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Distinctive features of classic and non-classic (Th17-derived) human Th1 cells

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Abbreviations: ROR: RAR-related orphan receptor C; UCB: umbilical cord blood; IL4I1: IL-4-induced gene 1; JIA: juvenile idiopathic arthritis; SF: synovial fluid

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Summary

T helper 17 (Th17) lymphocytes represent a third arm of the $CD4^+$ T-cell effector responses, in addition to Th1 and Th2 cells. Th17 cells have been found to exhibit high plasticity because they rapidly shift into the Th1 phenotype in inflammatory sites. In humans, Th1 cells derived from Th17 cells express CD161, whereas classic Th1 cells do not; these Th17-derived Th1 cells have been termed "non-classic Th1 cells. In this study, we examined similarities and differences between classic and non-classic human Th1 cells by assessing a panel of T-cell clones, as well as $CD161^+$ or $CD161^-$ $CD4^+$ T cells derived ex vivo from the circulation of healthy subjects or the synovial fluid of patients with juvenile idiopathic arthritis. The results show that non-classic Th1 cells can be identified based on CD161 expression, as well as the consistent expression of retinoic acid orphan receptor C, IL-17 receptor E, CCR6 and IL-4-induced gene 1, which are all virtually absent in classic Th1 cells. The possibility to distinguish these two cell subsets by using such a panel of markers may allow the opportunity to better establish the respective pathogenic roles of classic and non-classic (Th17-derived) Th1 cells in different chronic inflammatory disorders.

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Introduction

CD4$^+$ T helper (Th) cells can be classified into lineages on the basis of cytokine production, the expression of specific transcription factors and the immunological function they mediate: Th1 cells, which express the transcription factor T-box expressed in T cells (T-bet) and secrete IFN-γ, protect the host against intracellular infections; Th2 cells, which express GATA-3 and secrete IL-4, IL-5 and IL-13, mediate host defense against helminths [1, 2]. Recently, additional subsets that preferentially produce distinct cytokines have been described. The most studied subset includes cells that selectively produce IL-17A (Th17 cells), express the transcription factor RAR-related orphan receptor (ROR)γt, and protect the host against infection with extracellular pathogens [3, 4]. Human Th17 cells are, at least partially, different from murine Th17 cells [5, 6]. Indeed, human Th17 cells express not only distinctive Th17 molecules, such as IL-23 receptor (IL-23R) and RORC, but also those typical of the Th1 phenotype such as IL-12Rβ2 and T-bet [6]. Moreover, we have previously discovered a subset of human Th17 cells which are also able to produce IFN-γ, named Th17/Th1 cells; both Th17 and Th17/Th1 cells can be induced to become Th1 cells when cultured in presence of IL-12 [6]. Similar findings were then reported also in some murine models [7-9], thus suggesting that both human and murine Th17 possess a plastic phenotype [10, 11].

Human IL-17-producing cells originate from CD161$^+$ precursors present in umbilical cord blood (UCB) and newborn thymus, and maintain CD161 expression, which is detectable on memory circulating and tissue-infiltrating Th17 and Th17/Th1 lymphocytes [12, 13, 14]. Moreover, Th1 cells that derive from Th17

cells can be recognized in humans because of their expression, as Th17 cells, of CD161 [15]. The NK1.1 molecule, the murine equivalent of CD161, has not been described in mouse Th17 cells.

Based on this finding, we have selected human $CD161^-$ (classic) and $CD161^+$ (non-classic) Th1 cells and examined at both clonal level and in ex vivo-derived cells the main differences existing between them. The results showed that non-classic Th1 cells can be identified not only because of their CD161 expression, but also because they consistently express RORC, IL-17RE, CCR6 and IL-4-induced gene 1 (IL4I1), which are virtually absent in classic Th1 cells. The possibility to distinguish the two cell subsets by using this panel of markers offers the opportunity to better establish their respective pathogenic roles in chronic inflammatory disorders.

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Results

Establishment of classic and non-classic Th1 clones

Human Th17 cells are characterized by the surface expression of CCR6, the IL-23 receptor (IL-23R), and CD161 [6, 12]. As a consequence, a high proportion of clones derived from the $CD161^+CCR6^+$ cell fraction of PBMCs from healthy donors, produced IL-17A alone or IL-17A plus IFN-γ, thus exhibiting a Th17 or Th17/Th1 phenotype, respectively. In a subsequent study [16], we found that a proportion of clones generated from the $CD161^+CCR6^+$ cell fraction showed the ability to produce IFN-γ alone, i.e they exhibited a Th1 phenotype, supporting the concept that CD161 expression is a peculiar, but not exclusive, property of the Th17 phenotype. Accordingly, we have recently demonstrated the presence of $CD161^+$ Th1 lymphocytes in the synovial fluid (SF) of patients with juvenile idiopathic arthritis (JIA), that derived from an in vivo shifting of Th17 to Th1 cells in presence of IL-12 [15]. These cells named non-classic Th1 cells to distinguish them from the classic $CD161^-$ Th1 counterpart [15].

