DIFFERENTIATION OF HUMAN NAIVE CD4+ T CELLS INTO Th17 CELL PHENOTYPE

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ABSTRACT

DIFFERENTIATION OF HUMAN NAIVE CD4+ T CELLS INTO Th17 CELL PHENOTYPE

Th17 cells are recently identified CD4+ T cell subset and best defined as their secretion of IL17, an inflammatory cytokine, and their role in host defense and autoimmunity. Further information on Th17 cell subset is strongly correlated with the differentiation and maintenance of these cells in culture. Although the master regulators and culture conditions in mouse Th17 differentiation are defined, the requirements and maintenance of human Th17 cell cultures remain unclear. Here, we suggest a new culture condition that maximizes IL17 expression and gives minimum IL17+IFNg+ and IL17+Foxp3+ among human studies. Our data define the doses and combinations of various cytokines that rise IL17 expression, as well as regulatory molecules in human Th17 cell differentiation. Various combinations of cytokines reveal that IL1 is the most important cytokine that primes Th17 differentiation. Also it was observed that TGF positively regulates Th17 differentiation in a dose-dependent manner by inhibiting IFN expression thus represses Th1 differentiation and impairs Treg polarization at transcription factor state by tightly regulating Foxp3. Although it was suggested that Th17 cells must express RORC2, the master regulator, a minor cell population that expresses IL17, but does not co-express RORC2 was observed.

ÖZET

NSAN KAYNAKLI NA F CD4+ T HÜCRELER N N Th17 HÜCRE FENOT P NE FARKLILA TIRILMASI

Th17 hücreleri IL17 enflamatuar sitokinini salgılayan ve konak savunması ve otoimmünitede olan rolleri ile son zamanlarda tanımlanmı CD4+ T hücre alt grubudur. Th17hücreleri ile ilgili bilgiler onların hücre kültürü artlarında farklıla maları ve devamlılı ı ile ilgilidir. Th17 hücrelerinin ana düzenleyici faktörleri ve kültür artları farede belirlenmesine ra men, insanda hala net olarak belirlenememi tir. Yapılan bu çalı mada, insan çalı maları arasında en yüksek IL17 ve en dü ük IL17+IFN +, IL17+Foxp3+ ekspresyonu artını sa layan kültür ko ulu belirlenmi tir. Sonuçlarımız göstermektedir ki, insan Th17 farklıla masında, farklı sitokin kombinasyonları ve dozlarının kullanılması, IL17 ekspresyonunun yanı sıra bazı düzenleyici moleküllerin de artmasını sa lamaktadır. Farklı sitokin kombinasyonlarının denenmesi sonucu IL1 'nın Th17 farklıla masında en etkili molekül oldu u tespit edilmi ir. Ayrıca, TGF molekülü doza ba lı olarak Th17 farklıla masını, Th1 farklıla masını IFN , Treg farklıla masını Foxp3 molekülünü baskılayarak olumlu etkiledi i belirlenmi tir. Son olarak, Th17 hücrelerinin ana düzenleyici molekül olan RORC2 molekülünü eksprese etmeleri gerekti i bilgisine ra men küçük bir grup hücrenin IL17 eksprese ederken RORC2'yi eksprese etmedi i gözlenmi tir.

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ABBREVIATIONS

CD	Cluster of Differentiation
APC	Antigen Presenting Cells
CD4+	Helper T Cell
IL	Interleukin
Th	T helper
Treg	Regulatory T Cell
IFN	Interferon Gamma
Foxp3	Forkhead box P3
RORC2	Retinoic acid receptor-related orphan receptor C2
TGF	Transforming Growth Factor Beta
CD25	Alpha Chain of the IL-2 Receptor
STAT	Signal Transducer and Activator of Transcription
T-bet	T-box Expressed in T cells
GATA3	GATA Binding Protein 3
DC	Dendritic Cell
GMCSF	Granulocyte-macrophage Colony-stimulating Factor
Runx1	Runt-related Transcription Factor 1
IRF-4	Interferon Regulatory Factor 4
ANN	Annexin
7AAD	7-Amino-actinomycin D
IMDM	Iscove's Modified Dulbecco's Medium

CHAPTER 1

INTRODUCTION

1.1. T Cells

The immune system has developed to defend the body against invading pathogens, internal and external factors that disrupt homeostasis. The defense mechanism is so complicated and requires immune cells to have an unlimited repertoire of receptors to recognize pathogens. The recognition process is antigen-specific and is accomplished by the unique cell surface receptors of lymphocytes. The engagement of receptor with its complement antigen leads to clonal expansion of antigen-specific lymphocytes which have the capacity to clear the invading pathogen directly or indirectly. The key point is to distinguish self from non-self molecules by the immune system to maintain homeostasis. Receptors of immune cells are generated from random joining of gene segments. This random event gives rise to a huge repertoire of receptors which can harbor self-reactive ones that are needed to be eliminated by mechanisms of self tolerance. Otherwise self-reactive immune cells that are not eliminated by control mechanisms can cause development of autoimmunity (Jager and Kuchroo, 2010).

