Alitretinoin treatment reduces ILC2 numbers and influences lymphocyte homing properties in chronic hand eczema patients

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Retinoic acid (RA) is a vitamin A derivative known for its pleiotropic effects on cells of the immune system. Currently, systemic administration of 9-cis-RA, better known as alitretinoin, is used as a second-line treatment for chronic hand eczema, a common skin disease, when patients are refractory to topical treatment modalities. Although alitretinoin often alleviates the symptoms, its exact mechanism of action is currently unknown. Here, we show that during the course of treatment expression of α4β7 on T cells is increased, while expression of cutaneous lymphocyte antigen (CLA) is reduced on both T cells and innate lymphoid cells (ILCs). While migration patterns on B cells did not seem to change, antibody titers increased for all isotypes investigated. Also, treatment with alitretinoin caused a decrease in the numbers and percentage of ILC2 in the circulation, implying that systemic treatment with RA causes a shift in the migration pattern of hematopoietic cells, increased antibody production and affected ILC composition in peripheral blood.

**Abstract**

Retinoic acid (RA) is a vitamin A derivative known for its pleiotropic effects on cells of the immune system. Currently, systemic administration of 9-cis-RA, better known as alitretinoin, is used as a second-line treatment for chronic hand eczema, a common skin disease, when patients are refractory to topical treatment modalities. Although alitretinoin often alleviates the symptoms, its exact mechanism of action is currently unknown. Here, we show that during the course of treatment expression of α4β7 on T cells is increased, while expression of cutaneous lymphocyte antigen (CLA) is reduced on both T cells and innate lymphoid cells (ILCs). While migration patterns on B cells did not seem to change, antibody titers increased for all isotypes investigated. Also, treatment with alitretinoin caused a decrease in the numbers and percentage of ILC2 in the circulation, implying that systemic treatment with RA causes a shift in the migration pattern of hematopoietic cells, increased antibody production and affected ILC composition in peripheral blood.
INTRODUCTION

Retinoic acid (RA), a vitamin A metabolite, is a pleiotropic molecule that plays crucial roles during mammalian development in general, and the development of the immune system in particular1-3. In adults, it is known to be important for maintaining immune homeostasis, mainly in mucosal tissues4. Depending on the context in terms of other pro-inflammatory signals, cytokines and local and/or intracellular dose, RA signaling can be either pro- or anti-inflammatory5. It induces lymphocyte homing to the intestine and mucosal tissues via up-regulation of the homing molecules CCR9 and α4β7 on activated lymphocytes as well as IgA class-switching in intestinal B cells6,7. In vitro, RA inhibits interleukin (IL-) 4-dependent isotype switching to immunoglobulin (Ig)G1 in mice and IgE production by human B cells upon stimulation with CD408,9. On the flip side, production of both IgG and IgM and plasma cell differentiation were induced in presence of RA when B cells were stimulated via toll like receptor (TLR) 9 in vitro or following in vivo immunization with tetanus toxoid10,11 in humans and mice respectively. These discrepancies accentuate the context-dependent differences in the effects of RA. In mice T cells, RA promotes differentiation of FOXP3+ inducible regulatory T cells (iTregs), while inhibiting differentiation of Th17 cells12-14. Furthermore, the balance between IL-5 and IL-13 producing type II innate lymphoid cells (ILC2s) and IL-22 producing type III ILCs (ILC3s) in the intestine was shown to shift depending on the presence or absence of RA15. In both mouse and humans (in vivo and in vitro respectively), RA was shown to enhance skewing of ILCs towards ILC3s rather than ILC2s16,17, amongst others, by directly inducing transcription of the ILC3 lineage defining transcription factor, retinoic acid-related orphan receptor (ROR)yt18.