To investigate the main features of these two different Th1 subsets, $CD4^+CD161^+$ and $CD4^+CD161^-$ T-cell populations were purified from peripheral blood (PB) of healthy human subjects and then cloned under limiting dilution conditions. As expected, clones derived from the $CD4^+CD161^+$ T-cell fraction exhibited a Th17, a Th17/Th1, or a Th1 phenotype, whereas virtually all clones generated from the $CD4^+CD161^-$ T-cell subset had a Th1 phenotype and none of them was able to produce IL-17A (Fig. 1A and B). $CD161^-$ Th1 clones were considered as classic Th1 cells, whereas $CD161^+$ Th1 cells were considered as non-classic (Th17-derived) cells.

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Transcription factors

The expression of the transcription factors T-bet and RORC by classic and non-classic Th1, as well as by Th17 or Th17/Th1, cells were compared by assessing 20 randomly selected T cell clones from each phenotype with quantitative RT-PCR. In agreement with previously published data [15, 16], the Th1-related transcription factor T-bet was found to be expressed by all four types of T cell clones analysed, reaching the highest mRNA levels in both classic and non-classic-Th1 cells (Fig.2A). By contrast, the Th17-associated transcription factor RORC was distinctively expressed by Th17, Th17/Th1 and non-classic Th1, but not by classic Th1, clones (Fig.2A).

Cytokine receptors

The expression of mRNA levels for cytokine receptors that have been previously associated to Th17 cells, such as IL-23R, IL-1RI, IL-12Rβ2, and IL-17RE [6, 12, 17], was also evaluated. As shown in Fig. 2B, mRNA levels for IL-23R and IL-1RI (the receptors of two cytokines deeply involved in human Th17-cell differentiation) were significantly higher in Th17, Th17/Th1 and non-classic Th1 when compared with that of classic Th1 cells, while IL-12Rβ2 mRNA levels were comparable in all four types of clones. Of note, IL-1RI mRNA levels were significantly lower in non-classic Th1 in comparison to both Th17 and Th17/Th1 clones (Fig. 2B). Finally, IL-17RE mRNA, a member of IL-17 receptor family, whose full-length isoform is selectively expressed by murine Th17 cells and potentiates their function in

response to IL-17C [17], was clearly detectable in Th17, Th17/Th1 and non-classic Th1 clones, but virtually absent in classic Th1 clones (Fig. 2B).

Chemokine receptors

In addition to transcription factors and cytokine receptors involved in the differentiation/modulation of classic Th1 and Th17 cells, some chemokine receptors have also been reported to be preferentially associated with one or the other T-cell subset. In particular, CXCR3A have been found to be expressed by Th1 cells [5], whereas CCR6 preferentially associates with Th17 cells [5, 6]. As shown in Fig. 3A, CCR6 mRNA expression was virtually absent in classic Th1 cells, whereas it was expressed at comparable levels by Th17, Th17/Th1 and non-classic Th1 cells. Conversely, CXCR3A mRNA levels were significantly higher in classic Th1 cells when compared with those of Th17, Th17/Th1 and non-classic Th1 cells (Fig. 3A). Interestingly, CXCR3A expression was significantly lower in Th17 than in Th17/Th1 and non-classic Th1 cells, the latter showing significantly higher CXCR3A mRNA levels than Th17/Th1 cells (Fig. 3A). Single cell analysis of CXCR3A and CCR6 protein expression performed by flow cytometry revealed that classic Th1 and Th17 cells had a clear CXCR3A and CCR6 single positive (SP) phenotype respectively; Th17/Th1 cells comprised of both CCR6 SP and $CXCR3A^{+}CCR6^{+}$ double positive (DP) phenotypes; non-classic Th1 were mainly characterized by a $CXCR3A^{+}CCR6^{+}$ DP phenotype (Fig. 3B).

Il4I1 expression and proliferative response

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Th17 cells, differently from Th1 cells, show poor proliferative responses to anti-CD3 plus anti-CD28 stimulation, mainly because of their inability to produce IL-2 [18]. This defect in IL-2 production is due to the reduced c-Fos, c-Jun and nuclear factor of activated T-cells activity. The reduced activity of these transcription factors associated with high expression of the IL4I1 mRNA, which encodes for a L-phenylalanine oxidase able to down-regulate CD3ζ expression in T cells [19]. As shown in Fig. 4A, IL4I1 mRNA was expressed not only by Th17, but also by Th17/Th1 and by non-classic Th1 cells, but never by classic Th1 cells. In order to establish whether non-classic Th1 cells also exhibited poor, if any, proliferative response to TCR triggering that is characteristic of Th17 cells, 20 clones of each of the four different Th phenotypes (Th17, Th17/Th1 and classic and non-classic Th1 cells) were stimulated in vitro for 5 days with anti-CD3 plus anti-CD28 mAbs and their proliferative response was assessed. In accordance with previously published results [18], Th17 clones showed significantly reduced ability, when compared with classic Th1 cells, to proliferate in response to TCR triggering (Fig. 4B). Non-classic Th1 cells showed a significantly higher proliferative response to TCR triggering when compared with Th17 cells, but significantly lower than classic Th1 cells. As expected, Th17/Th1 cells exhibited a proliferative response comparable to that of Th17 cells (Fig. 4B). Finally, in order to exclude any link between the frequency of cytokine-producing cells of the different clones and cell proliferation, correlation between the level of ^{3}H uptake in response to anti-CD3 plus anti-CD28 in the presence or absence of IL-2, and the frequency of IFN-γ- and IL-17-producing cells was performed. As shown in Fig. 4C, no significant correlation was present between the frequency

of cytokine-producing cells and the degree of proliferation for any of the Th-cell subsets analysed.

