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MCAM-expressing CD4⁺ T cells in Peripheral Blood Secrete IL-17A and are Significantly Elevated in Inflammatory Autoimmune Diseases

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Abstract

Th17 cells are a subset of CD4⁺ T cells characterized by production of IL-17 and are known to be key participants in inflammatory reactions and various autoimmune diseases. In this study we found that a subset of human CD4⁺ T cells expressing MCAM (CD146) have higher mRNA levels of *RORC2*, *IL-23R*, *IL-26*, *IL-22*, *IL-17A*, but not *IFN-γ*, compared to CD4⁺ T cell not expressing CD146. Upon TCR stimulation with CD3/CD28, CD4⁺CD146⁺ T cells secrete significantly more IL-17A, IL-6, and IL-8 than do CD4⁺CD146⁻ T cells. Low frequencies of CD4⁺CD146⁺ T cells are found in the circulation of healthy adults, but the frequency of these cells is significantly increased in the circulation of patients with inflammatory autoimmune diseases including Behcet's, sarcoidosis and Crohn's disease. Patterns of gene expression and cytokine secretion in these cells are similar in healthy and disease groups. In Crohn's disease, the increase in CD4⁺CD146⁺ cells in the circulation correlates with disease severity scores. These data indicate that expression of CD146 on CD4⁺ T cells identifies a population of committed human Th17 cells. It is likely the expression of CD146, an endothelial adhesion molecule, facilitates adherence and migration of Th17 cells through the endothelium to sites of inflammation.

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7. Contributions

P.D. prepared the original draft of the manuscript and was involved in all aspects of the experimental design and research, including execution of all experiments, analysis, as well as interpretation of results and preparation of the manuscript and figures. A.B. was involved in data analysis, interpretation of results and preparation of the manuscript and figures. L.W., N.S. and M.Y. provided reagents and clinical specimens for some experiments. W.S. and R.B.N. were involved in the experimental design and preparation of the manuscript and figures. J.P.M. was involved in all aspects of study design, data analysis, interpretation of results and preparation of the manuscript and figures. All authors discussed the results presented in the manuscript.

Declaration of conflicts of interest

None to declare

Keywords

CD146/MCAM; Th17; inflammation

1. Introduction

Over the past several decades, CD4⁺ T lymphocytes have been assigned to subsets based on patterns of cytokine secretion and associated function. The initial subsets described were Th1 and Th2 cells. Th1 cells were characterized by production of interferon-gamma (IFN- γ) and interleukin-12 (IL-12), and were recognized as playing an important role in protection against intracellular pathogens. Th2 cells were found to produce interleukins-4, -5, -10 and -13 and were found to be involved in responses to extracellular parasites [1, 2]. Subsequently, T regulatory cells (Tregs) were identified as a subset of CD4⁺ T cells capable of suppressing immune responses and thus maintaining self-tolerance and immune homeostasis [3, 4]. A number of additional CD4⁺ T cell subsets have putatively been identified, including Th3 (TGF- β -producing) [5, 6], Tr1 (IL-10-producing) [7], Th9 (IL-9-producing) [8], T follicular helper (Tfh, IL-4-producing) [9] and Th22 (IL-22-producing) [10].

One important subset of CD4⁺ T cells described in the past decade is the Th17 lymphocyte population. These T cells are characterized by their production of IL-17A, IL-17F, IL-21, IL-22 and IL-26. Human Th17 cells have also been reported to express CCR6, CCR4, IL-23R, and CD161 [11]. Th17 differentiation involves IL-1 β , IL-6 and IL-23, and possibly TGF- β , although the role of the latter in humans is debatable [12]. The transcription factors RUNX1 and RORC2 are expressed in human Th17 and the transcription factor aryl hydrocarbon receptor (AHR) is known to enhance Th17 cell responses [12, 13]. Th17 cells are regarded as major cellular participants in tissue inflammation and play an important role in recruitment of other leukocytes to sites of inflammation. In addition to leading to up-regulation of chemokine production by local tissues through release of their cytokines, Th17 cells also produce a number of chemokines, such as CXCL-8 and CXCL-13. These chemokines actively recruit neutrophils and B cells to sites of inflammation. In both mouse models and in humans, Th17 cells have been widely implicated in the pathogenesis of inflammatory autoimmune diseases such as multiple sclerosis, Crohn's disease, and autoimmune uveitis, and have been found in elevated levels in the peripheral circulation of patients with these diseases [14-17]. Increasing effort is being directed toward developing therapies that can address the inflammation arising from Th17 function. Such therapies currently under investigation include tocilizumab (anti-IL-6R) and ustekinumab (anti-IL-12/IL-23p40).