Thymus derived lymphocytes (T cells) are major players in immune response against invading pathogens. In order to accomplish a proper immune response, T cells must be activated to become effector and so take part in the immune system. T cell activation is a complex event that is initiated by an immune response. For a naive CD4+ T cell to become activated, a complex signal transduction that leads to transcription factor activation, cytokine production, T cell proliferation and differentiation is required. Many molecules transcribed during T cell activation are rapidly and transiently expressed and play essential roles in the development (Valle-Rios et al., 2009).

Upon interaction with antigen by antigen presenting cells, T helper cells are activated, expand and differentiate into various effector T-cell subsets. Depending on cytokine profiles, these T-cell subsets were defined and they carried out distinctive features of their subsets. The identified effector T cell lineages are Th1, Th2, Tfh, Th9,

Th17 cells and also multiple subsets of regulatory T cells including natural Foxp3^{+}CD4^{+}CD25^{+} regulatory T cells (nTreg), induced Foxp3^{+} or Foxp3^{-} regulatory T cells (iTreg) including Tr1 and Th3 cells (Jager and Kuchroo, 2010).

1.2. Th1 and Th2 Cell Subsets

Th1 cells are a subset originated from naïve CD4+ T cells and important for host defense against intracellular pathogens. Th1 cells predominantly secrete interferon (IFN)- and lymphotoxin, which mainly serve to activate macrophages and provide recruitment at the site of inflammation and also provide help for B cells to produce isotypes of immunoglobulins especially IgG2a (Coffman, 2006, Mosmann and Coffman, 1989). In addition, Th1 cells express genes associated with cytotoxicity, such as Fas-ligand and granzymes. Uncontrolled Th1 responses against self-antigens can lead to the development of autoimmunity (Yu et al., 2003).

Th1 cells are generated from naive T helper cells by TCR engagement and STAT1 signaling, induced by activation of the IFN- R with IFNs. Phosphorylated STAT1 induces expression of the transcription factor T-bet, which then drives Th1 differentiation by trans-activating the Th1 signature cytokine IFN- and the specific subunit of the receptor for interleukin (IL)-12, IL-12R 2. Thus, the cell becomes responsive to IL-12, which is produced by activated APCs, and subsequent IL-12 signaling through STAT4 further stabilizes the Th1 phenotype (Yang et al., 1999).

Th2 cells, on the other hand, were described to have very different effector functions, as clearing extracellular organisms like parasites and helminths. In addition, Th2 cells play a very important role in eosinophilic inflammation and IgE production in allergic reactions and asthma (Ho and Glimcher, 2002, Murphy and Reiner, 2002).

Differentiation of naive T cells into Th2 cells is driven by activation of T cells via TCR and IL-4 receptor, which leads to phosphorylation of STAT6. pSTAT6 is critical for the induction of the Th2 transcription factor GATA3, which in turn trans-activates by driving epigenetic changes in the cluster of Th2-specific cytokines such as IL-4, IL-5 and IL-13 (Weaver et al., 2006), while down-regulating Th1-related factors such as STAT4 and IL-12R 2 (Cua et al., 2003). In addition, the transcription factor c-maf contributes to Th2 differentiation by trans-activating IL-4 transcription (Zhang et al., 2003).

Briefly, cytokines and transcription factors expressed by Th1 and Th2 show stimulatory effect on themselves but act as suppressor for each other. The Th2 cytokine IL-4 strongly inhibits Th1 differentiation and, vice versa, the Th1-cytokines IL-12 and IFN- inhibit Th2 differentiation (Jäger and Kuchroo, 2010). T-bet directly inhibits Th2 differentiation by binding to its master regulator GATA-3, preventing it from DNA binding and activation of Th2 specific genes (Chen et al., 2006, Hwang et al., 2005). Likewise, GATA-3 can down-regulate STAT-4, a transcription factor important for Th1 differentiation (Usui et al., 2003). Binding to GATA-3 target sites on Foxp3 promoter, GATA-3 also negatively regulates Foxp3 expression thereby inhibits Treg differentiation (Mantel et al., 2007). Immature myeloid DCs express Jagged-1, which triggers Th2 polarization in CD4+ T cells, whereas stimulation of Toll-like receptors on DCs upregulates the Delta-4 ligand, which triggers an opposite Th1 polarizing program (Liotta et al., 2008).

1.3. Regulatory T Cells

Lymphoid cell subsets represent suppressive or immunomodulatory properties. Foxp3-expressing Treg cells represent the only currently known population of lymphocytes whose suppressor function is crucial for the maintenance of immune homeostasis. Treg cells suppress immune responses through various mechanisms including the production of anti-inflammatory cytokines, direct cell-cell contact, and modulation of the activation state and function of antigen presenting cells (Shevach, 2009).

