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Migratory capacity and function of dendritic cells from mesenteric afferent lymph nodes after feeding a single dose of vitamin A

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Running head: Dendritic cells of afferent lymphatics upon vitamin A feeding

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Key words: Vitamin A-afferent lymphatic-dendritic cell- chemoattractant-homing receptor-tolerance.

Abstract

Lamina propria dendritic cells (DCs) have a permanent turnover with constitutive migration to mesenteric lymph nodes (MLN) and replenishment by progenitors. Luminal bacteria and dietary constituents provide key signals that endow DCs their unique properties in vivo. Taken into account that the intestinal immune system is greatly influenced by retinoids, we evaluated in B6 mice 3, 8, 16 and 24 h after feeding a single dose of vitamin A, phenotype and function of cells present in mesenteric afferent lymph nodes as well as signals involved in migration. We studied the frequency of CD11c+MHC-II+CD103+CD86+ and RALDH+ DCs by flow cytometry, we determined CCL-21 and D6 levels in tissue homogenates by Western blot and we co-cultured cells isolated from afferent lymphatics with sorted CD4+ lymphocytes to assess Foxp-3 induction and homing receptor expression. Sixteen h after vitamin A administration, DCs isolated from afferent lymphatics were able to induce homing receptors and Foxp3 expression in CD4+ lymphocytes. Our results show that a single dose of vitamin A generated a stream of signals and amplified the tolerogenic activity of DCs migrating to lymphoid tissue.

Introduction

One micronutrient of great importance is vitamin A (VA) that enters the body with the diet and achieves its highest concentration in proximal intestines and mesenteric lymph nodes (MLN). VA is metabolized by intestinal epithelial, stromal and dendritic cells (DCs) to the active form all-trans retinoic acid (RA), which regulates transcription through its nuclear receptors [1,2]. The intestinal immune system is greatly influenced by retinoids in many aspects of the homeostasis, including the control of inflammatory or tolerogenic immune responses, the epithelial differentiation and the barrier function. Still, many of the events related to VA feeding, remain to be determined. The specific effects of RA and its receptors on the immune response are not always predictable, and depending on the dose, receptor isoform and cell type the result can be very different [3]. In fact, physiological and pharmacological doses of RA (exogenous RA concentration above 100 nM is considered a pharmacological concentration) may bring dissimilar conclusions [4-6]. Moreover, differences between signaling pathways induced by physiological and pharmacological RA doses have not still been established [5]. RA appears to have a central role in eliciting immune responses both under homeostatic as well as inflammatory conditions but the effector outcome of its signaling depends on local RA levels, additional cytokines, and other microenvironmental factors [3,4,6].

The RA has a key contribution to the intestinal immunity in the maturation and differentiation of innate and adaptive immune cells [2,4]. RA promotes the maturation of DCs and induces retinaldehyde dehydrogenase (RALDH) expression in intestinal migratory $CD103^+$ DCs that seem to be initially exposed to RA in the lamina propria [7]. These unique subsets of RALDH+ DCs

efficiently convert CD4+ T cells into inducible Foxp3+ regulatory T cells in concert with TGF-β stimulation [8]. Consequently, at steady state, migratory RALDH+ DCs, which present gut-derived antigens and secrete RA, promote and maintain immune tolerance [9,10]. In adult mice MLN drain continuously the gut and receive large numbers of $CD103^+$ $RALDH^+$ DCs [10,11]. Inside MLN, RA drives regulatory T cell induction, lymphocyte gut homing imprinting (α4β7 and CCR9 on T cells) and IgA production [12-15]. Nevertheless, if DCs migrating in afferent lymphatic vessels own already their unique tolerogenic properties is not completely understood.

The lymphatic endothelium secretes CCL-21, causing chemotaxis of mature DCs with increased expression of CCR7 receptor [16]. On the other hand, intestinal lymphatics also express "decoy" receptors that promiscuously remove inflammatory chemokines [17]. Herein we hypothesized that vitamin A stimulated the lymphatic vascular network, triggering the production of chemoattractants and promoting the recruitment of tolerogenic DCs. As VA metabolites can be normally found in the gut or lymph vessels, we evaluated in normal B6 mice if feeding a single dose of VA could amplify signals in afferent lymphatic vessels involved in DC recruitment and imprint DCs tolerogenic properties before reaching MLN.

Materials and Methods

Ethical considerations

Animal experiments were approved by and conducted in accordance with guidelines of the Committee for Animal Care and Use of the Facultad de

Ciencias Químicas, Universidad Nacional de Córdoba (Approval Number HCD 15-09-69596), and in strict conformity with the recommendation of the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (OLAW Assurance number A5802-01). Our animal facility obtained the NIH Animal Welfare Assurance (Assurance No. A5802-01, Office of Laboratory Animal Welfare, NIH, Bethesda, MD, USA).