Interestingly, in recent years a pan-RA receptor (RAR) agonist, alitretinoin (Toctino), has entered the clinic, in particular for the treatment of chronic hand eczema (CHE). Hand eczema (HE) is a common disorder with a 1-year prevalence of up to 10% amongst adult in the general population19. Of all HE patients, 4-7% develops severe chronic hand eczema (CHE), which is defined as a long lasting, relapsing form of HE or HE that is unresponsive to emollients and topical corticosteroid treatment20, impairs quality of life and can result in economic loss due to occupational disability21,22. Initial treatment of HE is based on avoidance of eliciting factors, supplemented with topical corticosteroids and emollients. Patients who remain unresponsive can be treated with alitretinoin, which is proven effective, although its exact mechanism of action is still unknown. Considering the pleiotropic effects of RA on leukocytes, a number of possibilities arise, ranging from altered cytokine and specific Ig isotype production to increased presence of Tregs and/or altered migration pattern.
of cells. In a previous study performed in peripheral blood (PB) and skin samples of CHE patients, it was shown that during the course of treatment with alitretinoin, levels of plasmablast and circulating IgE as well as activated CD40L+ IL-17 producing CD4+ T cells were reduced, while in the skin total CD4+ T cell counts, and more specifically Treg counts were increased.23.

**Figure 1: PBMC composition of CHE patients during alitretinoin treatment.** PBMCs were analyzed by flow cytometry upon isolation. (A) gating of the different PBMC populations. A gate was set on lymphocytes, followed by gating on single cells and live cells. For T cells a gate was set on CD3+ cells followed by gating on lineage negative cells. For B cells a gate was set on CD19+ cells followed by gating on lineage negative cells. For gating on ILCs, CD3-CD19- cells were gated on, followed by gating on CD127+ cells. The lineage cocktail contained monoclonal antibodies against CD11c, CD14, CD34 and CD94. Plots representative of multiple individual stainings. (B) PBMC count in CHE patients prior to treatment (t = 0) compared to healthy donors (both groups n = 6). (C) PBMC count, percentage of lymphocytes (PBL) as part of total PBMCs and absolute PBL counts per patient in time. (D) Percentage of total lymphocytes and absolute counts of T cells (CD3+ Lin-), B cells (CD19+ Lin-) and ILCs (CD3 CD19 Lin CD127+) per patient in time.
However this study focused mainly on B- and T cell effector function, while ILCs were disregarded, as was leukocyte homing. This might be important as ILC derived cytokines were shown to play a role in the skin in the context of atopic dermatitis and psoriasis\textsuperscript{24,26}. Additionally, as RA triggers gut homing of lymphocytes\textsuperscript{6,27} while reducing skin-homing\textsuperscript{28,29}, altered leukocyte homing can be an important working mechanism of alitretinoin.

Here, we report a study on six patients, in which we assessed the composition of the lymphocyte population in PB, as well as for a number of homing molecules, during treatment with alitretinoin. We show that expression of the skin homing molecule CLA drops on B- and T cells as well as on ILCs, while the expression of the gut homing molecule α4β7 is induced. Unexpectedly and in contradiction to a previous report\textsuperscript{23}, IgE levels in the plasma of patients were increased, while the same was seen for IgA, IgG1, 2, 3 and 4. Also, the ILC2 population was decreased in these patients during the course of treatment showing that alitretinoin influenced both lymphocyte migration patterns and ILC differentiation.

**RESULTS**

**Alitretinoin treatment does not alter lymphocyte composition in PB**

To assess the effects of alitretinoin treatment on the composition of the PB lymphocyte population (B cells, T cells and ILCs), we analyzed patient PBMCs by flow cytometry (for gating, see Fig. 1A). No difference was seen between total PBMC count of patients at the start of treatment (t = 0) as compared to healthy donors (Fig. 1B). Although PBMC counts fluctuated during treatment, no clear trends were observed regarding the total counts of PBMCs or the percentage or number of PB lymphocytes (PBL, Fig. 1C). Further assessment of PBL composition revealed no clear differences regarding the percentage and number of circulating T and B lymphocytes (Fig. 1D). A slight decrease in the percentage of ILCs was seen in 5 of the 6 patients, while this was not reflected by a change in the absolute size of the population in peripheral blood (Fig. 1D).

