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The Bromodomain and Extra-Terminal Protein Inhibitor OTX015 Suppresses T Helper Cell Proliferation and Differentiation

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Abstract: Background: Dynamic epigenetic alterations accompanying CD4⁺ T helper cell differentiation have been implicated in multiple autoimmune diseases. The bromodomain and extra-terminal (BET) proteins are epigenetic regulators that recognize and bind to acetylated histones in chromatin and are targets for pharmacological inhibition. In this study we tested a new BET inhibitor under clinical development, OTX015, to interrogate its effects on key CD4⁺ T cell subsets associated with autoimmunity.

Methods: Naïve and memory murine and human CD4⁺ T cells were isolated and differentiated into populations characterized by the expression of interferon (IFN)- γ and interleukin (IL)-17. Cultured cells were then exposed to varying concentrations of OTX015 *in vitro*, and its impact on cytokine expression was quantified by flow cytometry. In parallel, the expression of the transcription factors TBX21 and RORC was quantified by PCR. A previously studied BET inhibitor JQ1 was used as a pharmacological control.

Results: OTX015 suppressed both murine and human CD4⁺ T cell proliferation. Its impact on cytokine expression varied in murine and human naïve and memory subsets. Higher concentrations of OTX015 also had a greater impact on the viability of murine versus human cells. IL-17 and IFN- γ expression was not altered in murine memory CD4⁺ T cells, whereas in human memory CD4⁺ T cells, OTX015 inhibited IL-17, but not IFN- γ . Across all human T cell subsets OTX015 suppressed IL-17 more effectively than IFN- γ .

Conclusion: Our studies demonstrate that OTX015 has anti-inflammatory effects by suppressing murine and human CD4⁺ T cell proliferation and subset-dependent proinflammatory cytokine expression, including the selective suppression of IL-17 in human memory CD4⁺ T cells.

Keywords: Autoimmune disease, CD4⁺ T cells, OTX015, bromodomain and extraterminal domain (BET) inhibitors, epigenetics, immunosuppression, antiproliferation

1. INTRODUCTION

Aberrant activation and expansion of CD4⁺ T helper (Th) cells and their secreted effector cytokines underlie the pathogenesis of many autoimmune diseases. The mechanism by which CD4⁺ T cells are abnormally activated and proliferate in these disorders remains elusive, but epigenetic dysregulation has been implicated in a range of conditions, including systemic lupus erythematosus rheumatoid arthritis, uveitis, systemic sclerosis and type 1 diabetes [1-4]. Epigenetic modulation empowers the differential expression of lineage-specific signature genes in T cells to dictate T cell fate and function and regulate the immune system [5]. Consequently, therapeutics targeting epigenetic proteins are being developed to fine-tune the immune response with a view to clinical application in the treatment of autoimmune disorders [6,7].

Chromatin regulators are a class of proteins that affect gene expression by influencing the accessibility of transcription factors and co-factors to chromosomal DNA. Typically, chromatin regulators include writer proteins that add post-translational modifications (PTMs) to histones, reader proteins that recognize PTMs, and eraser proteins that remove PTMs from histones [8]. Bromodomain extra-terminal (BET) family proteins belong to chromatin readers. They are comprised of four members: BRD2, BRD3, BRD4, and BRDT [9]. BET proteins recognize acetylated lysine residues through their bromodomains, recruit transcriptional coactivators, and promote pro-inflammatory and oncogenic gene expression [9,11], and it has been reported that BET inhibition

promotes cell differentiation, inhibits proliferation, and suppresses inflammation [10]. Targeted inhibition of BET proteins has therefore been shown to be protective in several autoimmune disease models [12-15].

In 2010 the first two BET inhibitors, JQ1 and I-BET, were reported simultaneously from two different groups [15,16]. JQ1 suppresses inflammatory responses mediated by Th17 through disruption of BRD4 functioning and its downstream signaling cascades [14,17]. However, its systemic toxicity and insufficient pharmacokinetic effect *in vivo* have limited its potential for clinical application [18,19]. A new BET inhibitor, OTX015, has been reported to be better tolerated in clinical trials for hematologic malignancies and solid tumors [20,21]. Given the potential to extend its application to autoimmune conditions, we sought to interrogate the effect of OTX015 on the classical proinflammatory CD4⁺ T cell subsets *in vitro* as the first stage in its pre-clinical evaluation for therapeutic application. In this study we show that murine and human subsets of CD4⁺ T cells respond differently to OTX015 and that OTX015 preferentially acts on human Th17 cells.

