued using the RAR antagonist Ro-41-5253. When added into the MoDC-lymphocyte cocultures, 100 nM Ro-41-5253 impaired the up-regulation of $\beta_7$ integrin expression induced by the RA-treated MoDC; 1000 nM of the RAR antagonist was needed to abolish $\beta_7$ up-regulation (Fig. 4C). Intriguingly, the basal $\beta_7$ integrin expression levels—induced by untreated MoDC—were also impaired in the presence of the RAR antagonist (Fig. 4C). CCR9 mRNA expression was also affected by the RAR antagonist. Concentrations as low as 1 nM impaired the expression, with 100 nM of Ro-41-5253 effectively abolishing CCR9 mRNA induction (Fig. 4D). A cross-titration of RA and Ro-41-5252 on SEB-stimulated PBMC revealed that a 1000× molar excess of the RAR antagonist was required to counteract the effects of RA with respect to induction of $\beta_7$ integrin expression (Fig. 4E). Thus, according to the findings presented in Fig. 4C, substantial amounts of RA were still present in the cocultures. Because RA may regulate its own synthesis by positive feedback activation of retinal dehydrogenase enzymes (RALDH) and RARs (22–24), the possibility of de novo synthesis of RA by RA-treated MoDC was considered. However, addition of the RALDH inhibitor citral into cocultures did not significantly affect the ability of RA-treated MoDC to induce up-regulated $\beta_7$ integrin expression (Fig. 4F). These findings suggested that RA-treated MoDC appear to be able to function as efficient reservoirs and carriers of bioactive RA.

Potential role of additional MoDC-derived factors in the regulation of $\beta_7$ integrin expression

The experiments described above indicated a role for a cell-mediated carry-over of RA into the cocultures. Accordingly, RA-treated MoDC might efficiently “deliver” RA to lymphocytes during immunological synapse formation. However, the induction of $\beta_7$ integrin expression on lymphocytes separated from RA-treated MoDC by a transwell insert would rather argue for a release of RA or other soluble factors from the RA-treated MoDC into the culture medium (Fig. 3B). Indeed, serially diluted supernatants from 4-day-old cocultures of lymphocytes and RA-treated MoDC still induced increased $\beta_7$ integrin expression in SEB-stimulated PBMC (Fig. 5A). Such an activity seemed to be in conflict with the well-acknowledged instability of RA in aqueous solutions. Moreover, the levels of $\beta_7$ integrin expressed by lymphocytes cocultured with RA-treated MoDC were substantially higher than the levels induced on PBMC by saturating (100 nM) concentrations of exogenous RA (Fig. 5B). This could not be explained by an increased stimulatory potential of the RA-treated MoDC (Fig. 5C). To assess a potential function for a soluble factor produced by RA-treated MoDC in up-regulating $\beta_7$ integrin expression by cocultured lymphocytes, MoDC were fixed with PFA before addition of the lymphocytes. Although the fixation of SEB-loaded MoDC did not abrogate their ability to stimulate proliferative responses (Fig. 5D), $\beta_7$ integrin expression by the cocultured lymphocytes was strongly impaired (Fig. 5E). This was noted with both the RA-treated and untreated fixed MoDC. In contrast, CCR9 mRNA induction by the RA-treated MoDC was less affected by the fixation of the MoDC (Fig. 5F). Because fixation of the MoDC likely prevented the synthesis and release of cytokines, but not the release of RA, these findings would indicate an additional potential role for MoDC-derived factors other than RA in the regulation of $\beta_7$ integrin, but not of CCR9 mRNA, expression.