**Alitretinoin caused altered homing profile in adaptive lymphocytes and increased Ig titers**

As RA is known to induce the gut homing receptor α4β7, thereby allowing lymphocyte homing to mucosal tissues\textsuperscript{6}, we analyzed expression of the secondary lymphoid organ (SLO) homing marker CD62L, the mucosal homing marker α4β7 and the skin homing marker CLA on circulating CD3⁺ T cells (Fig. 2). The portion of CD62L expressing T cells was reduced, in all but one patient. As expected, relatively more T cells expressed α4β7 in 4 of the
5 patients analyzed (for one patient no t = 0 value for this marker could be included). Similar as for CD62L, CLA expressing T cells were reduced during the course of treatment. Interestingly, one patient showed an initial increase in the presence of CLA expressing T cells at the start of treatment, although this was reduced at later time points (Fig. 2A). As CHE is at least partially typified by a type-II immune response²¹, we assessed the expression of the chemokine receptor of Th2 cells (CRTH2), which is expressed on Th2 cells and ILC2²⁰,²¹. However, no uniform effect of treatment for the expression of CRTH2 on T cells could be observed, as the levels varied in the different patients (Fig. 2B). In mice, RA has also been shown to play a role in skewing T cells to a FOXP3⁺ Treg phenotype, while inhibiting Th17 differentiation both in vitro and in vivo¹²-¹⁴. For this reason, we analyzed patient PBMCs for transcription of FOXP3 by rtPCR. In all patients we observed an increase in FOXP3 expression during the course of treatment. At the start of the treatment FOXP3 levels were similar between healthy donors (HD) and patients, suggesting that RA treatment specifically results in FOXP3 induction (Fig. 2C). On B cells, no difference was seen regarding relative expression of CD62L.

Figure 2: Phenotypic analysis of CD3⁺ T cells in the PB of CHE patients during alitretinoin treatment. PBMCs of CHE patients were analyzed using flow cytometry for the expression of homing molecules on CD3⁺ T cells. (A) Percentage of T cells expressing CD62L, α4β7 and CLA per patient in time, relative to t = 0. (B) Percentage of T cells expressing CRTH2 per patient in time relative to t = 0. (A, B) Percentages are represented as part of the total cells in the T cell gate (CD3⁺ Lin). (C) Relative expression of FOXP3 as determined by rtPCR, in time, relative to t = 0 (left) and relative expression of FOXP3 in the PBMCs of patients at the start of treatment (t = 0) as compared to healthy controls (HD, n = 3) (right).
and α4β7, while CLA expression was reduced in all patients (Fig. 3A). As expected based on previously published reports, an increase in α4β7+ B cells was found in 4 out of 6 patients during the course of treatment. No changes were observed in the expression of CD62L on B cells, while a consequent reduction was seen in the size of the CLA+ B cell population. To further assess B cell function and as RA was shown to influence class switching and plasma cell differentiation of B cells in SLOs, we analyzed plasma levels of the different immunoglobulin isotypes (IgA, IgE, IgG1-4). As expected based on previous reports, IgE levels at the start of treatment (t = 0) were elevated in

![Graphs showing CD62L, α4β7, and CLA expression over time.](image)

![Graphs showing IgA, IgE, IgG1, IgG2, IgG3, and IgG4 levels in plasma at start of treatment compared to healthy controls.](image)

**Figure 3. Phenotypic analysis of PB CD19+ B cells and assessment of plasma Ig in CHE patients during alitretinoin treatment** (A) Percentage of B cells expressing CD62L, α4β7 and CLA per patient in time relative to t = 0. Percentages are represented as part of the total cells in the B cell gate (CD19+ Lin). (B) IgA, IgE and IgG1, 2, 3 and 4 titers in plasma of CHE patients at start of treatment (n = 6) as compared to healthy controls (HD, n = 2). (C) IgA, IgE and IgG1, 2, 3 and 4 titers in plasma of CHE patients during the course of alitretinoin treatment per patient in time, relative to t = 0.
patients as compared to HD, while a similar trend was observed for the other isotypes (Fig. 3B). In line with a role for RA in plasma cell differentiation, Ig production in general\textsuperscript{10,11} and IgA class switching in particular\textsuperscript{7}, we observed a consistent increase in IgA, IgG1, 2, 3 and 4 titers during treatment (Fig. 3C). Unexpectedly and in contrast with a recent report\textsuperscript{23}, a similar trend was seen also for IgE (Fig 3C).

These data show that alitretinoin treatment causes a shift in the expression of homing receptors, allowing the migration of circulating adaptive lymphocytes away from the skin and towards the MALT, and an increase in plasma Ig titers.