Differences between classic and non-classic Th1 clones detectable on ex vivo-derived cells

In order to exclude the possibility that all these findings were an artifact due to the use of long-term in vitro-cultured T cells (T-cell clones), the mRNA levels of all the above-mentioned molecules were also measured on ex vivo-derived IFN-γ-producing T cells, which had been purified from the sorted $CD161^+$ (non-classic Th1 cells) and $CD161^-$ (classic Th1 cells) PB $CD4^+$ T-cell fractions by capture secretion assay, as already reported [15]. In agreement with data obtained using T-cell clones, ex vivo-derived non-classic Th1 cells could be clearly distinguished from classic Th1 cells not only for their expression of CD161, but also of RORC, IL-17RE, CCR6, and IL4I1 (Fig. 5A). Moreover, comparable results were obtained when the above mentioned markers in classic and non-classic Th1 cells isolated from SF of three JIA patients were evaluated (Fig. 5B).

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Discussion

Classically, Th1 cells have been defined as effector Th cells which secrete IFN-γ but not IL-4, express the transcription factor T-bet and protect the host against intracellular infections [1, 2]. Th1 cells have also been thought to be involved in chronic inflammation, both in murine experimental models and human diseases, especially organ-specific autoimmune disorders [1, 2]. Classic Th1 cells can be derived in vitro and in vivo from naïve Th cells under conditions characterized by the presence of IL-12, IL-18, and IFNs, and in absence of IL-4 [20]. More recently, a new lineage of effector $CD4^+$ Th cells has been described that selectively produces IL-17A (Th17 cells), expresses the transcription factor RORγt and is critical for the host defense against extracellular pathogens [3, 4]. This subset has been initially thought to be pathogenic in many chronic inflammatory disorders, including both experimental animal models and human autoimmune disorders [6, 12, 15, 21]. According to findings mainly obtained in models of knockout mice [22-24], and also because of the counteracting activity of IFN-γ on the development of Th17 cells [3], Th1 cells were thought to be rather protective than pathogenic in the same disorders [3]. Our group was the first to demonstrate that part of human IL-17A-producing cells were also able to produce IFN-γ, and more importantly, to provide evidence that human Th17 cells exhibit plasticity and can be shifted to produce IFN-γ in presence of IL-12 [6]. The shifting of Th17 cells towards the Th1 phenotype was then demonstrated even in different murine experimental models and the concept of Th17 cell plasticity now represents an established fact in both species [10, 11]. In a recent study, we found that Th17 cells are rare in the SF of patients with JIA whereas Th1

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cells were highly predominant, which was at least partially due to the property of Th17 cells to shift into Th1 cells in presence of IL-12 [15]. The Th17-derived Th1 cells expressed CD161, while the other Th1 cells present in the SF did not, and we named the former cells as non-classic, as compared with classic CD161$^-$ Th1 cells [15]. However, the similarities and differences between classic and non-classic (Th17-derived) Th1 cells were not extensively investigated. More recently, we also demonstrated the existence of another mechanism responsible for the rarity of Th17 cells in inflamed tissues. Indeed we showed that Th17 lymphocytes are, on one hand unable to proliferate in response to TCR triggering due to abnormalities in the molecular pathways that allow IL-2 production, and, on the other hand, possessed a reduced ability to respond to IL-2 [18]. The inability of Th17 cells to produce IL-2 in response to TCR triggering appeared to be related to the up-regulation of the L-amino acid oxidase IL4I1 which, in turn, was strictly dependent upon the activity of the Th17 master gene, *RORC* [18].

Based on all these findings, in this study, we investigated and compared the similarities and differences among Th17 cells, classic Th1 cells, and non-classic Th1 cells. In order to approach this question we took advantage from our previous observation that human Th17 cells all are contained in a subset of memory Th cells that express CD161 (or NKR-P1A), the human homologue of the murine NK1.1 [12, 14], which is not only expressed on almost all NK cells, but also by a subset of T cells, which have been named NKT cells [25]. In contrast, murine Th17 cells do not seem to exhibit such a marker. More importantly, in the same study, we also found that human Th17 cells originate from a small subset of thymic CD161$^+$ precursors, which also express RORC, which in turn is critical for