Role for TGF-β secreted by RA-treated MoDC

Although no IL-10 was detectable in supernatants from either untreated or RA-treated MoDC (data not shown) 1000 nM RA clearly induced TGF-β at 24 and 48 h posttreatment (Fig. 6A). Pan-TGF-β neutralizing mAb significantly reduced the capacity of
the RA-treated MoDC to induce β7 integrin up-regulation on cocultured lymphocytes while not affecting the basal level of β7 integrin expression (Fig. 6B). In contrast to β7 integrin expression, the up-regulation of CCR9 mRNA induced by RA-treated MoDC was not affected by the presence of the TGF-β-neutralizing mAb (Fig. 6C). TGF-β has been implicated in up-regulating αEβ7, but not α4β7 integrin expression, on T cells entering the mucosal compartment (25, 26). As Abs against the αEβ7 heterodimer were not available for the porcine system, our studies routinely used a cross-reactive anti-mouse β7 mAb. To certify that the anti-TGF-β-neutralizing mAb indeed impaired α4 integrin chain expression with a mAb specific for α4 (Fig. 6, D–F). RA-treated MoDC in fact induced increased levels of α4 integrin chain expression on cocultured lymphocytes compared with untreated MoDC (Fig. 6E). However, this was only a 2-fold increase compared with the several-fold augmented expression of the β7 integrin chain. Moreover, α4 integrin chain expression was not affected by the anti-TGF-β mAb (Fig. 6E). This may relate to pairing of the α4 integrin chain with the β1 chain and the ubiquitous expression of the α4β1 integrin by activated lymphocytes. Nonetheless, when selective gating on CFSElow α4β7 double-positive, high-expressing cells was used, the impairing effect of the anti-TGF-β mAb was again apparent (Fig. 6F), similar to the observations for β7 integrin chain expression (Fig. 6D).

**FIGURE 7.** RA is an inducer and potent amplifier of IL-6 production in MoDC. Four-day-old MoDC were stimulated for 48 h with 1000 nM RA, TLR ligands (Pam3Cys, 10 μg/ml; pIC, 10 μg/ml; LPS 1 μg/ml), or 1000 nM RA combined with TLR ligands. Supernatants were assessed for IL-6 by ELISA. One representative experiment of five independent experiments is shown.

**FIGURE 8.** RA-treated MoDC are potent inducers of FMDV-specific IgG and IgA responses. A, PBMC and APC-depleted CD172a− lymphocytes were isolated from FMDV-immune pigs and cultured with UV-inactivated FMDV in the absence or presence of RA (100 nM) or RA plus IL-6 (10 ng/ml). Where indicated in A and B, lymphocytes were cocultured with 48 h differentially pretreated MoDC and UV-inactivated FMDV in the absence (A) or presence (B) of exogenous RA (100 ng/ml), RA plus IL-6 (10 ng/ml), IL-6, anti-IL-6 Ab (5 μg/ml), or the RAR antagonist Ro-41-5253 (1 μM). FMDV-specific IgG and IgA was assessed in supernatants from 5-day-old cultures by sandwich ELISA. Mean values of three in vitro culture replicate wells are presented with error bars showing the range. Experiments were repeated three times with similar results.
RA synergizes with TLR ligands to promote IL-6 production in MoDC

The above results demonstrating a partial role for RA-induced TGF-β in the induction of αβ2 integrin expression led us to investigate the role of additional cytokines potentially produced by the RA-treated MoDC. Along with TGF-β, IL-6 is considered an important cytokine in the functioning of mucosal immune homeostasis. Indeed, RA treatment of MoDC for 48 h induced levels of IL-6 similar to those obtained after TLR ligand stimulation (Fig. 7). Intriguingly, when MoDC were simultaneously treated with RA and TLR ligands, up to 10-fold increased levels of IL-6 were detected (Fig. 7). MoDC cotreated with RA and TLR ligands were thus compared with the RA-treated MoDC for their capacity to induce increased β2 integrin expression. The cotreated MoDC were in fact more potent inducers of β2 integrin expression in certain experiments, but addition of an anti-IL-6-neutralizing Ab had no influence (data not shown). Hence, while IL-6 production by RA-treated, and particularly RA- and TLR-ligand cotreated MoDC represents a striking feature, IL-6 does not contribute to the capacity of MoDC to promote mucosal homing receptor expression.