2. MATERIALS AND METHODS

2.1. Healthy Controls

CD4⁺ T cells were obtained from up to 80 ml peripheral blood from healthy controls (HCs) following informed consent in accordance with National Health Service Research Ethic Committee approved protocols at the University

Hospitals Bristol Foundation Trust, United Kingdom (04/Q2002/84). Written informed consent was obtained from all study participants.

2.2. Materials

Both OTX015 and JQ1 (Strattech Scientific, UK) were dissolved in DMSO to make a stock solution of 10 mM. Aliquots were kept at -80°C for up to 6 months and diluted with appropriate culture media for *in vitro* use. Murine antibodies for cell culture (anti-CD3 ϵ , anti-CD28, anti-IL-4, anti-IFN- γ) and flow cytometry (anti-IL-17, anti-IFN- γ) were purchased from eBioscience (USA) and human antibodies (anti-IL-17, anti-IFN- γ) were purchased from Biolegend (USA) or eBioscience (USA). Sorting antibodies (anti-CD3, anti-CD4, anti-CD44, anti-CD62L for murine and anti-CD45RA, anti-CD45RO, anti-CCR7, anti-CCR6, anti-CCR4, anti-CXCR3 for human) were purchased from Biolegend (USA). Recombinant murine IL-6 and IL-12 were purchased from PeproTech (USA) and recombinant human TGF- β was purchased from R&D systems (USA).

2.3. Cell Isolation

CD4⁺ T cells from HCs were enriched by negative selection using the RosetteSep® Human CD4⁺ T cell Enrichment Cocktail (Stemcell Technologies, Canada) according to the manufacturer's instruction. CD4⁺ T cells were removed from the interface between the plasma and density gradient and washed twice with RPMI-1640 containing 10% (v/v) fetal calf serum (FCS) (Gibco, USA). The purity of CD4⁺ T cells achieved was greater than 95%. For murine CD4⁺ T cell isolation, cells from lymph nodes and spleens were isolated

and processed into single cell suspension. CD4⁺ T cells were isolated using the CD4 (L3T4) Microbeads (Miltenyi Biotec, Germany) according to manufacturer's instruction. The purity of CD4⁺ T cells achieved was greater than 95%.

2.4. Fluorescence-Activated Cell Sorting (FACS)

Non-specific murine antibody binding was blocked by incubating cells with 24G2 supernatant for 10 minutes at 4°C followed by incubation with the proliferation tracking dye, CellTrace™ Violet (ThermoFisher Scientific, USA) for 20 minutes at 37°C. The cell surface expression of CD44 and CD62L was used to phenotypically discriminate murine naïve (CD44^{low}CD62L^{high}) from memory (CD44^{high}CD62L^{low}) cells. Similarly, human CD4⁺ T cells were stained with CellTrace™ Violet, and then either sorted for naïve, memory T cells or Th subsets (Supplementary Figure 1) according to the following cell surface expression: naïve (CD45RA⁺CD45RO⁻CCR7⁺), central memory (CD45RO⁺CD45RA⁻CCR7⁺), effector memory (CD45RO⁺CD45RA⁻CCR7⁻), Th1 (CCR6⁻CXCR3⁺CCR4⁻), Th17 (CCR6⁺CXCR3⁻CCR4⁺) or IFN- γ & IL-17 double positive cells (CCR6⁺CXCR3⁺CCR4⁻). All FACS was performed using either the BD Aria (murine) or the BD Influx Systems (human) (BD Biosciences, USA) and routinely > 95% purity was achieved.

2.5. Flow Cytometry

As for FACS surface staining, nonspecific antibody binding was blocked with 24G2 supernatant followed by incubation with primary antibodies against

surface markers. Intracellular cytokine analysis was carried out by incubating the cells with 20 ng/ml PMA, 1 μ M ionomycin and 1 μ l/ml Golgistop (BD Bioscience, USA) for the last 4 hours of culture. Cells were fixed, permeabilized with the Cytofix/perm solution (BD Bioscience, USA) and stained with intracellular cytokine antibodies. Cells were washed with PBS containing 2% FCS twice. The LIVE/DEAD™ fixable dead cell stain kit (Thermofisher Scientific, USA) was used to exclude dead cells from analysis. All samples were acquired using the BD™ LSR II or BD LSR Fortessa™ X-20 (BD Bioscience, USA) and data were analyzed with FlowJo v10 (Tree Star Inc, USA).