\textit{Alitretinoin treatment influences the composition of the PB ILC population}

ILCs are mainly known to be tissue resident effector cells, yet a population of ILCs is present in the circulation\textsuperscript{32}. While they only form a very small population, the ILCs in PB have been shown to change their phenotype in response to ongoing inflammation in a number of inflammatory diseases including skin conditions\textsuperscript{30,33-36}. Hence, we set out to investigate whether the ILC population showed changes during treatment with alitretinoin. Although no changes were apparent in expression of either CD62L or α4β7, alitretinoin treatment caused a reduction in the expression of CLA by ILCs, as was seen in T and B lymphocytes. Of note, the patient that showed an initial increase and later decrease in CLA expressing T cells, showed the same trend for ILCs (Fig. 2A and 4A). ILCs can be subdivided into three subsets based on the expression of transcription factors and production of effector cytokines (ILC1, 2 and 3)\textsuperscript{37}. In humans, ILC1, 2 and 3 can be distinguished using the membrane markers cKit and CRTH2 (cKit\textsuperscript{−}/CRTH2\textsuperscript{−}, cKit\textsuperscript{+}/CRTH2\textsuperscript{−} and cKit\textsuperscript{+}/CRTH2\textsuperscript{−} for ILC1, 2 and 3, respectively)\textsuperscript{32}. In all patients, the three ILC populations were readily detected in PB (for gating, see Fig. 4B). A decrease was seen in the percentage of ILC1s and ILC2s in 5 and 4 of the 6 patients during the course of treatment, respectively, while ILC3 percentages were increased in 5 out of 6 patients (Fig. 4C). Further assessment of the different ILC populations in the blood revealed that alitretinoin treatment did not influence the percentage of CD62L expressing ILC 1 and 3, (Fig. 5A-C). Furthermore, while expression of α4β7 is induced on T and B cells by RA, alitretinoin treatment did not affect the percentage of α4β7\textsuperscript{−} ILC1, while α4β7\textsuperscript{−} ILC2 and 3 were reduced in all but two patients during treatment, one of which showed an initial increase before returning to baseline levels (Fig. 5A and C). Unlike what we observed for T cells and the total population of ILCs, no clear effects were seen in terms of CLA expressing ILC1s, 2s and 3s (Fig. 5). Finally, expression of the ILC3 activation marker NKp44 seemed to be slightly reduced in patients during the course of treatment, with one exception in which NKp44\textsuperscript{+} ILC3...
were increased at later time points after an initial decrease. Of note, NKp44+ ILC3s are nearly absent in the circulation of healthy donors \(^{32,33}\) (data not shown) and were also undetectable in 2 out of the 6 patients both at the start of treatment or at any of the time points at which PB of the patients was analyzed.

Collectively, alitretinoin treatment seems to reduce the circulating population of ILCs and more specifically ILC2s, while the ILC3 population increases in size. While CLA expressing ILCs are reduced in the circulation, alitretinoin treatment seems to also reduce \(\alpha 4\beta 7\) expression on ILC2 and 3.

**Figure 4: Expression of homing markers and composition of the ILC population during alitretinoin treatment.** PBMCs of CHE patients were analyzed using flow cytometry for the expression of homing molecules. (A) Percentage of ILCs expressing CD62L, \(\alpha 4\beta 7\) and CLA per patient in time, relative to \(t = 0\). Percentages are represented as part of the total cells in the ILC gate (CD3-CD19-Lin-CD127\(^+\)). (B) Gating for the different ILC populations (ILC1, 2 and 3) in human peripheral blood. Depicted are the cells in the ILC gate (CD3 CD19 Lin-CD127\(^+\)). Plot representative for multiple individual stainings performed. (C) Percentages of the total ILC gate (CD3 CD19 Lin-CD127\(^+\)) of ILC1 (cKit\(^-\)CRTH2\(^-\)), ILC2 (cKit\(^+\)CRTH2\(^-\)) and ILC3 (cKit\(^+\)CRTH2\(^-\)) per patient in time, relative to \(t = 0\).
Retinoids are used in the clinic for the treatment of a number of conditions, amongst which CHE. Although alitretinoin alleviates symptoms of CHE, the exact mechanisms by which it works are not clear. Considering the pleiotropic effects of RA, a number of mechanisms, ranging from cell differentiation, Ig class-switching and cell migration possibly cause the clinical improvements seen in patients. Here we show that in CHE patients treated with alitretinoin, expression of CLA decreased, implying a redirection of lymphocyte migration away from the skin towards mucosal tissues. These observations seem to be in line with the reported role of RA in mouse studies where it is described to induce migration towards the mucosa, via up-regulation of α4β7 and CCR9⁶⁻⁷.