MCAM (melanoma cell adhesion molecule, CD146) is an adhesion molecule found primarily on endothelial cells and which plays a role in the homotypic binding of endothelial cells to one another [18]. In previous studies we identified a small subset (roughly 2-3% in healthy donors) of T cells in human peripheral blood that express MCAM, but whose function remains largely unknown. These cells have an effector memory phenotype, showing expression of CD45RO and lack of CCR7 expression [19, 20]. Patients with arthritic diseases have been shown to have significantly higher frequencies of CD146⁺ T cells, both in the peripheral circulation and in synovial effusions, than in healthy individuals [21]. The current work was performed to further understand the nature of CD146-expressing T cells and to determine their role in a variety of inflammatory autoimmune diseases. A previous study found that clones of Th17 cells isolated from patients with multiple sclerosis were more likely to express MCAM than were Th1 clones generated from the same patients [15], but these data were not demonstrated in freshly isolated cells from the circulation. We

thus sought to determine the relationship between CD146 expression on CD4⁺ T cells and Th17 function in the peripheral circulation from both healthy individuals as well as patients with inflammatory autoimmune diseases.

2. Material and methods

2.1 Flow cytometry for immunophenotyping

Venous blood was collected in sodium heparin vacutainers (Becton Dickinson (BD), San Jose, CA) from patients attending the clinics of NIAID (Crohn's (n=20)) (protocol-82-1-0183), NEI (Behcet's (n=20) sarcoidosis (n=24)) (protocol-08-ei-0169) as well as from healthy volunteers (n=73) registered with NHLBI (protocol-07-H-0113). Erythrocytes were first lysed from the whole blood using ACK Lysing buffer (Quality Biologicals, Gaithersburg, MD), then cells were washed, counted, and incubated with fluorochrome-conjugated antibodies. The following antibodies were obtained from BD: CD3, CD4, CD8, CD19, CCR2, CCR4, CCR6, CCR7, CD33, CD14, CD45, CD45RO, CD161, CD146 (Clone P1H12). After incubating the cells with antibody for 30 minutes, the cells were washed three times with PBS and were acquired on a LSRII™ flow cytometer equipped with 405, 488, 532 and 638 laser lines using DIVA™ 6.1.2 software (Becton Dickinson, San Jose, CA). For certain experiments, PBMCs were subjected to PMA/Ionomycin stimulation for 16 hours, and treated with brefeldin-A 4h prior to staining for intracellular cytokines. Intracellular cytokine staining for IL-17A (Biolegend) and IFN- γ (BD) was performed using BD Cytofix/Cytoperm buffer as per the manufacturer's guidelines. Data were analyzed with FlowJo™ software version 9.3.1 (Treestar, San Carlos, CA).

2.2 Cell sorting

For sorting of cells, samples of peripheral blood were subject to lysis of erythrocytes using ACK Lysing buffer, then cells were incubated for 30 minutes at room temperature in the dark with antibodies appropriately diluted in sterile PBS supplemented with 10%FCS. Stained cells were sorted into CD4⁺ CD45RO⁺CD146⁺ and CD4⁺CD45RO⁺CD146⁻ subpopulations. Fluorescence minus one (FMO) controls were used to established positive staining for CD146. Sorting was performed on a FACSARIA SORP™ sorter equipped with 405, 488, 532 and 638 laser lines using DIVA™ 6.1.2 software (BD). To study cytokine production, CD4⁺ CD45RO⁺CD146⁺ and CD4⁺CD45RO⁺CD146⁻ T-cells were stimulated or polarized as described below. After stimulation or polarization, these cells were further stimulated for 4 hours with PMA/Ionomycin in presence of brefeldin-A (Leukocyte Activation Cocktail (BD)). These cells were then stained for both surface and intracellular antigens and analyzed as described above.