2.6. Murine CD4⁺ T Cell *In Vitro* Assays

To measure cell proliferation, isolated CD4⁺ T cells were stained with CellTrace™ Violet and seeded into 96-well plates (0.1 million cells/well) pre-coated with 5 μ g/ml anti-CD3 and 2 μ g/ml anti-CD28. RPMI1640 Culture media was supplemented with polarization cytokines and BET inhibitor: Th1 (20 ng/ml mIL-12, 100 ng/ml anti-IL-4) and Th17 (20 ng/ml mIL-6, 1 ng/ml TGF- β 1, 100 ng/ml anti-IL-4, 100 ng/ml anti-IFN- γ). All cells were cultured with RPMI-1640 containing 10% FCS, Pen/Strep and 2-mercaptoethanol in a humidified incubator containing 5% CO₂ at 37°C.

2.7. Human CD4⁺ T Cell *In Vitro* Assays

CD4⁺ T cells or FACS sorted naïve, memory T cells or Th subsets were seeded in 96 well microplates (0.1 million cells/well), stimulated with Dynabeads™ Human T-Activator CD3/CD28 (Gibco, USA) and BET inhibitor.

Proliferation and intracellular cytokine expression were assessed on day 5 following 4 hours of PMA and ionomycin stimulation. All cultures were incubated in a humidified incubator containing 5% CO₂ at 37°C.

2.8. Quantitative Real-Time PCR (qPCR)

Total RNA was extracted using RNeasy Plus Mini Kit and micro Kit (Qiagen, Germany) dependent on input cell number. RNA was reverse transcribed using GoScript reverse transcriptase (Promega, USA) in ProFlex PCR system (Applied Biosystems, USA). qPCR was performed using QuantStudio 3 System (ThermoFisher Scientific, USA). The following Taqman probes from Applied Biosystems were used: Actin-Beta (Hs_0160556_g1), RORC (Hs_01076122_m1) and TBX21 (Hs00203436_m1).

2.9. Statistics

The statistical analyses were performed with Prism Graphpad 7.0 (GraphPad Software, USA). Mann-Whitney U test, Kruskal-Wallis test or two-way ANOVA test were used accordingly.

3. RESULTS

3.1. OTX015 differentially affects murine naïve and memory CD4⁺ T cells

JQ1 has previously been shown to suppress cytokine expression in Th17 cells [14]. As OTX015 is a new BET inhibitor, we first tested whether OTX015 could suppress Th1 and Th17 subsets induced from FACS sorted murine naïve CD4⁺ T cells. We polarized these cells into Th1 or Th17 subsets in the presence

or absence of 50 nM of OTX015 for 72 hours (Figure 1A and 1B), and found that in our *in vitro* system OTX015 had a more profound effect on IFN- γ from induced Th1 than on IL-17 from Th17 cells. The optimal concentration of OTX015 and treatment duration were selected according to the cell viability and the expression of cytokines from dose-dependent and time-course studies (Supplementary Figure 2). At 50 nM, no significant viability change was observed between OTX015 treated or untreated control cultures (Supplementary Figure 2). However, at higher concentrations, OTX015 was more detrimental to the viability of murine than human CD4⁺ T cells. In addition, we confirmed that OTX015 and JQ1 had equivalent inhibitory effects on murine CD4⁺ T cell subsets, as indicated by the cytokine production and cell proliferation of Th1 and Th17 subsets after treatment (Supplementary Figure 3). These results indicated that OTX015 could efficiently suppress differentiation of CD4⁺ T cells from naïve cells.

Naïve and memory CD4⁺ T cells have different roles in the immune system. Their epigenetic status and epigenetic alterations toward stimulation are also different [22-24]. We therefore investigated the effect of OTX015 on stimulated memory CD4⁺ T cells. Murine CD4⁺ memory T cells were also sorted by FACS and stimulated with anti-CD3/CD28 in the presence or absence of OTX015 for 24 hours. Strikingly, OTX015 had no effect on the production of IFN- γ or IL-17 in murine memory CD4⁺ T cells (Figure 1C and 1D). These results indicate that different subtypes of CD4⁺ T cells may respond differently to epigenetic

regulators.