Regulatory T cells (Treg) control the balance of T cell repertoire against self-reactive and non infectious non-self reactive lymphocytes with a potential to cause autoimmune disease and allergy (Akdis, 2006, Hori et al., 2003). Tregs maintain immune homeostasis with distinct subsets include naturally occuring, tymus selected $CD4^+CD25^+Foxp3^+$ and inducible type 1 IL10 secreting Treg (Tr1) cells. Natural Tregs play a key role in controlling immune responses in autoimmune diseases, allergic disorders, infections, transplantation and cancer (Hori et al., 2003, Sakaguchi, 2000, Takahashi et al., 2000).

Foxp3 is the master transcription factor for Treg cell development and function. Its expression is important for Treg function to prevent immune homeostasis. Recent

studies show that loss or diminished Foxp3 expression in Treg cells leads to effector cells produce immune response promoting cytokines including IL-2, IL-4, IL-17 and IFN-   (Wan and Flavell, 2007, Williams and Rudensky, 2007).

Foxp3 induction leading to Treg cell differentiation occurs relatively late during thymic differentiation. In addition, peripheral naive CD4+ T cells are capable of Foxp3 upregulation during differentiation into so called induced Treg cells or iTreg cells (Curotto de Lafaille and Lafaille, 2009).

1.4. Th17 Cell Subset

CD4+ Th cells are accepted as the important actors in immune responses due to their capacity to regulate and coordinate other cells of the immune system. Since they are the major actors in immune response the characterization of these cells were crucial. Scientists characterized CD4+ Th cells into two subsets as Th1 and Th2, approximately 20 years ago (Mosmann and Coffman, 1989). This characterization and further experiments carried out on to these two helper subsets reveal the criteria required for defining Th cell lineages. The required criteria are T helper cells have ability to differentiate independently into each of Th subsets under both *in vitro* and *in vivo* conditions and are the cells that carry their own specific, distinctive and heritable markers (Dong and Flavell, 2001, Glimcher and Murphy, 2000).

Until recently, Th cells were thought to be a binary system, consisting of Th1 and Th2 cells. But in 2005, Harrington and Park discovered a new subset of CD4+ effector T cell family (Harrington et al., 2005, Park et al., 2005). This phenotype was characterized by IL-17 production and so called Th17. Th17 cells produce IL-17, IL-17F, and IL-22 that make them characteristic cytokines for Th17 cell subset. Expression of the IL-23R (Annunziato et al., 2007, Wilson et al., 2007) and the chemokine receptor CCR6 (Acosta-Rodriguez et al., 2007b, Annunziato et al., 2007, Hirota et al., 2007, Lim et al., 2008, Singh et al., 2008) further define this subset.

Cytokines and chemokines secreted from Th17 cells regulate various types of immune cells. Major cytokine of Th17 cell, IL-17, induces an inflammatory tissue response that results in pathogenesis of several autoimmune diseases and cancer (Eyerich et al., 2010). Th17 cytokines mainly induce expression of other cytokines such as IL-6, GMCSF and granulocyte colony stimulatory factor. On the other hand they act

on chemokines such as IL-8, CXC chemokine ligand 1, 10 and 20 and metalloproteases. According to these effects, IL-17A and IL-17F are key players on recruiting, activating and migrating neutrophils (Liang et al., 2007).

After Th17 subset was discovered scientists try to elucidate characteristics and differentiation pathway of the subset. At the beginning, most of the data were collected from mouse T cells which are more prone to differentiating into Th17 phenotype and when these results were compared with human T cells, it was observed that some of the mediators are not taking the same role as in mouse species. In contrast to mouse, human Th17 *in vitro* culture conditions and transcription factors that take role in the differentiation are still not clear.

1.4.1. Culture Conditions of Th17 Differentiation

Murine Th17 studies showed TGF- is the key cytokine with IL-6 in order to differentiate naive CD4 T cells into Th17 phenotype (Bettelli et al., 2006, Mangan et al., 2006, Veldhoen et al., 2006). This data make us to think that murine Th17 cells share similar features with Treg cells (Annunziato et al., 2008) due to the fact that TGF- alone initiates Treg differentiation by up-regulating Foxp3 transcription factor which negatively regulates Th17 differentiation, but together with IL-6, leads to Th17 phenotype (Bettelli et al., 2006, Veldhoen et al., 2006, Zhou et al., 2007). Moreover, some studies indicate that IL21 has a positive role in murine Th17 differentiation as producing autocrine signal, but in human its role is unclear and based on the observations, IL21 up-regulates IL17 and down regulates Treg cells function (Onoda et al., 2007, Peluso et al., 2007).