2.3 Human Th17 stimulation and polarization

Sorted CD4⁺CD45RO⁺CD146⁺ and CD4⁺CD45RO⁺CD146⁻ cells were cultured *in vitro* under either simple stimulation with CD3 and CD28 or under Th17 polarizing conditions as described by Veldhoen et al [22]. For polarization, cells were cultured in 96 well plates coated with 1 μ g/ml anti-human CD3 (clone OKT-3) and 2.5 μ g/ml anti-human CD28 (clone CD28.2) in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10%FCS, 1X antimycotic and antibiotic solution, and human recombinant 0.5ng/ml TGF- β , 30ng/ml IL-6, 10ng/ml IL-1 β , and 10ng/ml IL-23 (R&D Systems, Minneapolis, MN). In select experiments cells were plated on wells coated with 1 μ g/ml anti-human CD3 (clone OKT-3) and 2.5 μ g/ml anti-human CD28 (clone CD28.2). Cells were cultured in IMDM supplemented with 10%FCS and one of the following: a) no supplemental cytokines; b) with either IL-1 β or IL-23; c) with both IL-1 β and IL-23; or, d) with a combination of IL-1 β , IL-23, IL-6 and TGF- β . Cultures were incubated in a humidified chamber at 37°C supplemented with 5%

CO₂ for 5 days. Wherever possible an equal number of cells from each sorted subset was kept in IMDM supplemented with 10%FCS and 1X antimycotic and antibiotic solution but without stimulation to serve as controls. For select experiments, CD45RO was not used in the staining; therefore CD4⁺ CD146⁺ or CD4⁺ CD146⁻ cells were sorted and cultured as described above.

2.4 Supernatant Cytokine measurements

Cytokines were measured in the culture supernatants of sorted CD4⁺CD45-RO⁺CD146⁺ cells and CD4⁺CD45-RO⁺CD146⁻ cells stimulated with plate bound CD3/CD28 for 5 days. These assays were performed using the HCYTOMAG-60K-11 Human Cytokine Magnetic kit for Luminex assays (Millipore, Bellerica, MA) as per the manufacturer's instructions.

2.5 Gene expression

Total RNA from freshly sorted, Th17 polarized or stimulated cells was extracted using RNAqueous micro kit (Ambion, Austin, TX) according to the manufacturer's instructions. First strand cDNA synthesis was performed using Superscript cDNA synthesis kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. cDNA was preamplified using a pool of Taqman gene expression assays using TaqmanPreAmp master mix (Applied Biosystems, Foster city, CA) according to the manufacturer's instructions. This amplified cDNA served as the template for the amplification of genes of interest and the housekeeping genes (β -Actin # Hs99999903) by real time PCR in a 7900-sequence detector (PE-Applied Biosystems, Norwalk, CT). Samples were analyzed in duplicate and, after normalizing the Ct values to housekeeping genes, fold changes in expression were calculated using $\Delta\Delta C_t$ (cycle threshold) method [23]. The primers obtained from Applied Biosystems were as follows: IL-17A #Hs99999082_m1, IFN- γ #Hs00989291_m1, CCR-6 #Hs00171121_m1, ROR-C #Hs01076112_m1, T-bet (T-box 21) #Hs00894392_m1, IL-23R #Hs00332759_m1, IL-22 #Hs01574154_m1, IL-26 #Hs00218189_m1 AHR #Hs00907314_m1, RUNX-1 #Hs01021970_m1, MCAM/CD146 #Hs00174838_m1, CXCL-13 #Hs00757930_m1 and IL-21 #Hs00222327_m1.

2.7 Statistical analyses

Data obtained with cells from one donor were considered as one experiment (*n*). Statistical analysis performed on the results using Graphpad Prism 5.0d software (La Jolla, CA), included the calculation of mean, SE, and *p* values by use of a two group comparison Mann-Whitney. Within one experiment, data were analyzed using a paired two-tailed student's *t* test. The significance level was set as $p \leq 0.05$, and the *p* values are given for each series of experiments.