3.2. OTX015 preferentially inhibits IL-17 over IFN- γ in human CD4⁺ T cells

To test the potential to translate our murine findings to human disease, we applied OTX015 on human CD4⁺ T cells for 5 days. OTX015 suppressed, in a dose-dependent manner, both human CD4⁺ T cell proliferation (Figure 2A and 2B) and the frequency of IFN- γ single-positive, IL-17 single-positive, and IL-17/IFN- γ double-positive subsets in a mixed population of human CD4⁺ T cells (Figure 2C – 2F). Unlike murine CD4⁺ T cells, the cell viability change was less than 10% at the highest dose of OTX015 (Supplementary Figure 4). Between the two main proinflammatory cytokines IFN- γ and IL-17, OTX015 preferentially inhibited IL-17. At 312.5 nM, OTX015 restrained more than 50% of IL-17 expression but failed to significantly suppress IFN- γ . Next, we next conducted a comparison of JQ1 and OTX015, and found that there was no significant difference between these two BET inhibitors in either affecting cell proliferation or cytokine production (Supplementary Figure 5). These results were different from that of murine cells, prompting us to question whether OTX015's differential effect on naïve and memory T cells would be consistent across species.

3.3. OTX015 preferentially suppresses IL-17 in human memory CD4⁺ T cells.

Next, we tested OTX015 on different human CD4⁺ T cell subpopulations. Human naïve and memory CD4⁺ T cells subsets were isolated by FACS and activated *in vitro* with or without OTX015 for 5 days. As shown in Figure 3A and 3B, OTX015 dose-dependently suppressed the proliferation of all subsets without significantly affecting the cell viability of naïve T cells and memory cells at lower doses of OTX015 (Supplementary Figure 6). As for cytokines, IL-17 was barely detectable in the naïve CD4⁺ T cells, while IL-17 single-positive cells accounted for approximately 3.8% and 5.7% in the effector (TEM) and central (TCM) memory T cells, respectively. OTX015 significantly suppressed the proliferation and frequency both of IL-17 single-positive and IL-17/IFN- γ double-positive in TEM and TCM in a dose-dependent manner (Figure 3C, 3D and 3F). However, OTX015 had limited suppressive effects on the production of IFN- γ from naïve cells and even caused a slight increase in IFN- γ production from TEM (Figure 3E). Consistent with this, for naïve CD4⁺ T cells, there was no significant change in the level of *TBX21* and *RORC* between OTX015 treated and control group. But in TCM, *RORC* was significantly inhibited by OTX015. In TEM cells, we saw an increase in *TBX21* and a decrease in *RORC* by OTX015 (Figure 3G).

3.4. OTX015 inhibits Th1, Th17 and IFN- γ & IL-17 double positive cell proliferation but preferentially acts on Th17 cytokines in human Th subsets

To study the effect of OTX015 on human CD4⁺ T cell subsets in more detail, Th1, Th17 and IFN- γ & IL-17 double positive cells were sorted based on the expression of chemokine receptors and activated for 5 days with 2,500 nM OTX015. As shown in Figure 4A and 4B, OTX015 significantly suppressed the proliferation of all three cell subsets. On day 5, Th1 and IFN- γ & IL-17 double positive cells primarily produced IFN- γ , while Th17 cells primarily produced IL-17 (Figure 4C). OTX015 was able to significantly reduce IL-17 production from Th17 cells but had limited ability to suppress the production of IFN- γ from Th1, or to suppress either IL-17 or IFN- γ from IFN- γ & IL-17 double positive cells (Figure 4D-4G). This result is highly consistent with that in human memory T cells.