Contrary to mice, human Th17 differentiation condition is different so thinking of human Th17 in a different origin seems logical. According to recent studies, the role of TGF- in human is controversial. Various studies rejected the role of TGF- in human Th17 differentiation, especially they suggest IL1 , IL-23 and IL-6 are required for the process (Acosta-Rodriguez et al., 2007a, Chen et al., 2007, Wilson et al., 2007). Contrary to these papers, from the logic of being TGF- in human serum or bovine serum, in Th17 cultures, different groups showed that TGF- is necessary for human Th17 differentiation (Manel et al., 2008, Volpe et al., 2008, Yang et al., 2008a). The results about TGF- are contradictory, because these groups use different cell origin

such as, cord blood, PBMC, naive or memory cells and also the doses of the cytokines in cultures are mostly different. This makes the case difficult to evaluate because of various unstable parameters.

Among human studies Manel et. al. proposed that TGF- , IL-1 and IL-6, IL21 or IL23 were necessary and sufficient to induce IL17 expression in naive umbilical cord blood (UCB) human CD4+ T cells (Manel et al., 2008). Volpe et. al. found that TGF- , IL-23, IL-1 and IL-6 were all essential for human Th17 differentiation, individually affect Th17 produced cytokines (Volpe et al., 2008). TGF- , up-regulates RORC2 expression but inhibits its ability to induce IL17 expression because TGF- induces expression of Foxp3 transcription factor that competes with ROR t about their binding targets (Li and Greene, 2007). Another important observation is that, in the absence of TGF- , Th17 profile shifts into Th1 like phenotype (Volpe et al., 2008). Yang et.al. found that TGF- and IL21 promote Th17 differentiation of human naive CD4+ T cells and RORC2 up-regulation (Yang et al., 2008a) whereas Rodriguez et.al. observed that IL-1 and IL-6 enhance but TGF- inhibits the Th17 differentiation in human naïve CD4+ T cells (Acosta-Rodriguez et al., 2007a).

Finally, it was shown that TGF- positively regulates human Th17 differentiation in a dose-dependent manner (Zhou et al., 2008). High TGF- inhibits Th17 differentiation because of Foxp3 expression and low levels of TGF- favor Th17 differentiation indirectly by inhibiting Th1 differentiation (Bettelli et al., 2006, Zhou et al., 2008).

According to these results, TGF- may not play critical role in human Th17 cell differentiation directly, but indirectly favors their development by inhibiting the development of Th1 cells, which are more susceptible than Th17 cells to its suppressive activity on cell proliferation (Annunziato et al., 2008).

1.4.2. Mechanisms of Th17 Differentiation

Murine naive CD4+ T cells differentiate into Th17 subset directed by ROR t which is called master regulator of Th17 differentiation (Ivanov et al., 2006). ROR t is up-regulated by STAT-3, and recent data indicates that STAT-3 deficient cells represent greatly impaired ROR t expression and Th17 differentiation (Laurence et al., 2007). Normally, STAT-3 is activated in response to IL-6, IL-1 and IL-21 which are key

cytokines that take role in differentiation and maintenance of the Th17 phenotype and it directly binds to *Il17A/F* promoters (Sands, 2007, Yang et al., 2007). However, this STAT-3 signaling is not enough to promote Th17 differentiation only by itself. *Rorc* gene deficient mice studies showed that these cells are unresponsive to IL-23 stimulation which results with reduced numbers of Th17 cells and also are resistant to autoimmune diseases (Yang et al., 2008b).

In recent publications, high levels of ROR -another retinoid acid related nuclear orphan receptor- induced by TGF- and IL-6 (also STAT-3 dependent) were observed. According to their data they suggest that ROR and ROR t work synergistically to promote differentiation of Th17 cell subset (Yang et al., 2008b). IFN regulatory 4 was also shown as a player in murine Th17 differentiation (Brustle et al., 2007). Its absence in mice results in reduced ROR t expression, increased Foxp3 expression, loss of IL-17 production, and protection from experimental autoimmune encephalomyelitis (EAE). *Irf4* was also identified as a direct target of Foxp3, and its selective loss in Treg cells selectively impaired suppression of Th2 cell responses (Zheng et al., 2009). Like IRF4, retinoic acid (Mucida et al., 2007) and aryl hydrocarbon receptor (Quintana et al., 2008) have also been shown to take role in regulation of murine Th17 differentiation although their exact roles are not clear. Runx1 was recently shown to form a complex with ROR t and to cooperate with it to promote Th17 cell differentiation (Zhang et al., 2009). In Treg cells, interaction between Runx1 and Foxp3 was reported to be required for suppression of IL-2 and IFN- production, up-regulation of Treg cell-associated molecules, and suppressive activity (Ono et al., 2007).