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CD161 expression, whereas Th17 cells could never have been derived from CD161$^-$ Th cells [12, 14]. We therefore evaluated the expression of different cytokines, transcription factors, cytokine and chemokine receptors, as well as the expression of IL4I1 and the ability to proliferate in response to TCR triggering, by classic Th1 T-cell clones derived from CD161$^-$ circulating CD4$^+$ T cells from healthy donors and non-classic Th1, as well as Th17 and Th17/Th1 clones, derived from CD161$^+$CD4$^+$ T cells from the same donors. The results show that non-classic Th1 cells, differently from classic Th1 cells, express the majority of typical Th17-associated molecules. In particular, in addition to CD161, non-classic Th1 cells express the master transcription factor for Th17 cells RORC, the chemokine receptor CCR6, the cytokine receptor IL-17RE and IL4I1. At the same time, non-classic Th1 cells show increased expression of some classic Th1-associated molecules. In particular, in addition to IFN-γ, non-classic Th1 cells showed increased expression of T-bet, the master transcription factor for Th1 cells, which has been found to be expressed also by Th17 cells [6], as well as of IL-12Rβ2, and acquire the ability to express CXCR3A. The data obtained at clonal level were confirmed not only on classic and non-classic Th1 cells freshly isolated from the circulation of healthy subjects, but also from CD4$^+$ T cells from the SF of children affected by JIA.

Thus, the results of the present study provide convincing evidence that non-classic Th1 cells can be easily distinguished from classic Th1 cells by using a combination of the surface markers CD161, CCR6, CXCR3A and IL-17RE, as well as by their ability to express RORC and IL4I1. These findings are of particular relevance because the possibility to easily distinguish classic from non-classic

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(Th17 derived) Th1 cells may allow to define the role of these Th cell subsets in the pathogenesis of chronic inflammatory disorders. Indeed, these disorders have been in the past associated to Th1 responses and more recently to Th17 cells, even because of the ability of the latter to polarize towards the Th1 phenotype. Accordingly, increased numbers of non-classic Th1 cells are present not only in the inflamed joints (SF) of JIA children [15], but also in the gut of Crohn's disease affected patients [12], the numbers of Th17 cells in both sites being very usually low [12, 15].

One intriguing question emerging from this study was the demonstration that despite non-classic Th1 cells maintained the IL4I1 expression at levels similar to those of Th17 cells, they showed an increased ability to proliferate in response to TCR triggering, a condition which was more similar to that of classic Th1 cells. This finding can account for the high number of non-classic Th1 cells in inflamed tissues, but the reason why non-classic Th1 cells at least partially re-acquire the ability to proliferate, still mantaining significantly higher mRNA levels of RORC and IL4I1 when compared with classic Th1 cells, is at present unclear. One possibility is that the inability of Th17 cells to expand is also due to abnormalities other than those related to the up-regulation of IL4I1. In our previous study, we showed indeed that Th17 cells also exhibit a block in the activation of PI3K/AKT pathway in response to IL-2 [18]. Therefore, the existence of additional inhibitory mechanisms cannot be excluded and their changes following the shifting of Th17 cells into non-classic Th1 cells, leading to better expansion of these latter, might be hypothesised.

In conclusion, the results of this study demonstrate that non-classic (Th17-derived) Th1 cells can be clearly distinguished from classic Th1 cells in both the circulation and in inflamed tissues by using a panel of different markers. This finding may be of great help in better understanding the respective pathogenic roles of non-classic and classic Th1 cells in several chronic inflammatory disorders, thus allowing more appropriate therapeutical interventions.

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Materials and methods

Subjects

PB samples were obtained from 8 healthy volunteers. The procedures and all the experiments of the study were in accordance to the ethical standards of and approved by the Regional Committee on Human Experimentation. SF samples were also obtained from 3 children affected by oligoarticular JIA according to the ILAR classification.

Reagents

The culture media used was RPMI 1640 (Seromed) supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 2 x 10^{-5} M 2-mercaptoethanol (2-ME; all from Invitrogen), and 10% FBS HyClone (Gibco Laboratories, Grand Island, NY). Unlabeled or fluorochrome-conjugated anti-CD3, CD4, CD8, CD28, CD161, CCR6, IFN-γ, and isotype-matched control mAb were purchased from BD Biosciences (San Jose, CA). The fluorochrome-conjugated anti-IL-17 mAb was obtained from eBioscience (San Diego, CA) and the fluorochrome-conjugated anti-CXCR3 mAb was obtained from R&D Systems (Minneapolis, MN). PMA, ionomycin and brefeldin A were purchased from Sigma Chemical Co. (St. Louis, MO).

Establishment of T-cell clones and their characterization

$CD4^+$ T cells, derived from PBMNCs of 4 healthy donors by using the CD4 isolation kit II (Miltenyi Biotec, Bergisch Gladbach), were further divided into $CD161^+$ and $CD161^-$ T-cell fraction by a staining with an anti-CD161–PE mAb,

followed by incubation with an anti-PE microbead mAb (Miltenyi Biotec). Then the two cell subsets $CD4^+CD161^+$ and $CD4^+CD161^-$ were cultured under limiting dilution (0.3 cell/well) in presence of 10^5 irradiated (9,000 rad) allogeneic PBMCs as feeder cells, 1% PHA (vol/vol), and 50 U/ml rIL-2 (Proleukin, Prometheus, Inc. San Diego, USA), in order to obtain T-cell clones. Recovered $CD4^+$ T-cell clones were classified on the basis of their ability to produce IFN-γ and/or IL-17 and to express surface marker CD161, as previously described [16]. Briefly, T cells were polyclonally stimulated with PMA plus ionomycin, fixed in formaldehyde and then analysed for intracellular cytokines production on a BDLSR II flow cytometry (BD Biosciences). Selected T-cell clones of each phenotype were further analysed by flow cytometry for surface expression of CXCR3A and CCR6.