Mice

Female C57BL/6 (WT) and Foxp3-GFP mice from the Jackson Laboratory were used in these studies. For each group of experiments, mice were matched by age (six- to eight-week-old) and body weight (20-25g). Animals were maintained in specific pathogen-free conditions and housed in collective cages at 22 ± 1 °C under a 12-h light/dark cycle (lights on at 7:00 a.m.). Mice were fed *ad libitum* with free access to laboratory chow and drinking water throughout the study. For VA administration, we used a dose scaled to mouse weight which is comparable to the one used by the WHO in clinical trials (50,000 IU/2.5 kg body weight) on infant survival [18]. Mice were feed once 50 μl DMSO (control group) or 5 mg/1625 IU VA/50 μl DMSO (~three times the WHO dose) and killed later to isolate mesenteric afferent lymphatic vessels (MLAV). For experiment in Fig. 1, mice were fed al 4 p.m. and killed 3,8,16 or 24 h later; the following studies were done feeding VA at 4 p.m. and killing mice 16 h later.

Reagents and antibodies

Anti CD4 (GK 1.5), anti MHC-II (25-9-17), anti CD11c (HL3), anti CD86 (GL1) and anti $\alpha 4\beta 7$ integrin (DATK32) were provided by BD PharMingen (San Diego,

CA, USA); anti mouse CD199 (CCR9) and CD103 (2E7) by Biolegend (San Diego, CA, USA). Corresponding isotype-matched mAbs were used as controls in flow cytometry experiments. Anti D6 (ab1656) and anti CCL-21 (ab9903) were from Abcam (Braunschweig, Germany). CFSE and eFluor 670 were from eBioscience (San Diego, CA, USA). The RALH activity was evaluated with the ALDEFLOUR kit (StemCell Technologies, Grenoble, France). Stocks of VA (325000 IU/g; Todo Droga, Córdoba, Argentina) were prepared in DMSO.

Cell isolation

To isolate MLAV cells, small intestine together with mesentery and appendix were extracted from the peritoneal cavity. MLN and the small intestine were dissected and the intestine placed in a fan-shape [19]; the adjoining sections of the mesentery containing afferent lymphatic vessels were removed and incubated in 5% FCS-RPMI to allow cells to migrate without further manipulation. After 3 h lymphatic vessels were removed carefully and homogenized for Western blot; cells were recovered by gently centrifugation. In different assays, total MLAV content (i.e, cells+ lymph), cell free lymph or cells were used.

Western blot

CCL-21 and D6 were determined in MLAV homogenates by Western blot as described [20]. Bands were analyzed with the Image Studio™ Lite Software (Lincoln, NE, USA), normalized with β-actin and expressed as Relative Units.

Cell culture

MLN single cell suspensions labelled with 1μM CFSE or sorted CD4+Foxp3-GFP$^-$ cells were cultured with MLAV cells for 24 h (assessment of homing receptors) or 96 h (induction of Foxp3) at a 1:1 ratio. When indicated, cells were harvested for flow cytometry and supernatants were collected and kept at -20 ºC to measure cytokines. For proliferation assays, MLN single cell suspensions labelled with 1μM eFluor 670 were cultured with a) MLAV total content, b) MLAV cell free content or c) cells recovered from MLAV. After 72 h the dilution of eFluor and homing receptor expression were evaluated by flow cytometry; in supernatants TGFβ was determined by ELISA.

Measurement of cytokines and chemokines

Murine TGFβ (BD Biosciences) or IFNγ and IL-10 (BD Pharmingen) were measured by ELISA kits, as specified by the manufacturers.

Flow cytometry

Cells (0.5-1×10^6) were resuspended in FACS buffer (PBS, 5% FBS, 0.02% NaN2) containing FBS 10% on ice for 15 min to prevent non-specific antibody binding. Thereafter FACS buffer containing the desired antibodies was added and incubated on ice for 30 min. On the basis of forward and side light scatter, debris and dead cells were gated out; 10,000 events were analyzed using a

FACSAria flow cytometer (BD Bioscience). The flow cytometry analysis was performed with the FlowJo software, as previously [21].

Statistical analysis

Data were expressed as mean ± SEM. Statistical differences between groups were determined by analysis of variance followed by a Student-Newman-Keuls test. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA). A p value of less than 0.05 was considered statistically significant.