3. Results

3.1 Frequency of CD4⁺CD146⁺ T cells in healthy individuals and patients with inflammatory diseases

CD4⁺CD146⁺ T cells were measured by flow cytometry in the peripheral blood from healthy donors and from patients with various inflammatory autoimmune diseases (Behcet's syndrome, sarcoidosis and Crohn's disease) (Figure 1A, 1B). In healthy donors, we observed an average of $3.04 \pm 0.2\%$ of CD4⁺ T cells expressing CD146 (*n*=73). In Behcet's patients, the frequency increased significantly to $5.7 \pm 0.9\%$ (*n*=20), ($p=0.0002$). In sarcoidosis patients, the frequency of CD4⁺ T cells expressing CD146 was $6.4 \pm 0.7\%$ (*n*=24) ($p<0.0001$). Finally, the frequency of CD4⁺ T cells expressing CD146 in patients with Crohn's disease was $5.2 \pm 0.4\%$ (*n*=22) ($p<0.0001$) (Figure 1C).

In Crohn's disease, the frequency of CD4⁺CD146⁺ T-cells demonstrated a significant correlation to disease severity scores (HBI scores, estimated as described by Yoshida [24]) (Figure 1D). It was not possible to perform this correlation in the other diseases, as severity scores were not available.

In both healthy donors, as well as the patient populations, the CD4⁺CD146⁺ T cells displayed an effector memory immunophenotype (expression of CD45RO and lack of expression of CD197 (CCR7)), consistent with our previous observations (data not shown). These increased frequencies of CD4⁺CD146⁺ T cells observed in the diseases analyzed in the current study suggest that an elevation of these cells is common in a broad array of human inflammatory autoimmune diseases.

3.2 Evaluation of mRNA from CD4⁺CD45RO⁺CD146⁺ and CD4⁺CD45RO⁺CD146⁻ T cells

Freshly isolated peripheral blood cells from healthy donors (n=7) were sorted into two subpopulations: viable, CD45⁺ CD4⁺ CD45RO⁺CD146⁺ and viable, CD45⁺ CD4⁺ CD45RO⁺CD146⁻ T cells. The mRNA levels of these populations were then assessed by RT-PCR for IL-17A, IFN- γ and CD146 (as a control) (Figure 2). In addition to IL-17A and IFN- γ mRNA, we measured mRNA level of transcripts specifically associated with a Th17 phenotype [12, 13] including *RORC2*, *CCR-6*, aryl hydrocarbon receptor (*AHR*), *Runx-1*, *CXCL-13*, *IL-21*, *IL-22*, *IL-23R*, *IL-26*.

We found that the mRNA levels of IL-17A, *RORC2*, *IL-22*, *IL-26*, *IL-23R*, *CXCL-13*, *CCR-6*, *CD146*, but not *IFN- γ* , were significantly higher in CD4⁺CD45RO⁺CD146⁺ T cells than the in CD4⁺CD45RO⁺CD146⁻ T cells from the same individual (Figure 2A). By contrast, in these individuals, the mRNA levels of IFN- γ were lower in CD4⁺CD45RO⁺CD146⁺ T cells than the in CD4⁺CD45RO⁺CD146⁻ T cells. The mRNA levels of *Runx-1* and *AHR* were also higher in CD4⁺CD45RO⁺CD146⁺ T cells than their CD4⁺CD45RO⁺CD146⁻ counterparts, but these findings did not reach statistical significance. To further confirm the Th17 molecular signature of CD4⁺CD45RO⁺CD146⁺ compared to CD4⁺CD45RO⁺CD146⁻ T cells, similar experiments were performed on stimulated cells. Cells were sorted from PBMCs after 16h of *in vitro* stimulation with PMA and ionomycin. We observed similar mRNA increases in the CD4⁺CD45RO⁺CD146⁺ T cells compared to CD4⁺CD45RO⁺CD146⁻ T cells as seen in freshly isolated cells, further confirming the association of a Th17 phenotype with CD146 expression (Figure 2B).

In vitro stimulation was also performed on PBMCs isolated from Behcet's patients (n=4) and in the CD4⁺CD45RO⁺CD146⁺ subset we found similar increases of the following mRNAs; *RORC2*, *CCR-6*, *AHR*, *Runx-1*, *CXCL-13*, *IL-21*, *IL-22*, *IL-23R*, *IL-26* (data not shown).

3.3 Expression of chemokine receptors and Th17-associated markers on CD4⁺CD146⁺ T cells

CD4⁺CD146⁺ T cells from fresh peripheral blood of healthy donors, and patients with Behcet's, sarcoidosis and Crohn's disease were examined for co-expression of surface markers known to be associated with a Th17 phenotype, including *CD161*, *CCR6*, *CCR4*, *CXCR-3* or *CCR2*, although none of these are specific for Th17 cells. Here we report that *CD146* is partially, but not completely, co-expressed with *CD161* (Figure 3A), *CCR6* (Figure 3B), *CCR4* (Figure 3C), and *CXCR-3* (Figure 3E) but not with *CCR2* (Figure 3D).