**Figure 5: Expression of homing markers on the different ILC subsets during alitretinoin treatment.** PBMCs of CHE patients were analyzed using flow cytometry. (A) Percentage of ILC1s expressing CD62L, α4β7 and CLA per patient in time, relative to t = 0. Percentages are represented as part of the total cells in the ILC1 gate (CD3 CD19 Lin CD127⁻ cKit CRTH2⁻). (B) Percentage of ILC2s expressing CD62L, α4β7 and CLA per patient in time, relative to t = 0. Percentages are represented as part of the total cells in the ILC2 gate (CD3 CD19 Lin CD127⁺ cKit⁺ CRTH2⁻). (C) Percentage of ILC3s expressing CD62L, α4β7, CLA and NKP44 per patient in time, relative to t = 0. Percentages are represented as part of the total cells in the ILC3 gate (CD3 CD19 Lin CD127⁺ cKit⁺ CRTH2⁻).

**DISCUSSION**

Retinoids are used in the clinic for the treatment of a number of conditions, amongst which CHE. Although alitretinoin alleviates symptoms of CHE, the exact mechanisms by which it works are not clear. Considering the pleiotropic effects of RA, a number of mechanisms, ranging from cell differentiation, Ig class-switching and cell migration possibly cause the clinical improvements seen in patients. Here we show that in CHE patients treated with alitretinoin in the course of treatment T cell expression of α4β7 was increased while expression of CLA decreased, implying a redirection of lymphocyte migration away from the skin towards mucosal tissues. These observations seem to be in line with the reported role of RA in mouse studies where it is described to induce migration towards the mucosa, via up-regulation of α4β7 and CCR9⁶⁻⁷.
In line with these reports more αβ7+ B cells were present in patient PB upon treatment with alitretinoin, while in all but one patient CLA expression was reduced. Further analysis of B cell function by means of plasma Ig titers revealed an increase of all isotypes investigated, while only IgE levels were increased compared to healthy individuals at the beginning of treatment. These results suggest that elevated RA signaling in these patients did not have specific effects on class switching to IgA as reported before for mouse B cells but rather a more general stimulation of antibody production. This is in line with reports of RA increasing Ig production and plasma cell differentiation in combination with TLR9 triggering or tetanus toxoid immunization, in human and mouse B cell respectively. The increase in IgA levels we observed is to be expected based on previous work in mice. However, the increase we measured in IgE and IgG1 levels seems at odds with a previous study in CHE patients that reported a decline in serum IgE, IgA and IgM levels and no changes in total IgG levels in patients at the end of alitretinoin treatment and reports showing that IL-4 dependent class switching to IgG1 and IgE was inhibited by RA. Importantly, the patient groups described here received additional medications during the course of alitretinoin treatment, which was not the case in the other report. Interestingly, a recent report shows that treatment of atopic dermatitis with topical corticosteroids leads to an increase in serum allergen specific IgE levels while hydrocortisone leads to T-cell dependent and independent IgE production human B cells in vitro. Importantly, considering the affected area (hands and feet only) of the patients described by us, the dosage of topical corticosteroids used is relatively low. Therefore, systemic effects of topical application seem unlikely although they cannot be ruled out. Whether this indeed is the cause of the differences observed here is a matter for future investigation.