Proliferation assay

5×10^4 cells of selected T-cell clones of each phenotype were stimulated for 3 days with 5 μg/ml anti-CD3 plus 5 μg/ml anti-CD28 mAb (BD Biosciences), to evaluate their proliferative response. Cultures were pulsed for the last 8 h with 0.5 μCi (0.0185 MBq) of ^{3}H-TdR (GE Healthcare), harvested, and radionuclide uptake was measured by scintillation counting.

Cytokine secretion assay and cell culture

MNC from PB of 3 healthy donors and from SF of 3 JIA patients were stimulated with PMA plus ionomycin. After three and half hours stimulated cells were recovered, washed and stained with IFN-γ and IL-17 catch reagents (Miltenyi Biotec), following manufacturer instructions. Following additional 45 minutes of

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incubation (37 °C, 5% CO2) cells were stained with anti-CD3-Pacific Blue, CD4-PE-Cy7, CD8-allophycocyanin-Cy7, CD161-PE, IL-17-allophycocyanin and IFN-γ-FITC conjugated mAbs, analysed and sorted by FACSAria (BDBiosciences) into $CD3^+CD4^+CD8^-CD161^+IL\text{-}17^-IFN\text{-}\gamma^+$ (non-classic Th1) and $CD3^+CD4^+CD8^-CD161^-IL\text{-}17^-IFN\text{-}\gamma^+$ (classic Th1), cells. The recovered cells were cultured for one week in presence of IL-2 (50U/ml) and then analysed for the expression of mRNA levels of RORC, IL-17RE, CCR6, and IL4I1.

Real-time quantitative RT-PCR

Taq-Man RT-PCR was performed, as described elsewhere [10, 12]. Primers and probes used were from Applied Biosystems (Foster City, CA 94404 USA). Quantitative PCR analysis of CXCR3A was performed by using the following: FAM probe, 5′-TGAGTGACCACCAAGTGCTAAATGACGC-3′; forward 5′-ACCCAGCAGCCAGAGCACC-3′; reverse 5′-TCATAGGAAGAGCTGAAGTTCTCCA-3′, were from Applied Biosystems.

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Figure legends

Fig. 1: Cytokine profile of T-cell clones generated from the CD161^{+} and CD161^{-} fraction of circulating CD4^{+} T cells.

(A) CD161^{+} (n=232) or CD161^{-} (n=145) T-cell clones, derived from PB of 3 different donors, were stimulated with PMA plus ionomycin and assessed by flow cytometry at single cell level for their ability to produce IFN-γ and/or IL-17A. Red dots represent CD4^{+}CD161^{+} clones and black dots represent CD4^{+}CD161^{-} clones. Arrows indicate the 4 T cell clones depicted in panel B. (B) A representative flow cytometric analysis of each Th cell population shown in panel A (classic and non-classic Th1, Th17, Th17/Th1 clones) is depicted.