3.4 Measurement of intracellular cytokines in CD4⁺CD146⁺ and CD4⁺CD146⁻ populations

We next examined the cytokine production of CD4⁺CD146⁺ T cells and CD4⁺CD146⁻ T cells obtained from healthy controls and Behcet's patients after *in vitro* PMA ionomycin

stimulation. PBMCs were stained as described in the methods, and viable CD3⁺CD4⁺ cells were gated for analysis. Then we assessed intracellular IFN- γ and IL-17A production in CD146⁺ and CD146⁻ subpopulations. Figure 4 shows the results of these experiments. Proportionally, IL-17A production was highly associated with CD146 expression, although not all CD146⁺ cells displayed IL-17A. Furthermore, IL-17A production could be found in lesser proportions outside of the CD146⁺ T cells, suggesting that CD146 identifies a subpopulation of Th17 cells, but is not a comprehensive marker for all cells of this lineage.

3.5 *In vitro* stimulation and Th17 polarization of sorted CD4⁺CD45RO⁺CD146⁺ and CD4⁺CD45RO⁺CD146⁻ T cells

Next, we investigated whether *in vitro* stimulation or polarization with Th17-inducing cytokines would further enhance the Th17 phenotype of CD4⁺CD45RO⁺CD146⁺ T cells and also what effect polarization would have on CD4⁺CD45RO⁺CD146⁻ T cells. To assess the effect of stimulation (with CD3/CD28 alone) and the effect of polarization on these cells, we looked at both the messenger level (mRNA measurement) and the translational level (intracellular and secreted cytokines). Sorted CD4⁺CD45RO⁺CD146⁺ and CD4⁺CD45RO⁺CD146⁻ populations from peripheral blood were subjected to *in vitro* stimulation for 5 days with plate-bound CD3/CD28 without the addition of exogenous cytokines. Examination of the mRNA levels of both subsets after stimulation revealed that a Th17 signature consisting of RORC2, CCR-6, AHR, Runx-1, CXCL-13, IL-21, IL-22, IL-23R, and *IL-26* was associated with the CD4⁺CD45RO⁺CD146⁺ subset (Figure 5). Staining of intracellular IL-17A and IFN- γ showed that the stimulated CD146⁺ cells yielded 10 times more IL-17A positive cells than did the stimulated CD146⁻ cells (Figure 6A).

In the next experiment, sorted CD4⁺CD45RO⁺CD146⁺ and CD4⁺CD45RO⁺CD146⁻ T cell populations from peripheral blood were stimulated with CD3/CD28 in the presence of IL-1 β or IL-23 alone or with both, or both of these cytokines plus TGF- β and IL-6 (Figure 6B, 6C, 6D, 6E). TCR stimulation of CD4⁺CD45RO⁺CD146⁺ or CD4⁺CD45RO⁺CD146⁻ cells in the presence of IL-1 β or IL-23 alone or in combination gave rise to a cytokine secretion profile similar to TCR-stimulated cells in the absence of exogenous cytokines (Figure 6B-6D). Finally, TCR stimulation of CD4⁺CD45RO⁺CD146⁺ or CD4⁺CD45RO⁺CD146⁻ T cells with IL-1 β , IL-23, IL-6, and TGF- β resulted in a cytokine secretion pattern similar to TCR-stimulated cells in the absence of cytokines, except that the CD4⁺CD45RO⁺CD146⁻ T cells demonstrated an increased number of cells producing only IFN- γ (Figure 6E). The addition of exogenous cytokine combinations together with *in vitro* CD3/CD28 stimulation also results in IL-17A production from the CD4⁺CD45RO⁺CD146⁻ subset, although the level of IL-17A production remained lower compared to the CD4⁺CD45RO⁺CD146⁺ subset.