Similar to their mouse counterparts, human Th17 cells also express RORC2, -mouse ortholog of Ror t- (Acosta-Rodriguez et al., 2007a, Annunziato et al., 2007, Wilson et al., 2007) and over-expression of RORC2 in cord blood CD4+ T cells induces expression of IL-17A, IL-17F, IL-26 and CCR6, but not IL-22, CCR4 or CCR2 (Manel et al., 2008). In adult CD4+ T cells, over-expression of RORC2 induces many aspects of the Th17 cell phenotype, including expression of CCR6, CCR4, CD161, down-regulation of CXCR3 and induction of a Th17-associated cytokine profile (Crome et al., 2009). Indeed, over-expression of RORC2 induced IL-17 production in only ~20% of transduced T cells, suggesting that expression of other transcription factors in addition to RORC2 is probably required to achieve human Th17 development fully. It is also showed that transduction of human cord blood cells with ROR- d increases IL-17

expression (Manel et al., 2008) suggesting that, as in mice, this factor may cooperate with RORC2 to induce Th17 cells.

STAT3 also appears to be critical for the development of human Th17 cells based on evidence from patients with hyper-immunoglobulin (Ig)E syndrome who carry autosomal dominant mutations in STAT3. T cells from these patients fail to differentiate into Th17 cells in vitro due to a lack of IL-6-stimulated STAT3 activation and consequently RORC2 expression (Holland et al., 2007, Ma et al., 2008). Expression of the AhR is also observed by human Th17 cells (Veldhoen et al., 2008) but it is not known if Ahr contributes to their development.

1.4.3. Importance of Th17 Cells

Th17 cells and their products are associated with the pathology of many inflammatory and autoimmune diseases. IL-17 was detected in the lesions of multiple sclerosis and also thought to compromise the blood brain barrier (Kebir et al., 2007, Tzartos et al., 2008). Surprisingly, blood brain-barrier endothelial cells express IL-17 and IL-22 receptors, treatment with IL-17 and IL-22 cause disruption of the gap junctions of these cells that increases their permeability. This allows Th17 cells to migrate across the blood brain barrier (Kebir et al., 2007). Similarly, a higher amount of IL-17 expression was detected in the synovium of rheumatoid arthritis patients than healthy population (Kotake et al., 1999). In patients of Crohn's disease and ulcerative colitis, IL-17 and IL-23 were also detected (Duerr et al., 2006, Fujino et al., 2003). In addition, recent papers revealed that Th17 products are also induced in some cancer types (Kryczek et al., 2009, Miyahara et al., 2008, Zhu et al., 2008).

Th17 cells take important roles in autoimmunity and cancer hence it is crucial to identify Th17 properties and differentiation mechanisms. In this aspect, we optimized culture conditions and expression patterns of Th cell related cytokines and transcription factors of human naïve CD4+ T cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. PBMC (Peripheral Blood Mononuclear Cells) Isolation

The whole blood was taken from healthy volunteers without no systemic disease into blood collection tubes with heparin (Becton Dickinson) by the help of IZTECH medical personnel under the consent of Ege University Hospital. PBMC isolation from whole blood was carried out by Ficoll – Hypaque Density Gradient Centrifugation (Boyum et al., 1991).

2.2. Sample Selection

PBMC samples were controlled for recent infection by activation markers CD25 and CD69. In order to get more naive population samples were analyzed by cell type specific surface and activation markers before naive CD4 isolation. According to this aim, cells were stained by fluorescence conjugated CD4, CD14, CD45RA, CD45RO and from the activation/apoptosis perspective CD25, CD69/ANN V, 7AAD antibodies. We specifically used samples that have ~25-35% CD4+, ~50-70% CD45RA+ and low amount of neutrophil, eosinophils in order to prevent plugging and non specific binding in column separation.

2.3. Naive CD4 Cell Sorting

Naive CD4+ T cells were isolated with "Human Naive CD4+ T cell isolation Kit II (Miltenyi Biotec, Bergisch Gladbach). In this method cells were separated in a negative selection manner. Appropriate amount of PBMC's incubated with biotin conjugated CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCR / , anti-HLA-DR, and CD235a (glycophorin A) antibodies for 10min. at 8ºC. These antibodies are all specific for unwanted cell populations. Then

cells were incubated with magnetic microbead conjugated anti-biotin antibodies for 15min. at 8ºC. After the antibody incubations, cells were washed with 1X PBS supplemented with 0,5% FBS and 2mM EDTA solution and centrifuged 10min at 300g in order to discard unbound antibodies. After all, cells were loaded into separation column in VarioMacs separation unit which has extremely magnetic field power. According to this magnetic field, antibody-bound unwanted cells were captured in the column and naive CD4+ T cells were collected as flow through. In order to increase efficiency of the separation collected flow through was passed through the column at least two times. After we collect the naive CD4 population, live/death cell number was determined by trypan blue staining by counting with hemocytometer. In addition, sorting efficiency was determined by immunofluorescence staining with CD4, CD14, CD45RA, CD25 antibodies.