Results

Cell traffic toward MLN upon VA feeding

To evaluate the effect of VA administration on the mobilization of immune cells toward MLN we feed WT mice 5 mg of VA and 3, 8, 16 and 24 h later we cultures MALV to recover migrating cell. No significant differences were found in controls after 3,8,16 and 24 h of DMSO gavage, therefore they were included as a single timepoint (Fig. 1A). A significant increment in cellularity was found 8 h after VA administration which was concurrent with the rise in the chemokine CCL-21 in MALV homogenates (Fig. 1B and C). Interestingly, levels of D6 -a decoy and scavenger receptor for inflammatory CC chemokines [17, 22 15,19] were significantly higher 16 h after VA feeding, when cellularity in MALV regained values of control group (Fig. 1C).

Phenotype of DCs in MLAV upon VA feeding

Then 3, 8 or 16 h after VA administration we evaluated in MLAV the frequency and phenotype of tolerogenic DCs. While no differences between control and VA groups were observed at 3 and 8 h (data not shown), a modest increment in the frequency of CD11c+MHC-II+CD103+ and CD11c+MHC-II+CD103+CD86+ DCs was found 16 h after VA feeding (data not shown), with higher expression of the co-stimulatory molecule CD86 (Fig. 2A and B). Hence, further studies were performed 16 h after the intake of VA (VA16).

Function of DCs in MLAV upon VA feeding

Mucosal DCs induce homing receptors during T lymphocyte activation in MLN [21,23,24]. Then, we studied the induction of the integrin α4β7 after 24 h of culture of mononuclear MLN cells, labeled with CFSE+ for tracking, with MLAV cells; as can be seen, in gated CFSE+ cells, the frequency of α4β7+ lymphocytes was higher when MLAV cells from VA16 mice were included in cultures (Fig. 3A and B). Accordingly, the VA16 group presented a significant increase in CD11c+CD103+RALDH+ cells compared with controls (Fig. 3C and D). To confirm that the result was due to RA derived from VA metabolism, a homing receptor induction assay was performed in presence of the retinoic acid receptor-β (RARβ) inhibitor LE135 at 10, 50 and 100 μM [25]. While a toxic effect was observed at 100 μM, 50 μM inhibited the induction of α4β7 and CCR9 receptors in cultures of MLAV and sorted CD4+ lymphocytes (data not

shown). Besides, LE135 inhibited the release of IL-10 but not IFNγ or TGFβ in these co-cultures (Fig. 3E).

Taking into account that in adult mice, $CD103^{+}RALDH^{+}$ DCs promote the generation of antigen-induced Foxp3+ Tregs [14], we evaluated if DCs from MLAV could induce Foxp3 expression. Interestingly, sorted CD4+ GFP-$Foxp3^{-}$ lymphocytes showed a significant increment in frequency and levels of Foxp3 (MFI) after 96 h of culture with cells from VA16 (Fig. 4A and B). Finally, as migratory RALDH+ DCs present gut-derived antigens promoting immune tolerance [10], we aimed to assess ex vivo the ability of DCs from MLAV to drive T lymphocyte proliferation without any further stimulation. We performed cultures of mononuclear MLN cells labeled with eFluor dye with a) cells and lymph of MLAV (Total MLAV content), b) MLAV lymph free of cells and c) MLAV cells, (Fig. 5A). In cultures where MLAV cells from VA16 mice were included, a reduced proliferation on CD4+ cells was observed; in fact, in the left column (Total MLAV content), the frequency of eFluor+ cells (R1 gate, 71.3 vs. 61.4%) was larger while the percentage of cells with diluted fluorescent dye (R2 gate, 7.75 vs. 18.1%) was lower. Similarly, in the right column (MLAV cells), VA mice showed higher frequency of R1 (68 vs. 50.5%) and lower proportion of R2 (8.65 vs. 27.4%) gated cells %. When the b) condition was tested (MLAV lymph) as shown in middle column, no differences between VA and control cultures were observed; in both groups, less than 15% of eFluor+ cell proliferated (R1 87.7 vs. 88.7; R2 3.17 vs. 3.56). Still, higher levels of TGFβ were found in supernatants of b) (Fig. 5B) and c) (Fig. 5C) cultures from VA mice, although in both conditions, the production of TGFβ was similar. In terms of homing receptors α4β7 and CCR9, both control and VA cells induced higher expression in R2

gate (Fig. 5A), as described for lymphocyte activation in intestinal inductive mucosal sites [21].