4. DISCUSSION

In this study, we have shown that the BET inhibitor OTX015 significantly inhibits CD4⁺ T cell proliferation and differentially suppressed their expression of IL-17 and IFN- γ across murine and human CD4⁺ T cell subsets. Our findings were confirmed with detailed dose-response and time-course studies in both murine and human CD4⁺ T cells. BET inhibitors have been extensively studied in cancer and inflammatory diseases. JQ1 is a thoroughly-researched representative BET inhibitor and is reported to selectively block IL-17A and IL-22 in human naïve CD4⁺ T cells under Th17 polarizing conditions and to block

IFN- γ under Th1 biased conditions [14]. I-BET-762 has been also reported to downregulate IL-17 and IFN- γ in murine T cells [25]. However, JQ1 has a relatively short half-life in the plasma: 0.9 hours with intravenous injection of 5 mg/kg or 1.4 hours with oral administration of 10 mg/kg [26]. Moreover, it has been shown to cause severe weight loss and leukopenia in mice [19]. These drawbacks limit the clinical application of JQ1. OTX015, as a new BET inhibitor, belongs to the azepine chemical family like JQ1. It is the first oral BET inhibitor which has been tested in clinical trials in oncology [20, 21, 27]. Our results have shown that OTX015 and JQ1 have similar inhibitory effects on the inflammatory cytokine production from murine and human CD4⁺ T cells. Given that OTX015 is much safer and more stable than JQ1, OTX015 therefore has the potential to also be developed for the treatment of autoimmune diseases.

Downregulation of IFN- γ in Th1 and IL-17 in Th17 cells is a hallmark of inflammatory suppression. Th17 cells secreting IFN- γ are considered more pathogenic and are more resistant to corticosteroid suppression [28]. Our results revealed that OTX015 reduced the frequencies of IFN- γ and IL-17 single-positive and IFN- γ /IL-17 double-positive populations in whole human CD4⁺ T cells, which indicates a protective role of OTX015 in the inflammatory context. But it remains a caveat that OTX015 did not significantly suppress IFN- γ & IL-17 double positive cells in polarized Th17 cultures. However, given that it is well-established that both the activation and proliferation of T cells have important roles in the pathogenesis of autoimmune disorders. Our data suggest

that the anti-proliferative effect of OTX015 may work synergistically with its anti-inflammatory effect to treat autoimmune diseases.

Although naïve and memory CD4⁺ T cells have over 95% similarity in global gene expression, they display different expression patterns of immune-related genes, which reflects the difference in epigenetic status of these two subsets [22]. In support of this notion, previous studies showed that T cells exhibited epigenetic remodeling between initial stimulation and subsequent re-stimulation [23, 24]. Our results demonstrate that naïve and memory T cells respond differently to the epigenetic “reader” inhibition. OTX015 could efficiently suppress IFN- γ in induced murine Th1 cells, but not spontaneously secreted IFN- γ from murine memory T cells. The results were replicated using human total and memory T cells. Our results therefore hint that the epigenetic context must be considered when applying BET targeting compounds. It is also interesting that IL-17 but not IFN- γ was suppressed in human memory CD4⁺ T cells. This may be therapeutically desirable in the context of chronic inflammation as persistent IFN- γ expression has potential benefit in the late stages of conditions such as autoimmune uveitis [29].

Our results showed that both Th cell proliferation and cytokine production were suppressed by OTX015. To exclude a general suppressive effect, we performed qPCR to quantify the mRNA expression of critical transcription factors in human CD4⁺ T cell subsets. *RORC* but not *TBX21* was suppressed in human central memory T cells by OTX015. In TEM, we even saw an elevated

expression of *TBX21*, which is different from that in naïve CD4⁺ T cell subsets. These results echo the protein changes reported for JQ1, in which there was reduction of RORC and RORA, but not TBX21, GATA3 or FOXP3 in corresponding CD4⁺ T cell subtypes [14].

CONCLUSION

In summary, we have shown that the BET inhibitor OTX015 can efficiently suppress the proliferation of murine and human Th cells as well as subset-specific production of their pro-inflammatory cytokines. In addition, we have also demonstrated that both murine and human naïve CD4⁺ T cells reacted differently to OTX015 when compared to murine and human memory CD4⁺ T cells. These results indicate the potential benefits and also immune context-dependent caveats of developing BET inhibitors to target inflammatory diseases.

ABBREVIATIONS

PTM = post-translational modifications

BET = Bromodomain extra-terminal protein

TCM = central memory T cells

TEM = effector memory T cells

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All the participants were informed consent in accordance with National Health Service Research Ethic Committee approved protocols at the University Hospitals Bristol Foundation Trust, United Kingdom (04/Q2002/84).