2.4. Stimulation of Naive CD4+ T Cell (Th17 Culture Condition)

Naive CD4+ T cells were cultured with IMDM (Lonza) supplemented with 5% FBS (Gibco), Penicillin (100IU/mL), Streptomycin (100ul/mL) and 5mM -mercaptoethanol. As stimulants 5µg/ml anti-CD3 and 5µg/ml anti-CD28 (BD Biosciences) were used for TCR activation, 10ng/ml IL-1 , 10ng/ml IL-23, 30ng/ml IL-6 and 0,5ng/ml TGF (eBioscience) were added for Th17 polarization. In order to block Th1 and Th2 specific cytokines, 10µg/ml anti-IFN and 10µg/ml anti-IL4 (eBioscience) pure recombinant antibodies were added into the culture. Cultures were refreshed in every 5 days with 10ng/ml IL-23, 10µg/ml anti-IFN and 10µg/ml anti-IL4. Based on IL-17 expression cultures were stopped on the 9th or 12th days.

Table 2.1 Culture conditions.

	IMDM	STIM FULL	STIM NEUT FREE	STIM IL1 FREE	STIM IL6 FREE	STIM IL6 HIGH
Anti-CD3	-	5µg/ml	5µg/ml	5µg/ml	5µg/ml	5µg/ml
Anti-CD28	-	5µg/ml	5µg/ml	5µg/ml	5µg/ml	5µg/ml
IL-1	-	10ng/ml	10ng/ml	-	10ng/ml	10ng/ml
IL-23	-	10ng/ml	10ng/ml	10ng/ml	10ng/ml	10ng/ml
IL-6	-	30ng/ml	30ng/ml	30ng/ml	-	**90ng/ml**
TGF-	-	0,5ng/ml	0,5ng/ml	0,5ng/ml	0,5ng/ml	0,5ng/ml
Anti-IFN	-	10µg/ml	**-**	10µg/ml	10µg/ml	10µg/ml
Anti-IL4	-	10µg/ml	**-**	10µg/ml	10µg/ml	10µg/ml

2.5. Cell Surface Staining

Cells were washed with 1X PBS to remove medium. The appropriate surface antibodies (CD4, CD25, CD69, CCR6) were added into cells and incubated for 30min. at dark. The cells were washed for 2 times (300g-10min) to discard unbound antibodies and analyzed by FACSArray.

2.6. Cytokine Detection of T Cells by Intracellular Cytokine Staining

Firstly, cells were cultured under appropriate conditions for different time points. For intracellular cytokine staining 4 hours prior to the predetermined culture time, Golgistop (1:1500) (Becton Dickinson) was added to the cultures. After the culture time ended, cells were washed and harvested with 1X PBS supplemented with 0,5% FBS. Then, cell surface labeling of the cell of interest was carried out as described above. Following that paraformaldehyde containing fixation buffer (Becton Dickinson) was added to the cells for fixation and incubated for 10min at dark. The cells were washed for 2 times. For permeabilization, perm solution (Biolegend) was added, gently mixed and incubated for 15min. at dark. The cells were harvested and washed at 400g for 8min. and supernatants were removed. The appropriate cytokine antibody combinations (IFN , IL-17, ROR t, Foxp3, T-bet) were added directly to the cells and were incubated

for 30min. Lastly, after incubation, cells were washed for 2 times and analyzed by FACSArray.

2.7. Data Analysis

Data acquired by FACSArray were analyzed by using FlowJo analysis software. Then, data was exported to MS Office Excel for further analysis. A standard two-tailed t test was performed for statistical analysis. P values equal to or smaller than 0,05 were considered as significant.

CHAPTER 3

RESULTS

3.1. PBMC Isolation and Sample Selection

PBMCs were isolated from healthy volunteers by using Ficoll – Hypaque Density Gradient Centrifugation. Cell number and viability were determined by trypan blue staining with hemocytometer. PBMCs isolated from donors were controlled with activation markers against any recent infection. Samples that had higher than 10% CD25 and CD69 expression were excluded in this study (Figure 3.1A and B).