Discussion

The intestinal immune system is greatly influenced by retinoids in numerous aspects such as IgA class switching, Treg induction and imprinting of trafficking programs to activated B and T cells [1,2]. Still, many of the events related to VA feeding, remain to be determined. RA is an exogenous factor that directly communicates with nuclear receptors inside the cell, thereby providing the intestinal immune system with a powerful rheostat to sense the environment and fine-tune the immune response [4]. Intracellularly, RA can induce the transcription of multiple genes, influence translation or evoke epigenetic effects [3-5]. Still, the specific effects of RA and its receptors on the immune response are not always predictable, and depending on the dose, the cell type or the environmental conditions, the outcome can be very different. For instance, the available concentration of RA is a critical factor for its effects in T cells [4,5]: 1 nM RA is sufficient to promote immune cell subset differentiation (i.e. Th17) in the presence of splenic DCs and 10 nM RA represses Th17 cell differentiation and enhances Treg differentiation in vitro [26]; 1 μM instead, reduces IL-17 and IFNγ production possibly due to high toxicity [27]. Evaluation of VA activity in adequate animal models has provided a way to assess the potential immunostimulatory or immunoinhibitory activity of supplemental VA. In some animal studies, VA has been incorporated into the diet at a level higher than the usual [28]; in others, it was administered as an oral supplement, usually given more than once. These differences could be significant because, although a

single large dose of VA can quickly restore plasma retinol to a normal level and replenish liver reserves, it does not provide VA continuously for absorption from the intestine [29], which is key for the education of DCs [1,15]. Herein we used a dose scaled to young adult mouse weight which is comparable to the one used by the WHO in clinical trials to examine the effect of large-dose VA supplementation (50,000 IU/2.5 kg body weight) on infant survival [18]. Accordingly, a 21-h study was conducted in non-immunocompromised rats of normal VA status to evaluate early changes in lymphocyte populations and gene expression [30].

In our study, 8 h after a single dose of VA, an increment of CCL-21 was detected in MLAV. In fact, lymphatic endothelial cells participate actively in the shaping of both innate and adaptive immunity because they can express and signal through a variety of cytokines, adhesion receptors and molecules such as CCL-21 and D6 that modulate cell trafficking [31]. Recently it has been shown that the stimulation of CCR7 by its ligands triggers the activity of two signaling modules that regulate independently the chemotaxis or the migratory speed of DCs [32]. Therefore, CCL-21 could have been favoring the migratory speed and consequent accumulation on the way to lymph nodes. The increase in CCL-21 was concurrent with the peak of cellularity, possibly due to the recruitment of other subsets of naive, central memory and regulatory T cells bearing CCR7, beyond DCs [33]. On the other hand, the chemokine-scavenging decoy receptor D6 raised later on, possibly owing to its regulatory activity that may help to ensure that lymphatic conduits are uncongested facilitating DC trafficking [34,35]. Moreover, D6 could enhance the selective interaction of CCR7 with DCs ensuring that only mature DCs

migrate to lymph nodes [35]. Herein, the increment in CD11c+MHC-II+CD103+CD86+ DCs occurred later on in VA16 group in agreement with the concept that mature DCs generated in the presence of RA have increased migration toward the lymphoid chemokines CCL-19 and CCL-21 [36]. In our experimental condition, changes on chemokine expression could be triggered directly by VA as RA activity modifies chemokine expression in adipocytes [37] or indirectly by TGFβ released by the lymphatic endothelium [38] which is able to upregulate D6 expression between others [35].

Intestinal migratory CD103+ DCs carry antigens from the commensal microorganisms and produce high amounts of IL-10, TGFβ and RA that shape their properties [39]. Lamina propria DCs have a fast turnover, with a constitutive migration to MLN and replacement by DC progenitors that rapidly acquire a tolerogenic phenotype. Interestingly, this trait of CD103+ DCs does not seem to be an intrinsic property but appears to be dynamically regulated by the local microenvironment. This capacity is attributed to the exposure to local conditioning factors prior to the migration to MLN [40]. In this work, culture supernatants from MLAV VA16 h were rich in TGFβ; moreover, RALDH+ DCs isolated from MLAV were able to induce homing receptors and Foxp3 expression in CD4+ lymphocytes. It is important to note that the activation of lymphocytes is an early event in tolerance induction, which leads to downregulation of TCR [41] and limited proliferation [13,14]. Accordingly, a reduced proliferation was observed in co-cultures with MLAV cells from VA group, in agreement with a previous report that showed, using a transgenic reporter model, that RA inhibits IL-2 gene expression and T lymphocyte proliferation without affecting cell viability [42]. Besides,

lymphocytes activated in intestinal inductive mucosal sites upregulate the homing receptors CCR9 and α4β7 integrin [21].