RA-treated MoDC are potent inducers of FMDV-specific IgG and IgA secretion

Mucosal DC are characterized by their ability to promote gutropic T cells, and by their preferential secretion of the cytokines IL-10, TGF-β, and IL-6 (27). Another unique attribute of mucosal DC is their capacity to induce IgA responses, which was recently shown to depend on RA, IL-6, or IL-5, as well as other unidentified DC-derived factors (8). Considering that RA-treated MoDC produced significant levels of IL-6, especially after concomitant TLR ligand stimulation (Fig. 7), we analyzed the capacity of RA-treated and RA plus LPS cotreated MoDC to induce FMDV-specific Ig secretion in cocultured, FMDV-stimulated lymphocytes. FMDV-specific Ig secretion depended on the presence of APC, as Ig levels were strongly abolished in CD172α− lymphocytes depleted from monocytes and DC compared with total PBMC (Fig. 8A). Addition of untreated MoDC to these CD172α− lymphocytes was unable to restore specific IgG production, whereas the presence of LPS-treated MoDC induced a small increase in FMDV-specific IgG (Fig. 8A). In contrast, coculture of CD172α− lymphocytes with RA-treated or RA plus LPS cotreated MoDC induced high levels of FMDV-specific IgG, which even exceeded the levels obtained using total PBMC (Fig. 8A).

For FMDV-specific IgA, a similar pattern of induction was observed. However, RA plus LPS cotreated MoDC were now more potent than RA-treated MoDC in inducing FMDV-specific IgA responses (Fig. 8A). This was in keeping with the acknowledged synergy of RA and LPS in inducing IL-6 production and the well-acknowledged role of IL-6 in the induction of IgA responses (Fig. 7 and Refs. 8 and 28). However, addition of RA or RA plus IL-6 to total PBMC cultures did not further boost their FMDV-specific IgA or IgG production (Fig. 8A).

An additional experiment was performed to address the impact of exogenous RA and IL-6 added to cocultures with untreated MoDC and LPS-treated MoDC. When RA and IL-6 were added to cocultures of lymphocytes with otherwise untreated MoDC, substantially augmented FMDV-specific IgG and IgA responses were observed (Fig. 8B). This effect was even more pronounced when LPS-treated MoDC were used. Intriguingly, when the MoDC were pretreated with RA or RA plus LPS, neither an anti-IL-6-neutralizing Ab nor the RARα antagonist Ro-41-5253 impaired their ability to augment the FMDV-specific Ig responses.

These findings indicate that, in contrast to what was observed for mucosal homing receptor induction, direct interaction of RA with lymphocytes was probably not involved. Rather, exposure of MoDC to RA resulted in their modulation leading to an enhanced capacity for promoting the induction of potent FMDV-specific IgG and IgA responses.

Discussion

Accumulating evidence suggests that DC play pivotal roles in shaping the migration pattern of lymphocytes (4–6, 10–12). Importantly, only DC from mucosa-draining lymph nodes will endow lymphocytes with the correct set of homing receptors for subsequent entry into mucosal effector tissues. This particular capacity of DC may account for the long-observed general failure of parental immunization to induce mucosal immunity. Although DC targeting for enhanced Ag delivery and presentation has become a prominent focus of vaccine research, few studies have systematically examined the potential of manipulating peripheral DC for vaccine-induced mucosal immunity. Based on the well-recognized plasticity of the DC-system, this study sought to investigate whether DC could be modulated toward “mucosa-type” DC. In this context, MoDC were analyzed for their capacity to imprint mucosal homing receptor expression on lymphocytes following targeting with selected immunomodulatory and adjuvant factors. Using an in vitro coculture model, the present work shows that MoDC pretreated with the vitamin A-derivative RA promoted up-regulated αβ2 integrin and CCR9 mRNA expression by activated lymphocytes.