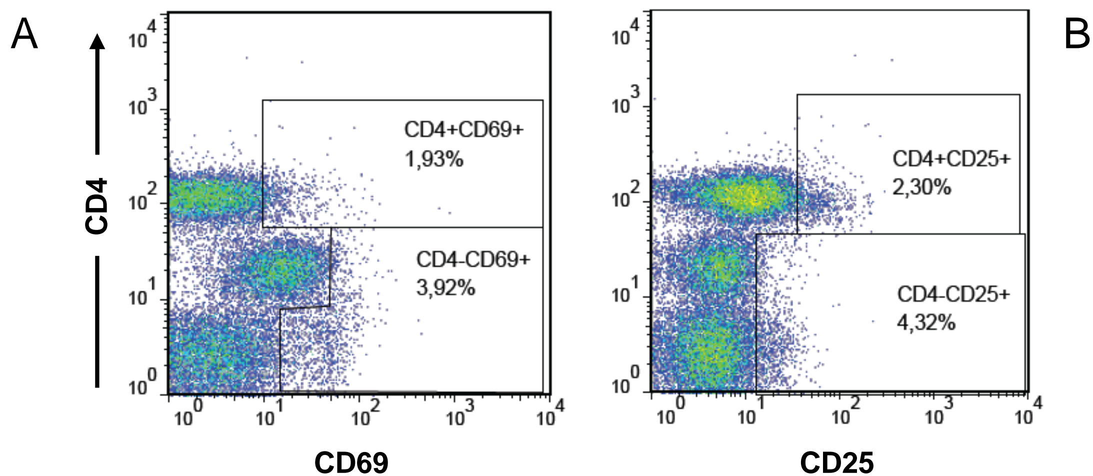

Figure 3.1.Sample selection or exclusion. Isolated PBMCs were controlled with activation markers CD25 and CD69 against any recent infection. Cell surface labeling was performed using flourescence conjugated anti-CD4, anti-CD69 and anti-CD25 antibodies and analyzed by FACSArray flow cytometry. (A) Early activation marker CD69 was observed among CD4+ and CD4- T cells. 1,93% CD4+CD69+ and 3,92% CD4-CD69+ T cells were detected. (B) Among PBMCs CD4+CD25+ cells were 2,30% and CD4-CD25+ cells were 4,32%.

3.2. Naive CD4+ T Cell Sorting

Naive CD4+ T cell sorting was carried out and efficiency was measured by immunofluorescence labeling by FACSarray. Sorted T cells were 96% naïve and no CD14 contamination was detected (Figure 3.2C and D). Also apoptosis rate was controlled by Annexin/7AAD staining. Cells that posses apoptosis rate <5% ANN+7AAD+ were used (data not shown).

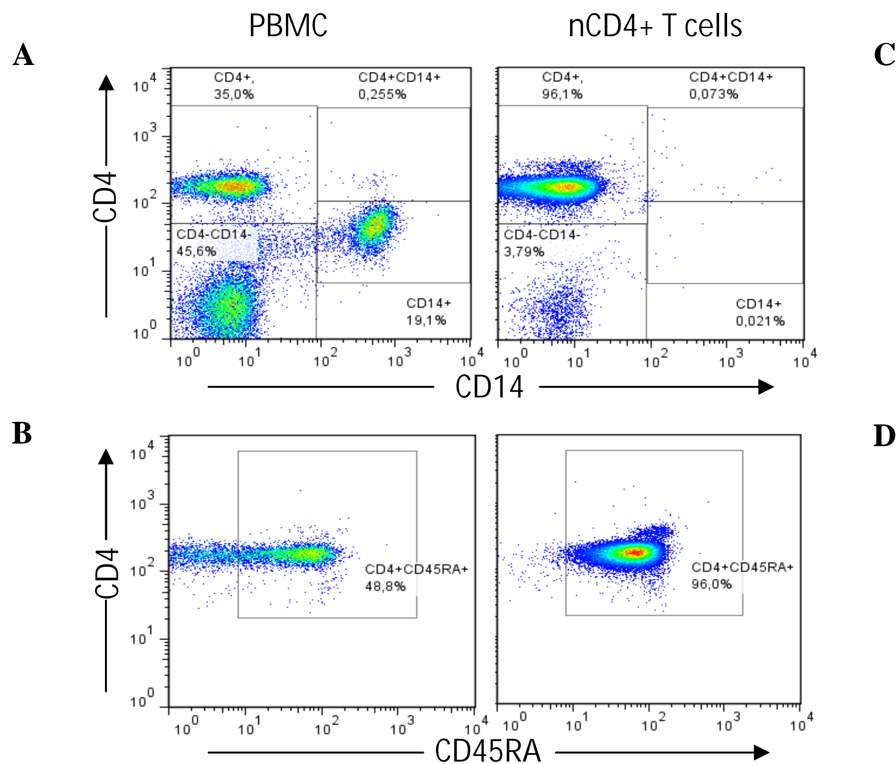

Figure 3.2. Detection of sorting efficiency. Naive CD4+ T cell sorting was performed from human PBMCs. Freshly isolated PBMCs were sorted with magnetic bead conjugated phenotypic antibodies by discarding unwanted cell populations. Cell surface labeling was performed using flourescence conjugated anti-CD4, anti-CD14 and CD45RA antibodies and analyzed by FACSArray flow cytometry. (A) CD4+ and CD14+ cells are shown among PBMCs and it has 35% CD4+ T cells. (B) CD4 gated cells are 49% CD45RA+ cells. (C) Naïve CD4 sorted cells have not any CD14+ cells and after sorting process CD4 cells are enriched to 96%. (D) CD4 gated cells reached to 96% CD45RA+ cells.