Induction of oral tolerance relies on MLN and antigen carriage by DCs [11] and inside MLN, stromal cells constitute a stabilizing element for mucosal tolerance generation [39, 43]. How CD103+ DCs acquire their unique and imprinting properties has been largely evaluated and factors such as luminal bacteria and dietary constituents seem to provide key signals [8-10]. For instance, lymph is rich in peptides derived from local catabolism that are relevant to prime and regulate effector immune responses under non-infectious conditions [31]. To point out, difficulties associated with isolation have hampered the study of intestinal DCs. Herein, we were able to evaluate DCs isolated from afferent lymphatic vessels without extensive manipulation. DCs located in MLAV 16 h upon VA feeding had the phenotype and competence of tolerogenic DCs, defined by the ability to drive the efficient de novo generation of Foxp3+ Tregs within MLN [11,14,23]. It has been suggested that MLN represent a meeting point that collects mature DCs able to trigger a tolerogenic immune response [44]. Our results show that a single dose of vitamin A was able to generate a stream of signals that amplified the tolerogenic activity of DCs migrating to lymphoid tissue

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Conflict of interest

The authors have no conflict of interest to declare.

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Legends for Figures

Figure 1. Cellularity and chemoattractants after vitamin A feeding. Mice were feed 5 mg vitamin A (VA) or DMSO (control group) and 3, 8, 16 or 24 h later mesenteric afferent lymphatic vessels (MALV) were removed and incubated in RMPI medium ay 37°C. After 3 h lymphatic vessels were removed carefully and homogenized for Western blot; cells were recovered by gently centrifugation. A) Cellularity normalized by mg of tissue from different groups; B) Representaive Western blot of CCL-21 in homogenates from control and VA 3, 8 and 16 h groups. C) CCL-21 and D6 values from control and VA 8 and 16 h measured by Western blot in MALV homogenates were normalized with β-actin and expressed as Relative Units. A) n=6-10 mice/group; B) and C) Representaive experiments of two similar with n=3/group. Data are mean ± SEM. *p<0.05 and **p<0.01 vs. control.

Figure 2. Phenotype of dendritic cells after vitamin A feeding. Mice were feed 5 mg vitamin A (VA) or DMSO (control group) and 16 h later mesenteric afferent lymphatic vessels (MALV) were removed and incubated in RMPI medium. After 3 h lymphatic vessels were removed carefully and cells were recovered by gently centrifugation for flow cytometry. A) Representative histogram of CD86 expression in CD11c+CD103+ cells in VA 16 h and control groups. b) Mean fluorescence intensity (MFI) of CD86 expression in control and VA 16 h groups. Experiments were performed three times with n=3/ group. Data are mean ± SEM. ***p<0.001 vs. control.

Figure 3. Induction of α4β7 integrin by cells obtained 16 h after VA intake. Mice were feed 5 mg VA or DMSO (control group) and 16 h later mesenteric afferent lymphatic vessels (MALV) were removed and incubated in RMPI medium. After 3 h lymphatic vessels were removed carefully and cells were recovered by gently centrifugation and co-cultured with CFSE+ mononuclear cells from MLN for 24 h. A) Gate strategy and representative histograms of α4β7 expression in CD4+CFSE+ lymphocytes induced after culture with control and VA MLAV cells. B) Average frequency of CD4+CFSE+$\alpha 4\beta 7$+ in both groups. C) Representative dot plots for CD11c+CD103+ DCs and histograms of RALDH activity in CD11c+ CD103+ DCs of MLAV from control and VA groups. D) Average frequency of CD11c+CD103+RALDH+ cells in both groups. E) Cytokines (IL-10, IFNγ and TGFβ) evaluated by ELISA in culture supernatants of induction assays with or without 50 μM the retinoic acid receptor-β inhibitor LE135. Experiments were performed three times with n=3/ group. Data are mean ± SEM. *$p<0.05$ vs. control.

Figure 4. Assessment of Foxp3 induction 16 h after vitamin A feeding. Mice were feed 5 mg VA or DMSO (control group) and 16 h later mesenteric afferent lymphatic vessels (MALV) were removed and incubated in RMPI medium. After 3 h lymphatic vessels were removed carefully and cells were recovered by gently centrifugation and co-cultured with sorted CD4+Foxp3-GFP- cells at a 1:1 ratio for 96 h. A) Gate strategy and representative histograms of Foxp3 expression in control and VA16 groups. B) Average frequency of CD4+Foxp3+ cells and MFI of Foxp3 expression alter co-culture

with MLAV cells from both groups. Experiments were performed three times with n=3/ group. Data are mean ± SEM. *p<0.05 vs. control.

Figure 5. Proliferation and homing receptors expression after co-culture with mesenteric afferent lymphatic vessel content. Mice were feed 5 mg VA or DMSO (control group) and 16 h later mesenteric afferent lymphatic vessels (MALV) were removed and incubated in RMPI medium. After 3 h lymphatic vessels were removed carefully and a) total content of MLAV (cells and lymph); b) cell free lymph or c) MLAV cells from control or VA groups were co-cultured with MLN cells from untreated mice labeled with eFluor 670; after 72 h the proliferation was evaluated as eFluor dilution by flow cytometry as well as the expression of the α4β7 and CCR9 homing receptors. (B and C) TGF-β levels were assessed in supernatants of co-cultures under the b) and c) conditions. Data are mean ± SEM. Experiments were performed three times with n=3/ group. * p<0.05.