HUMAN AND ANIMAL RIGHTS

All the procedures involving human subjects were compliant with the ethical guideline of the 1975 Declaration of Helsinki and its subsequent revisions. All animal work was performed in compliance with UK legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039).

CONSENT FOR PUBLICATION

Written informed consent was obtained from all study participants.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Moorfields Eye Hospital (National Health Service) Foundation Trust and University College London Institute of Ophthalmology. The views expressed are those of the authors and not necessarily those of the National Health Service, the National Institute for Health Research, or the Department of Health.

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FIGURE LEGENDS

Figure 1. Effects of OTX015 on murine naïve and memory CD4⁺ T cells.

Murine CD4⁺ T cells were treated with or without 50 nM OTX015 for 72 hours (naïve T cells) or 24 hours (memory T cells). (A) Representative dot plots of IFN- γ and IL-17 in induced Th1 and Th17 from murine naïve CD4⁺ T cells. (B) Frequency of IFN- γ -expressing Th1 or IL-17-expressing Th17 induced from murine naïve CD4⁺ T cell (n = 5). (C) Representative dot plots of IFN- γ and IL-17 in stimulated memory CD4⁺ T cells. (D) Frequency of IFN- γ - or IL-17-expressing cells in the stimulated memory CD4⁺ T cell population (n = 4). Data are shown as means \pm SD, **** $P < 0.001$.

Figure 2. Effects of OTX015 on human CD4⁺ T cells.

Human CD4⁺ T cells were isolated and stimulated with anti-CD3/CD28 for 5 days in the presence of escalating concentrations of OTX015 on day 5. (A) Representative histograms showing the proliferation of stimulated human CD4⁺ T cells. (B) Suppression of proliferation of the stimulated human CD4⁺ T cells (n=12). (C) Representative dot plots of IL-17- and IFN- γ - expressing cells in

stimulated human CD4⁺ T cells. (D-F) Frequency of IL-17 (D), IFN- γ (E) and IL-17 (F), IFN-g double positive in human whole CD4⁺ T cells treated with different concentration of OTX015 (n=12). Data are shown as means \pm SD, ** $P < 0.01$ *** $P < 0.005$ **** $P < 0.001$.

Figure 3. Effects of OTX015 on human naïve and memory CD4⁺ T cells.

Isolated human CD4⁺ T cells were sorted into naïve or memory subsets. The memory subsets were stimulated with anti-CD3/CD28 for 5 days. Cells were treated with or without varying doses of OTX015 on day 5. (A) Representative histograms showing the proliferation of different human CD4⁺ T cell subsets. (B) Representative dot plots of IL-17⁻ and IFN- γ ⁻ expressing cells in different human CD4⁺ T cell subsets. (C) Suppression of proliferation of different human CD4⁺ T cell subsets (n \geq 4). (D-F) Frequencies of IL-17⁻ (D) and IFN- γ ⁻ (E) expressing or double positive (F) cells in different human CD4⁺ T cell subsets (n \geq 4). (G) The mRNA levels of transcription factors in different human CD4⁺ T cell subsets (n=4). Data are shown as means \pm SD, * $P < 0.05$ ** $P < 0.01$ *** $P < 0.005$ **** $P < 0.001$. TCM: central memory T cells; TEM: effector memory T cells.

Figure 4. Effects of OTX015 on human Th1, Th17 and IFN- γ & IL-17 double positive cells subsets.

Human Th1, Th17 and IFN- γ & IL-17 double positive (DP) cells subsets were

sorted by corresponding chemokine receptors and treated with 2,500 nM

OTX015 for 5 days. (A) Representative histograms showing the proliferation

of different human T cell subsets. (B) Suppression of proliferation of different

human T cell subsets (n=6 for Th1 and n=7 for Th17/DP cells). (C)

Representative dot plots of IL-17- and IFN- γ -expressing cells in different

human T cell subsets. (D-G) Frequencies of IL-17- and IFN- γ -expressing cells

in different human T cell subsets (n=6 for Th1 and n=7 for Th17/DP cells).

Data are shown as means \pm SD, ** $P < 0.01$. DP cells = IFN- γ & IL-17 double

positive cells.