To confirm the results of the intracellular cytokine staining revealing that CD3/CD28 stimulation alone can induce IL-17A secretion from CD4⁺CD45RO⁺CD146⁺ T cells, we measured cytokine secretion by multiplex bead array (Luminex) in culture supernatants following CD3/CD28 stimulation. In healthy donors, we observed a significantly higher secretion of IL-17A by CD4⁺CD45RO⁺CD146⁺ T cells than by the CD4⁺CD45RO⁺CD146⁻ T cells ($p = 0.0095$). Furthermore, mean levels of IL-8 and IL-6 were also found to be higher in CD4⁺CD45RO⁺CD146⁺ subset compared to the CD4⁺CD45RO⁺CD146⁻ subset, and no difference in the secretion of IFN- γ was noted (Figure 7). Similar observations were made with CD4⁺CD45RO⁺CD146⁺ and CD4⁺CD45RO⁺CD146⁻ T cells from Behcet's and sarcoidosis (data not shown).

Overall, these data show that CD4⁺CD45RO⁺CD146⁺ T cells have the capacity to exhibit a Th17 cytokine profile that is independent of the effects of exogenous cytokines. In addition, these data show that the Th17 bias of CD4⁺CD146⁺ T cells compared to CD4⁺CD146⁻ T

cells is not merely the result of a memory phenotype, as CD45RO⁺ populations from both groups were used in these assays.

4. Discussion

The current data indicate that MCAM (CD146) identifies a population of circulating Th17 cells that are CD4⁺CD45RO⁺ and that frequently co-express CD161 [11, 25], CCR4 [11] and CCR6 [26]. Two of these markers, CD161 and CCR6, are associated with the migratory capabilities of Th17 cells [17, 25, 27]. The identification of these cells by CD146 is intriguing in this regard. In humans, CD146 is primarily expressed on the endothelium, although it has also been reported to be expressed on mesenchymal stem cells and certain cancers including melanoma [28]. CD146 on endothelial cells is found primarily at the endothelial junctions, and it has been shown that CD146 can facilitate binding of endothelial cells to one another in a homotypic fashion to establish cell cohesion [18]. The homotypic nature of this binding suggests that Th17 cells expressing CD146 might also be able to bind to the endothelium in a similar manner. This concept is supported by data from several previous studies. In a previous report, we demonstrated that CD146 expression on T cells facilitated binding of these cells to endothelial monolayers *in vitro* [20]. In similar studies, Brucklacher-Waldert et al reported that the binding of Th17 clones isolated from patients with multiple sclerosis that expressed CD146 could be inhibited from binding to endothelial monolayers by antibodies to CD146 [15]. Additionally, Guezguez et al using CD146 transfected human NK cells [29], reported that the expression of CD146 on these cells leads to rolling within the vascular bed. Further evidence for a role of CD146 in leukocyte-endothelial binding is also suggested by a study demonstrating the role of CD146 in monocyte trans-endothelial migration [18]. It is also worth noting that IL-17A and TNF- α are known to increase permeability of endothelial layers [30] and therefore might facilitate trans-endothelial migration. The identification of CD146⁺ Th17 cells strongly suggests that these cells can readily attach to, and possibly migrate across, endothelial layers to sites of inflammation. Indeed, our previous work has demonstrated that CD4⁺CD146⁺ T cells are present in high quantities in the synovial fluids of patients with arthritic disease. It would be most interesting to obtain biopsies from sites of inflammation in patients with inflammatory autoimmune diseases to determine the quantity and nature of CD4⁺CD146⁺ T cells at those sites.

Th17 cells have been shown to facilitate the migration of other leukocytes to sites of inflammation. The current finding of elevated mRNA levels of *CXCL-13* expression is consistent with previous reports associating this chemokine with Th17 cells [31, 32]. CXCL-13 is a potent B cell attractant and may enable T cells to function as B cell helpers, as reported by Mitsdoerffer et al [33] and Takagi et al [31]. Furthermore, we also observed higher secretion of IL-8 and IL-6 from CD4⁺CD146⁺ cells. IL-8 has been reported to enhance endothelial survival and angiogenesis [34] and IL-6 has been shown to enhance lymphocyte adherence to endothelial cells [35]. IL-17A has been demonstrated to induce blood barrier disruption by direct action on endothelial cells [30], possibly facilitating the migration of Th17 cells through the endothelial barrier [16]. The evidence of these cytokines acting directly on endothelial cells together with the finding of CD146 expression by Th17 cells, suggests a mechanism by which Th17 cells traffic through the endothelium, lending support to the hypothesis that these cells play a role in the early stages of inflammation.