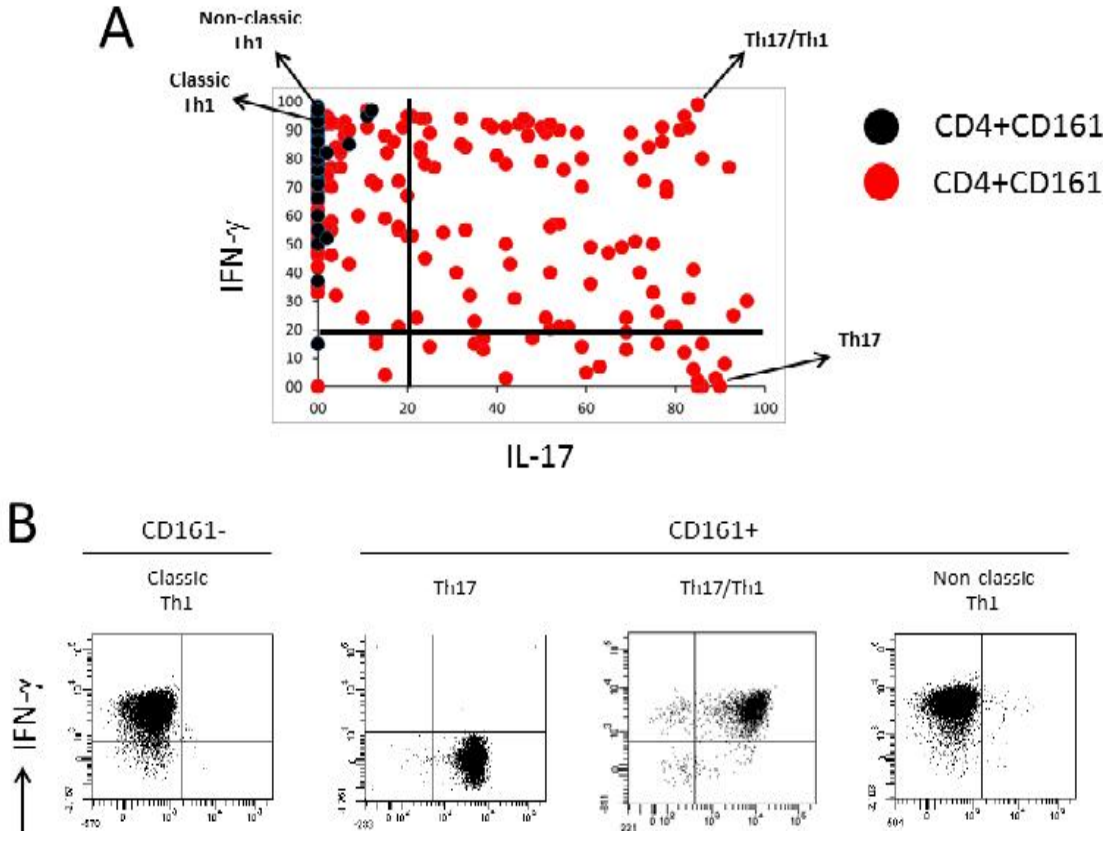

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Fig. 2: Transcription factor and cytokine receptor mRNA expression by classic, non-classic Th1, Th17/Th1 and Th17 clones.

(A) T-bet and RORC and (B) IL-12Rβ2, IL-23R, IL-1RI and IL-17RE mRNA expression by classic (n=15) and non-classic Th1 (n=15), Th17 (n=15) and Th17/Th1 (n=15) clones was evaluated by real time quantitative RT-PCR. Results were normalized to GAPDH mRNA and shown as mean values + SE. Data shown were obtained in 3 different experiments. A standard two-tailed unpaired t-test was used for statistical analysis.