When assessing the ILC population, it appeared that while no increase was seen in αβ7 expression, CLA expressing ILCs were diminished during treatment, implying a reduced ability to migrate to the skin. Also, while ILC1s and 2s were decreased, ILC3s were increased both in percentages and in absolute numbers (Fig. 4C). This suggests that rather than only change in migration properties, treatment may also result in a shift in differentiation of ILCs. RA has been reported to induce ILC migration to the intestine, to further regulate the balance between ILC2s and ILC3s in the intestine, and to promote in vitro differentiation of ILC3s in humans. While we did not observe an up regulation of αβ7, we did see an increase in the population of ILC3s at the expense of ILC2s during the course of treatment. As such, our results seem to agree with previously published reports that vitamin A and/or RA affect the differentiation of ILCs. Here, we propose a model in which treatment with alitretinoin causes an alteration in expression of homing receptors on T- and B cells and ILCs.
It seems, at least for the T- and B cells, that migration to the intestines and mucosal surfaces is induced, while skin homing is reduced, by means of up regulation of α4β7 and down regulation of CLA, respectively. This might be one of the mechanisms by which alitretinoin exerts its function, as potentially auto-reactive or allergen specific adaptive lymphocytes would not encounter their cognate skin-derived antigen in the intestines or the MALT. Subsequently, no overt inflammation would take place in the intestines upon migration of the cells.

The increase of ILC3s in PB at the cost of circulating ILC2s with skin homing properties raises the question whether ILC2s are involved in the pathophysiology of CHE. Considering the fact that ILC2s are involved in a number of diseases associated with type-II immunity in general and atopic skin conditions in particular24-26,30,43-46 these cells can be at least considered as suspects. ILC2s proliferate strongly upon stimulation with IL-25 and IL-3347 and migrate to inflamed tissues upon challenge48. Both IL-25 and IL-33 are expressed by keratinocytes and the production of these cytokines is enhanced in atopic dermatitis24. Interestingly, RA was shown to suppress activation induced IL-33 production by human keratinocytes in vitro49, which could also be one of the mechanisms of action of alitretinoin in CHE patients. Although ILCs are considered to be tissue resident effector cells48, the shift in expression of homing receptors following alitretinoin treatment implies that the cells can at least migrate from the blood to the tissues and specifically be recruited into tissues in case of inflammation. In parallel to T cells, ILC activation could take place in SLOs draining an inflamed area. Migration of ILCs from draining lymph nodes to tissues has been shown recently27,50 and along with reports on ILC plasticity17,51 and our data on a shift in expression of homing receptors on circulating ILCs it raises the possibility that a population of naïve-like ILCs exists. This population can be activated in SLOs and subsequently be recruited to inflamed tissues in case of an inflammation, in parallel to activation and subsequent directed migration of naïve T cells. Interestingly, at least in mice, RA has been shown to play a role in recruitment of ILCs to the intestine from mesenteric lymph nodes, although notably this was not true for ILC2s27.

In recent years, administration of retinoids has been shown to alleviate symptoms of CHE in patients which were refractory for topical corticosteroid treatment. We show here that alitretinoin treatment results in an altered expression of homing receptors on lymphocytes and possibly also affecting the differentiation of lymphocytes. In theory, these effects could provide an at least partial explanation for the beneficial effect of this treatment in CHE patients. Future research will have to assess whether the administered retinoids also directly influence the skin epithelium, thereby
decreasing inflammation, as was shown in vitro for human keratinocytes. Further dissecting the biological mechanism of the effect of alitretinoin will eventually lead to a better stratification of patients which may respond to the treatment. These insights will allow for a more personalized and presumably successful approach in the treatment of CHE in the future.

**Materials and Methods**

**Patient materials**

Patients diagnosed as suffering from corticosteroid refractory chronic hand eczema and subsequently started treatment with alitretinoin (9-cis-retinoic acid, Toctino, GSK, Brentford, Middlesex, UK) were recruited upon signing informed consent at the department of Dermatology, VU University Medical Center, Amsterdam, the Netherlands. Blood was drawn for routine laboratory checkups at intervals of one to two months. Patients were treated with varying dosages of 10-30mg, based on diagnosis, co-morbidities and clinical parameters (see Table 1). The study was approved by the Medical Ethical Committee of the VU University Medical Center, Amsterdam, the Netherlands in line with the declaration of Helsinki and according to Dutch law.

**PBMC isolation**

Plasma was separated from the cellular fraction of blood by centrifugation. PB mononuclear cells (PBMCs) were subsequently isolated by gradient centrifugation on lymphoprep (d = 1.077, Fresenius Kabi Norge AS). The PBMCs were subsequently centrifuged and washed twice in cold PBS (B. Braun Melsungen AG) supplemented with 10% (v/v) donor plasma.