In order to more rigorously investigate the adherence and migration of CD4⁺CD146⁺ T cells, we had intended to extend our observation in humans to mouse models of inflammatory diseases. We conducted studies of the peripheral blood in healthy (C57/B6) mice and in mice with experimental EAU [36] to examine CD146 expression on murine T cells. These experiments revealed that mouse T cells fail to express CD146, a finding

consistent with a previous report indicating that CD146 in the murine hematopoietic system is found primarily on NK cells rather than T cells [37]. In contrast to mice, CD146 is rarely found on human NK cells. Our inability to identify MCAM (CD146) as a marker of murine Th17 is therefore not an unusual discrepancy, as similar findings have been reported for other markers such as CD161 [38].

During the course of this study, we also observed elevated numbers of CD8⁺CD146⁺ T cells in the blood of patients with Behcet's disease and Crohn's disease, but not in sarcoidosis. The reason for this is unclear at present. We were unable to fully characterize these cells due to their relative paucity in the circulation, but it is tempting to speculate that these might also produce IL-17A.

In this and previous studies, we have observed that CD146 expression on T cells can be up regulated by mitogen stimulation and T cell receptor engagement. CD146 expression on other cell types has been shown to be upregulated by a variety of cytokines including TNF- α , IL-13, phorbol ester, and cAMP. We sorted CD4⁺CD146⁺ and CD4⁺CD146⁻ populations and co-cultured these with HDMECs, as performed by Taflin et al., [38]. These experiments demonstrated that CD146 expression was increased in both of these populations by co-culture, with CD146 negative population gaining expression on roughly 5% of the cells. IL-17 production was also observed in these cells (data not shown). Taflin et al showed that co-culture of T cells with endothelial cells promoted IL-17 expression by the T cells in an IL-6/STAT-3 dependent manner. Although not directly demonstrated in the current study, the up regulation of CD146 and IL-17 by the endothelium may occur in a coordinated manner and possibly through a common mechanism.

5. Conclusion

In summary, we have identified a novel subset of effector memory CD4⁺ T lymphocytes expressing the endothelial adhesion molecule CD146 that show multiple features of functional Th17 cells. These cells are found in low levels in the peripheral circulation of healthy individuals but are increased in patients with various inflammatory autoimmune diseases, and correlate with disease severity in Crohn's disease. We believe that this population of effector memory Th17 cells with enhanced endothelial binding capacity, and possibly with B cell facilitating properties, serves as early responders in a broad array of human inflammatory autoimmune diseases.

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CD146+ CD4+ T cells secrete IL-17A and display multiple features of Th17 cells
CD146+ CD4+ T cells do not require polarization to become TH17 cells
These cells are in low levels in the blood of healthy donors
CD146+ CD4+ T cells levels significantly increase in inflammatory autoimmune diseases
Lymphocytes expressing CD146 have been shown to have enhanced binding to endothelium

Figure 1. CD146 expression on CD4⁺ T-cells

Single-cell suspensions from healthy donors and patients with various inflammatory diseases were stained using a combination of antibodies as described in the material and methods.

A. Lymphocytes were identified based on their forward and side scatter properties. Subsequently, CD3 was used to identify T cells (CD3⁺) among the previously selected lymphocytes. CD4⁺ T cells and CD8⁺ T cells were identified as uniquely expressing CD4 or CD8 antigens. CD146⁺ expression was then determined on the gated CD4⁺ T cells.

B. Representative bivariate plots of CD146 expression in CD4⁺ T cells (as described above) in healthy, Behcet's, sarcoidosis and Crohn's patients.

C. The mean percentages ($\pm$ SEM) of CD4⁺ T cells expressing CD146 among healthy (n=73), Behcet's (n=20), sarcoidosis (n=24) and Crohn's (n=20) patients.

D. Correlation between frequency of CD4⁺CD146⁺ T cells in peripheral circulation and disease severity scores in Crohn's disease (Harvey-Bradshaw Index (HBI) [24]. With this index, active disease is defined as a HBI score of ≥ 4 . HBI scores were correlated to the frequency of CD4⁺CD146⁺ T cells. Represented is the linear regression between the 2 parameters.