Figure 1. Novotny Nuñez et al

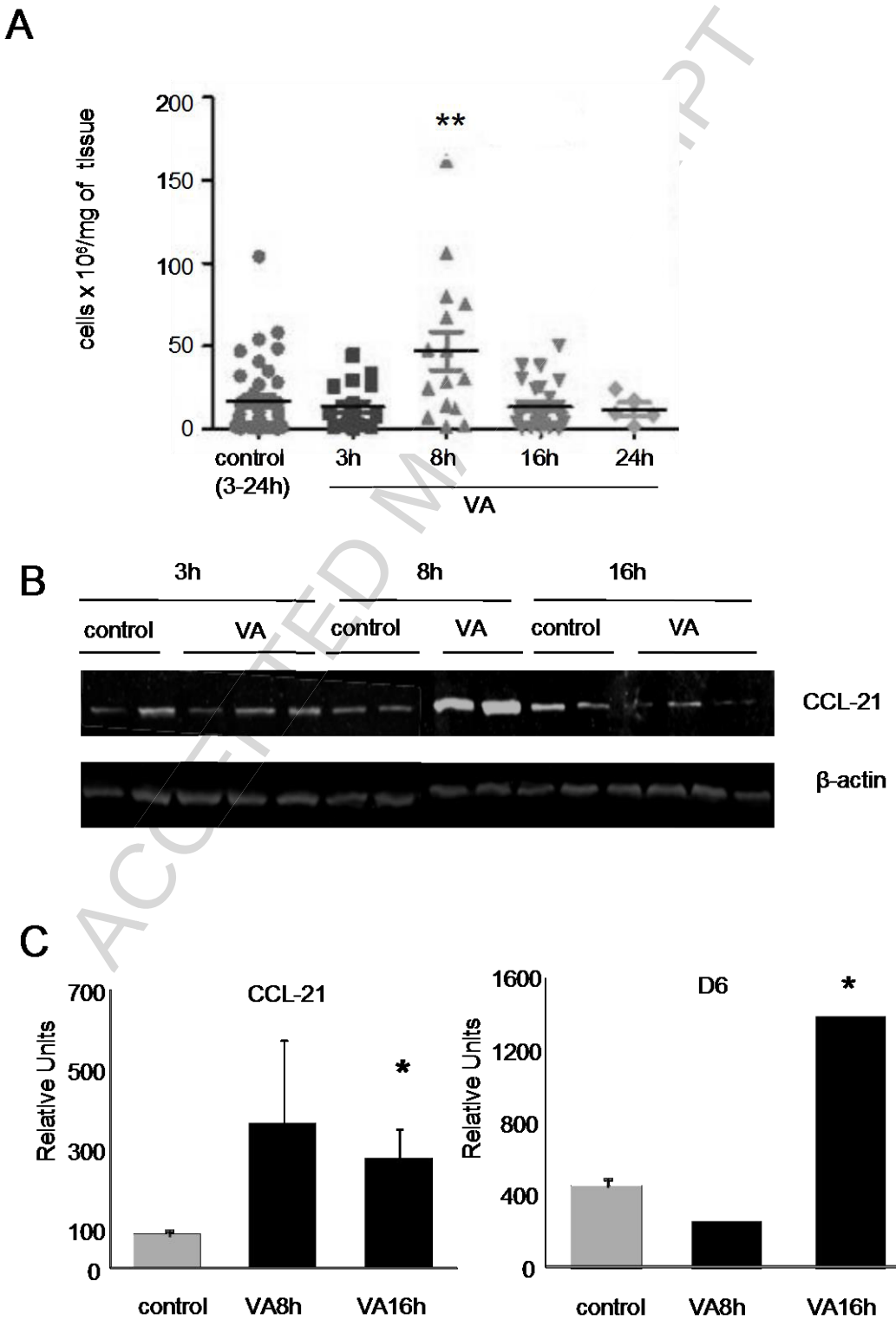


Figure 2. Novotny Nuñez et al

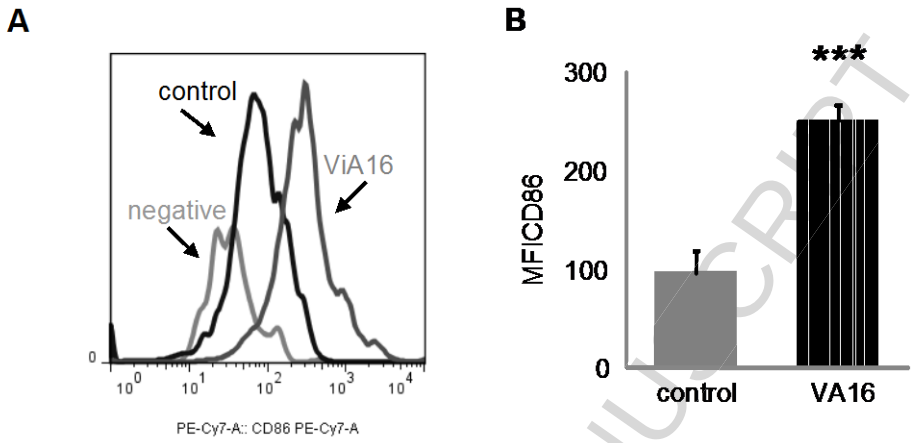