This apparently DC-mediated effect is intriguing considering that picomolar concentrations of RA can directly induce mucosal homing receptor expression by T cells in the absence of DC (7). In our porcine model, concentrations of RA of at least 1 nM were required for direct induction of β2 integrin and CCR9 mRNA expression in T cells. Hence, with the well-recognized instability of RA in aqueous solutions and the extensive washing of the MoDC a carry-over of RA at first seemed unlikely. Indeed, culture of SEB-stimulated PBMC in the presence of the final wash fractions of the RA-treated MoDC did not indicate any residual activity of RA in respect to the induction of β2 integrin expression (data not shown). However, the use of a RARα antagonist in cocultures demonstrated that RA was evidently responsible for the effects observed and that substantial amounts of RA, likely associated with the MoDC, still had to be present. This may explain why only MoDC pretreated with the supraphysiological dose of 1000 nM but not with the lower dose of 10 nM RA were capable of inducing mucosal homing receptor expression.

Although it has been demonstrated that RA can produce RA, little information is available regarding the cellular metabolism of RA in DC after exposure to RA (7, 29). At least in certain cell types, RA induces the expression of a specific RA-metabolizing P450 cytochrome enzyme (CYP26) and triggers its own metabolism toward less active and more polar forms, which can be released into the culture medium (30–32). In contrast, evidence also exists for a possible positive feedback loop of RA on its own synthesis by activation of specific RA response elements in promoters of RAR and RALDH genes (22–24). It was beyond the scope of this study and the possibilities of our laboratory to analyze the actual residual amounts of RA in the RA-treated MoDC or to assess the formation and release of metabolites. Another difficulty is that the biological activity of these metabolites with respect to the induction of mucosal homing receptors is unknown. Because addition of the RALDH inhibitor citral into cocultures did not abrogate the capacity of the RA-treated MoDC to induce increased mucosal homing receptor expression, a de novo synthesis...
of RA from serum-derived retinol appeared unlikely. In the absence of further evidence, we thus have to state that MoDC can act as surprisingly efficient reservoirs and carriers of bioactive RA.

Two observations nonetheless led us to investigate a role for additional potential factors involved in the functioning of the RA-treated MoDC. First, we and others noted a distinct suppressive effect of the RAR antagonist on basal or pre-existing β7 integrin expression, indicating that RAR antagonists may interfere with integrin expression in a RA-independent manner (8). Interference of certain RAR antagonists with AP-1 signaling has in fact been reported (33). Second, the increase in β7 integrin expression levels in cocultures with RA-treated MoDC was consistently greater than that observed with PBMC cultures supplied with saturating concentrations of RA, suggesting that other DC-derived factors may contribute to the induction of β7 integrin expression. Cytokine analyses revealed that RA treatment of MoDC induced substantial quantities of TGF-β and IL-6. Although addition of an anti-TGF-β-neutralizing mAb to the cocultures significantly impaired β7 integrin expression, an Ab against IL-6 had no influence. In addition, CCR9 mRNA expression was not affected. Thus, similar to what has been proposed in the mouse, β7 integrin and CCR9 expression appear to be regulated differentially (5, 6, 8, 9, 12). CCR9 expression may strictly depend on RA, whereas α4β7 integrin expression could be regulated by the cooperative action of RA and additional factors. In this respect, it is interesting to note that peripheral DC can induce low levels of α4β7 on T cells after long-term in vitro culture (11).

Although the results were unable to provide a clear pathway for cytokine-mediated control of α4β7 integrin regulation, secretion of TGF-β and IL-6 by RA-treated MoDC remains an important and novel feature. It is strongly reminiscent of the unique cytokine profile described for mucosal DC, promoting Th2 responses and supporting IgA switching (27).