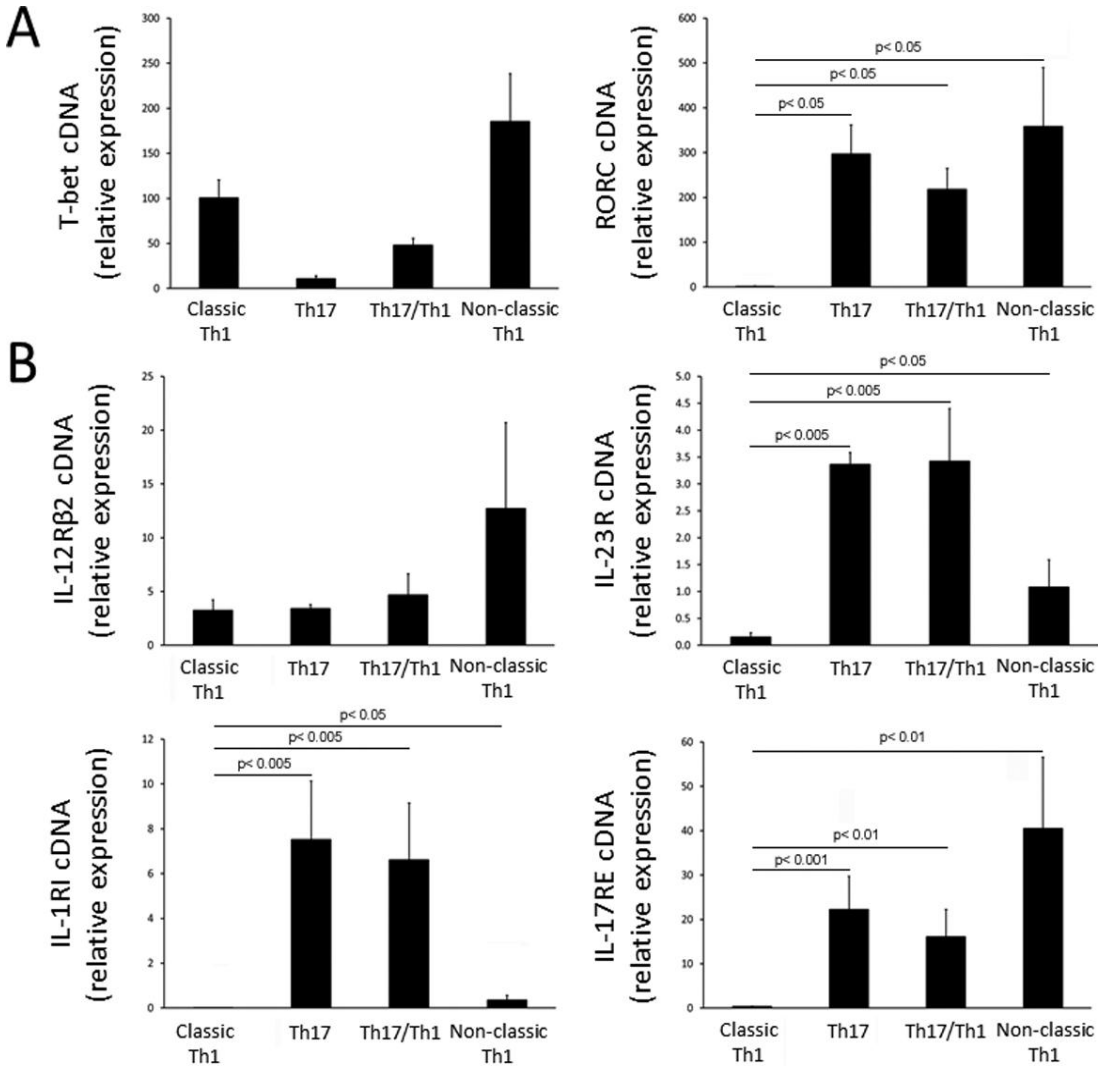

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Fig. 3: Chemokine receptors expression by classic and non-classic Th1, Th17/Th1 and Th17 clones.

(A) CCR6 and CXCR3A mRNA expression by classic (n=15) and non-classic Th1 (n=15), Th17 (n=15) and Th17/Th1 (n=15) clones was evaluated by real time quantitative RT-PCR. Results were normalized to GAPDH mRNA and shown as mean values + SE. A standard two-tailed unpaired t-test was used for statistical analysis.

(B) CCR6 and CXCR3A protein expression by classic (n=9) and non-classic Th1 (n=9), Th17 (n=9) and Th17/Th1 (n=9) clones was evaluated by flow cytometry and shown as mean values + SE. A standard two-tailed unpaired t-test was used for statistical analysis. A representative flow cytometric analysis of each Th-cell population (classic and non-classic Th1, Th17, Th17/Th1 clones) is depicted (bottom).

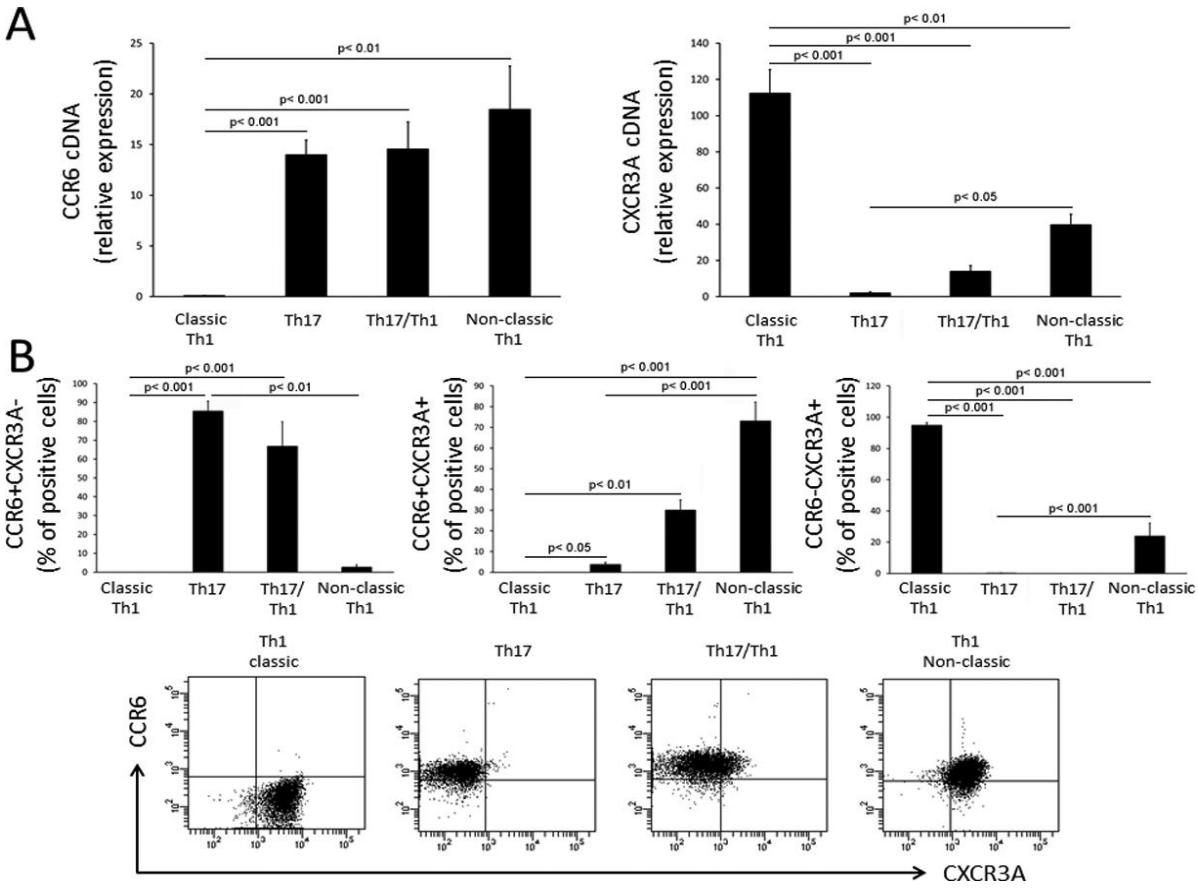

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Fig. 4. IL4I1 mRNA expression and proliferation in response to anti-CD3 plus anti-CD28 stimulation of classic and non-classic Th1, Th17/Th1, and Th17 clones.