**Table 1: Characteristics of the patients enrolled in this study**

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Flow cytometry
For FACS analysis, cells were stained with the following antibodies: CD14 (61D3), CD34 (4H11), CD94 (DX22, all FITC labeled), CD127-APC (eBioRDR5), CD117-PeCy7 (104D2, all eBioscience, San Diego, CA, USA), CRTH2-PE-CF594 (BM16), NKp44 (CD336)-PE (p44-8.1), CLA-BV421 (HECA-452), CD62L-BV421 (DREG56, all BD Bioscience, Mountain View, CA, USA), CD3-BV711 (OKT3), CD19-BV785 (HIB19, both Biolegend, San Diego, CA, USA), CD62L-biotin (145/15, Miltenyi Biotec, Bergisch Gladbach, Germany). The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: α4-β7 monoclonal antibody (Act-1, cat#11718) from Dr. A. A. Ansari. The antibody was biotin labeled in house using EZ-Link™ NHS Biotin (Thermo Fischer Scientific, Waltham, MA, USA) according to manufacturers’ protocol. Biotin labeling was detected using Streptavidin-eF605NC (eBioscience, San Diego, CA, USA). Exclusion of dead cells was done using staining with Fixable Viability Dye eFluor® 780 (ebioscience, San Diego, CA, USA). Analysis was performed using a LSR-Fortessa X20 (BD Bioscience, Mountain View, CA, USA) flow cytometer. Further analysis was done using Flowjo Software version 10 for Microsoft (Tree Star, San Carlos, CA). Gating was done based on Fluorescence Minus One (FMO) controls.

Enzyme linked immunosorbent assay (ELISA)
Patient plasma was isolated by centrifugation of whole blood and stored at -80ºC. ELISA for IgG1, IgG2, IgG3, IgG4, IgE and IgA on plasma samples were performed using the respective ELISA-Gold kits (eBioscience, San Diego, CA, USA) according to manufacturer’s protocol.

RNA isolation and real-time PCR
Cells were homogenized in Trizol reagent (Thermo Fischer Scientific, Waltham, MA, USA). RNA was isolated by centrifugation in Phase Lock Gel-heavy 1.5mL tubes (5 Prime GmbH, Hamburg, Germany), subsequently precipitated in 2-propanol (Sigma Aldrich, St. Louis, MO, USA) and finally washed with 75% EtOH (Cargill, Schiphol, the Netherlands). cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, Canada) according to manufacturer’s protocol. Real-time (rt)PCR was performed using SYBR Green mastermix (Foster City, CA, USA) on StepOne real-time PCR systems from Applied Biosystems (Bleiswijk, The Netherlands). Used primer sequences are listed in table 2.

Table 2: primer sequences used for rtPCR

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References


A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. 


Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. 


Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. 


Retinoic Acid Differentially Regulates the Migration of Innate Lymphoid Cell Subsets to the Gut. 


Vitamins A and D are potent inhibitors of cutaneous lymphocyte-associated antigen expression. 


CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. 


Activated innate lymphoid cells are associated with a reduced susceptibility to graft-versus-host disease. 


Composition of innate lymphoid cell subsets in the human skin: enrichment of NCR(+) ILC3 in lesional skin and blood of psoriasis patients. 


Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. 


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Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. 


Hydrocortisone and IL-4 induce IgE isotype switching in human B cells. 


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Glucocorticoids increase the synthesis of immunoglobulin E by interleukin 4-stimulated human lymphocytes. 


Effect of hydrocortisone on spontaneous IgE and IgG4 production in atopic patients. 


The role of T cells and the effect of hydrocortisone on interleukin-4-induced IgE synthesis by non-T cells. 


Hydrocortisone and IL-4 induce IgE isotype switching in human B cells. 


Potential preventive effects of proactive therapy on sensitization in moderate to severe childhood atopic dermatitis: A randomized, investigator-blinded, controlled study. 


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Potential preventive effects of proactive therapy on sensitization in moderate to severe childhood atopic dermatitis: A randomized, investigator-blinded, controlled study. 

Oral retinoic acid influences peripheral blood lymphocytes in CHE patients