Figure 2. mRNA level of CD4⁺CD45RO⁺CD146⁺ T lymphocytes in circulation compared to CD4⁺CD45RO⁺CD146⁻ T cells

A. Single-cell suspensions from healthy donors (n=7) were stained and sorted into 2 populations: CD4⁺CD45RO⁺CD146⁺ (Dark circles) and CD4⁺CD45RO⁺CD146⁻ (Open circles). After sorting, mRNA was extracted and RT-PCR was performed using gene expression assays as described in the methods.

B. PBMCs were stimulated with PMA/ionomycin for 16hrs as described in the material and methods, then sorted into CD4⁺CD45RO⁺CD146⁺ (Dark circles) and CD4⁺CD45RO⁺CD146⁻ (Open circles) subsets. mRNA isolation and RT-PCR, using the same gene expression as described previously, was performed following the sort.

Figure 3. Expression of chemokine receptors and Th17 phenotype-associated markers on CD4⁺CD146⁺ T cells

Single-cell suspensions from healthy donors and patients with various inflammatory diseases were stained with a combination of antibodies to study the co-expression of CD146 with other markers associated with a Th17 phenotype. The figure shows representative bivariate dot plots of CD146 co-expression with (A) CD161, (B) CCR-6, (C) CCR-4, (D) CCR-2 and (E) CXCR-3.

Figure 4. Intracellular cytokines in freshly isolated $CD4^+CD45RO^+CD146^+$ and $CD4^+CD45RO^+CD146^-$ T lymphocytes

PBMCs from healthy individuals ($n=7$) and patients with various inflammatory diseases ($n=4$) were stimulated using PMA/ionomycin and stained for intracellular cytokines IL-17A and IFN- γ . Data shown are the percentages of IFN- γ (left panel) and IL-17A (right panel) positive cells in $CD4^+CD45RO^+CD146^+$ and $CD4^+CD45RO^+CD146^-$ subsets of healthy donors (H) and patients (D).

Figure 5. m-RNA levels in $CD4^+CD45RO^+CD146^+$ T cells compared to $CD4^+CD45RO^+CD146^-$ T cells after *in vitro* stimulation

Single-cell suspensions from healthy individuals (n=7) were stained and sorted into 2 populations $CD4^+CD45RO^+CD146^+$ (Dark circles) and $CD4^+CD45RO^+CD146^-$ (Open circles). These subsets were stimulated with CD3/CD28 as described in the material and methods. mRNA isolation was performed after 5 days of culture. RT-PCR were performed using gene expression assay from Applied Biosystems measuring as described in the methods.

Figure 6. Intracellular cytokine expression of CD4⁺CD45RO⁺CD146⁻ T cells and CD4⁺CD45RO⁺CD146⁺ T cells in presence of Th17 polarizing conditions
Single-cell suspensions from healthy individuals (n≥3) were stained and sorted into 2 populations: CD4⁺CD45RO⁺CD146⁻ and CD4⁺CD45RO⁺CD146⁺. These sorted populations were subjected to different *in vitro* stimulations and the expression of IL-17A (x axis) and IFN-γ (y axis) was measured. CD4⁺CD45RO⁺CD146⁻ T cells are shown in the left panel and CD4⁺CD45RO⁺CD146⁺ T cells are shown in the center panel, and summary data are shown in the right panel.
The condition of stimulations were: (A) plate bound CD3 and CD28 (B) plate bound CD3 and CD28 and IL-1β, (C) plate bound CD3 and CD28 and IL-23, (D) plate bound CD3 and CD28 and IL-1β+IL-23 and (E) plate bound CD3 and CD28 and IL-1β+IL-23+TGF-β+IL-6.

Figure 7. Cytokine secretion of CD4⁺CD45RO⁺CD146⁺ T cells and CD4⁺CD45RO⁺CD146⁻ T cells

Single-cell suspensions from healthy individuals (n=7) were stained and sorted into 2 populations CD4⁺CD45RO⁺CD146⁺ and CD4⁺CD45RO⁺CD146⁻. These subsets were stimulated with CD3/CD28 as described in the material and methods. Secretion of IL-17A (A), IFN-γ (B), IL-8 (C) and IL-6 (D) were measured in culture supernatant using multiplex bead array assays in CD4⁺CD146⁻ or CD4⁺CD146⁺ subsets.