Figure 3. Novotny Nuñez et al

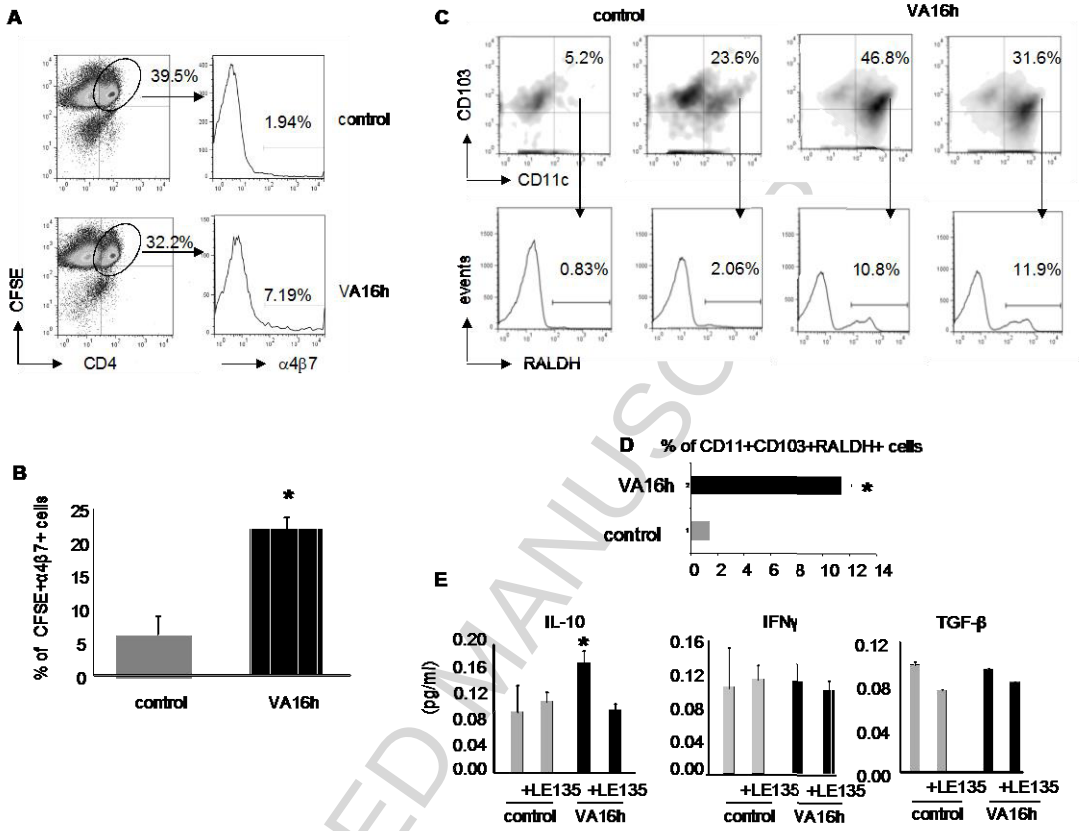


Figure 4 Novotny Nuñez et al.