(A) IL4I1 mRNA expression by classic (n=15) and non-classic Th1 (n=15), Th17 (n=15) and Th17/Th1 (n=15) clones was evaluated by real time quantitative RT-PCR. Results were normalized to GAPDH mRNA and shown as mean values + SE. (B) Classic (n=20) and non-classic Th1 (n=20), Th17 (n=20) and Th17/Th1 (n=20) clones were stimulated with anti-CD3 plus anti-CD28 and then assessed on day 5 for proliferation by evaluating ^{3}H-TdR uptake. Data are shown as mean values + SE. Data shown were obtained in 4 different experiments. A standard two-tailed unpaired t-test was used for statistical analysis. (C) Classic and non-classic Th1 clones and Th17 clones were evaluated for IFN-γ and IL-17 production at single cell level upon 5 hours activation with PMA plus ionomycin and assessed for proliferation upon stimulation with anti-CD3 plus anti-CD28, in absence or presence of IL-2, on day 5 by evaluating ^{3}H-TdR uptake. Correlation between frequencies of cytokines producing cells and ^{3}H-TdR uptake was performed by using Pearson's correlation coefficient.

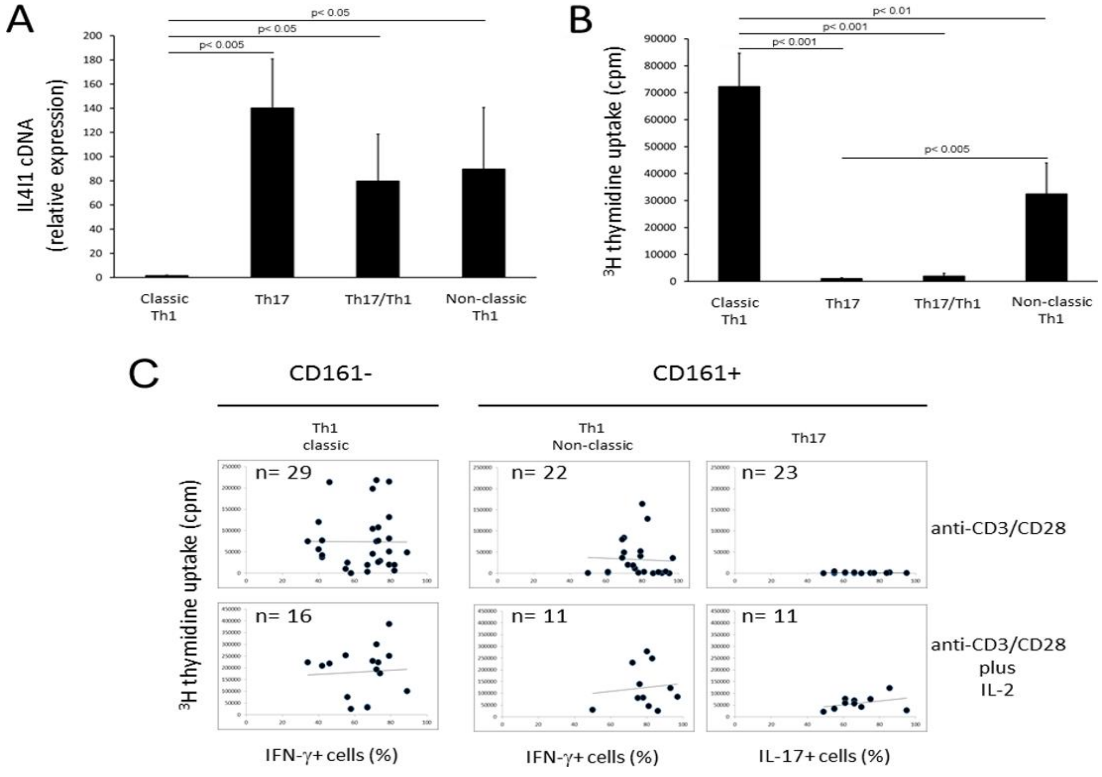

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Fig. 5: Differences between classic and non-classic Th1 clones are also present on ex vivo-derived cell subsets from both PB of healthy donors and SF of JIA patients

(A) PB of healthy subjects (n=3) and (B) SF of JIA patients (n=3) were stimulated with PMA plus ionomycin, and sorted by FACS into $CD3^+CD4^+CD8^-CD161^-IL\text{-}17^-IFN\text{-}\gamma^+$ (classic Th1) and $CD3^+CD4^+CD8^-CD161^+IL\text{-}17^-IFN\text{-}\gamma^+$ (non-classic Th1) cells. On day 7 each cell fraction was assessed for T-bet, RORC, IL4I1, IL-12Rβ2, IL-23R, IL-1RI, IL-17RE, CCR6 and CXCR3A, mRNA expression by real time quantitative RT-PCR. Results were normalized to GAPDH mRNA and shown as mean values + SE. Data were obtained in 3 different experiments. *p<0.05; **p<0.01; a standard two-tailed unpaired t-test was used for statistical analysis.