Interestingly, a recent study characterized the IgA-inducing ability of mucosal DC as occurring independently of T cell help but involving DC-derived RA and IL-6 or IL-5 (8). Notably, despite polyclonal activation of B cells in these in vitro cultures, the presence of DC could not be substituted with RA and IL-6 and/or IL-5, indicating that additional (unidentified) DC-derived factors or signals were crucially involved. Related to these observations, our studies demonstrated that MoDC pretreated with RA or with RA and TLR ligands were potent inducers of FMDV-specific IgG and IgA secretion by cocultured B cells compared with untreated MoDC or MoDC pretreated with LPS alone. Intriguingly, this effect did not appear to be dependent on the increased IL-6 production by the RA-treated MoDC, as an anti-IL-6-neutralizing Ab had no influence. Moreover, in contrast to what was observed for the induction of mucosal homing receptor expression by the RA-treated MoDC, the RAR antagonist had no impact on the capacity of the RA-treated MoDC to induce Ig production. Because addition of RA to cocultures of CD172a+ cells with untreated MoDC greatly boosted FMDV-specific Ig production, we can only speculate that DC themselves and not necessarily the B cells are the deciding targets of RA. Accordingly, exposure of DC to IL-6 and RA could lead to a generally increased stimulatory function of the DC and to the production of factors which are essential for the induction of B cell responses.

Based on our in vitro studies, we propose a novel role for RA as mucosal immune modulator directly targeting DC—a concept supported by the following notions: apart from mucosal DC, intestinal epithelial cells also possess the necessary enzymatic machinery to synthesize RA, and their direct metabolization of retinol to RA has been demonstrated (34). Due to the strategic location and multitude of enterocytes, these cells could represent a principal source of RA in the intestinal mucosa. Because epithelial cells cross-talk with DC by the production of chemokines and cytokines, LP DC may well be shaped by the presence of epithelial-derived RA. This could relate to the observation that the mucosal imprinting capacity of MLN DC is restricted to LP-derived CD103+ DC (9). Hence, exposure of LP DC to RA could result in the necessary induction of RALDH enzyme expression by positive feedback activation of the RALDH promoter (22). CD103 expression could either be directly induced by epithelial-derived TGF-β3, as proposed by Johansson-Lindbom et al. (9), or be mediated by RA-induced autocrine production of TGF-β3 by the DC (our own results). Unfortunately, no reagents were available to analyze porcine CD103 expression.

Taken together, we have identified RA as a factor endowing peripheral or “neutral” DC with attributes of mucosal DC. Although secretion of TGF-β3 and IL-6 and the ability to induce IgA responses by the RA-treated MoDC involved a true modulation of the DC by RA, their imprinting capacity of mucosal homing receptor expression is evidently associated with the release of RA and/or its metabolites acting on the lymphocytes. It will now be interesting to translate these in vitro findings into the in vivo situation. Although the overall importance of vitamin A in the functioning of immune responses is well-acknowledged from epidemiological data, most investigations on its potential adjuvant effect have focused on dietary supplementation. Nevertheless, a recent publication by Skountzou et al. (35) demonstrated that transcutaneous application of RA with cholera toxin and whole inactivated influenza virus augmented intestinal anti-influenza virus responses compared with a cholera toxin-virus combination. Although migration of peripheral DC to mucosal inductive sites may account for this effect (36, 37), our findings suggest that in situ modulation of DC and imprinting of mucosal homing receptor expression in local draining lymph nodes may also be involved. Such a mechanism has yet to be proven in vivo, but is at least supported by the notion that mucosal DC can potently override signals mediated by peripheral DC (12). Based on the modulation of DC by RA and the surprising capacity of these cells to store and carry RA, selective targeting of DC with RA shows promise for vaccine-induced mucosal immune responses via a parenteral route of immunization.

Acknowledgments
We thank H. Gerber for mAb preparations, V. Neuhaus and M. Alves for preparing recombinant cytokines, and M. Hofmann for assistance with CCR9 primer design. We further thank P. Barnett for the generous supply of the FMDV vaccine. Particular thanks go to D. Brechbühl and H. Lüthi for care and regular bleeding of the pigs.

Disclosures
The authors have no financial conflict of interest.

References