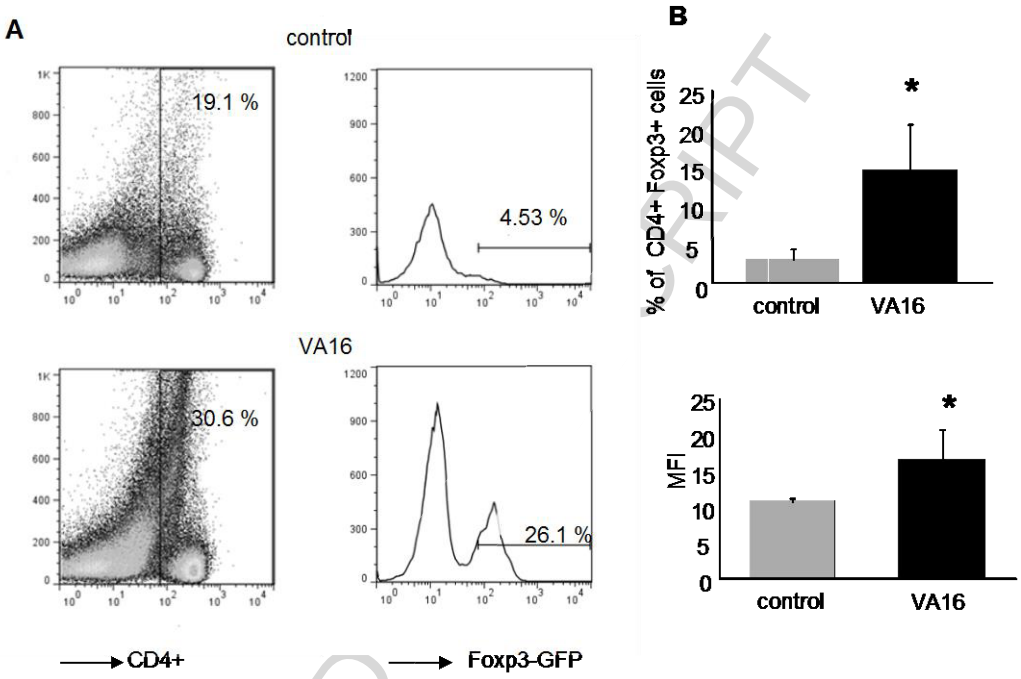
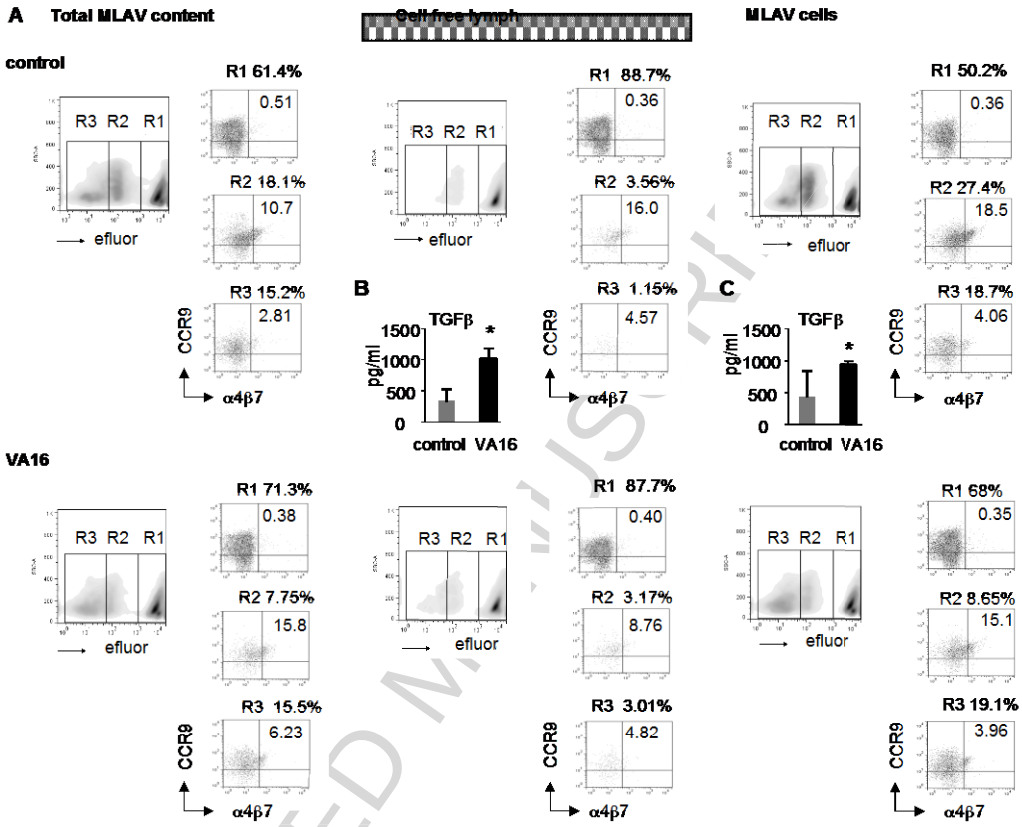


Figure 5 Novotny Nuñez et al



Novotny Nuñez et al Highlights

- Mice were fed a single dose of vitamin A
- At 3,8,16 and 24 h mesenteric afferent lymphatic vessels were isolated for studies
- The treatment amplified the tolerogenic activity of migrating DCs