3.3. Stimulation of Naive CD4+ T Cell

Sorted cells were immediately cultured with various conditions to assess optimum Th17 polarizing condition and determine effects of cytokines individually. Naïve CD4+ T cells were stimulated with anti-CD3 and anti-CD28 molecules for TCR activation in the presence of previously described Th17 conditions in methods section. All cultures were replaced with fresh medium on the 5th day supplemented with anti-IL23 and/or neutralizing IFN and IL-4 antibodies to capture unwanted cytokines which may inhibit Th17 differentiation. We detect 7-8 fold increase in IL-17 expression on the 9th day as compared to unstimulated culture which was only 2,76%. Highest expression was detected in high dose IL-6 cultures (24,7%) and lowest expression was observed in IL-1 free culture (18,1%). Cells that were cultured in Stim Full (21,4%), neutralizing free (21,1%) and IL-6 free (20,3%) conditions showed very similar IL-17 expression rates (Figure 3.3A). It is important to obtain pure Th17 cells so we investigated whether these cells also produced Th1 and Treg cells specific molecules. Except IL-6 high condition (5,37%), all the other conditions inhibited IFN expression compared to un-stimulated culture. We observed 2,3 fold inhibition in Stim Full culture (1,44%) means inhibition of Th1 type polarization. In spite of this, other cultures had low effect on IFN expression (Figure 3.3B). Finally cells were controlled whether they express Foxp3 which is Treg specific transcription factor. Although IL-6 high cultures had maximum IL-17 producing cells, 3,56% of them was also Foxp3+ which is a Treg specific transcription factor. Among these conditions, minimum IL17+Foxp3+ cells were detected in Stim Full condition which was only 1,40 percentage. Also Foxp3 expression was only induced in neutralizing free and IL-6 high cultures, contrariwise other conditions had very proximate levels according to un-stimulated culture (Figure 3.3C).

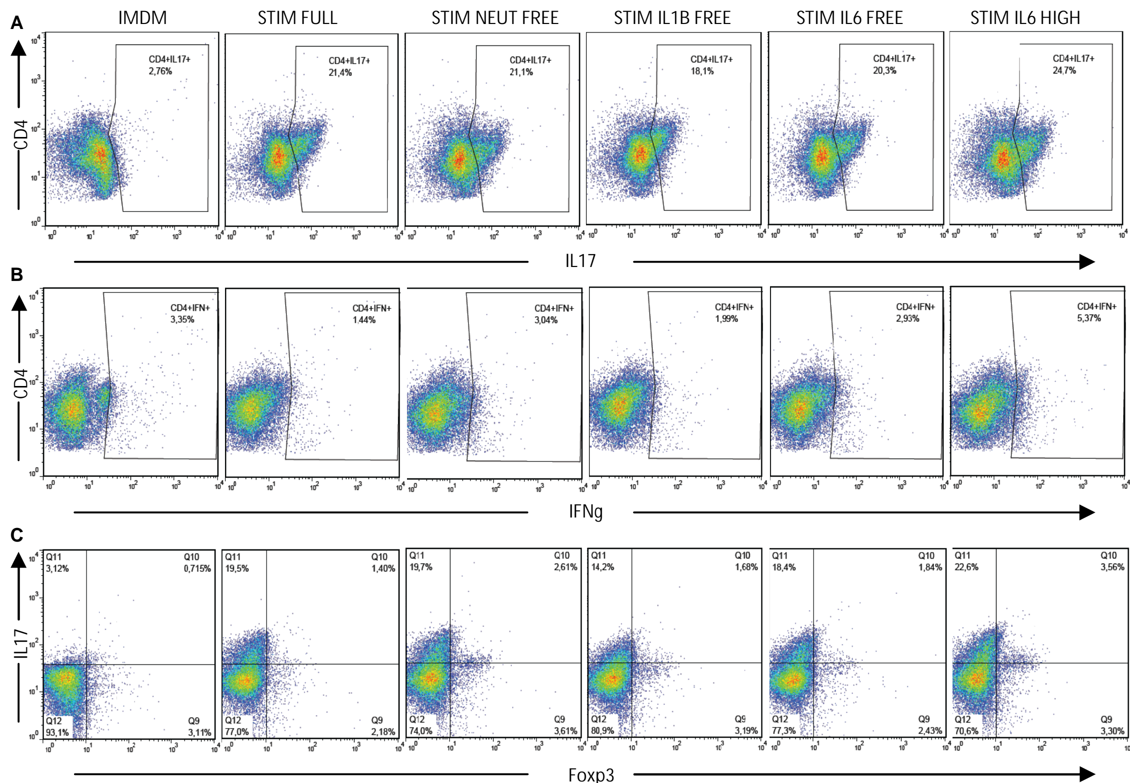

Figure 3.3. Cytokine expression profile of different culture conditions in 10th day. Naïve CD4+ T cells were cultured with various combinations of cytokines as discussed in methods section. Golgi Stop was added to the cultures 4h before intracellular immunofluorescence staining. After 4h incubation cells were washed and stained with cell surface CD4+ antibody. After fixation and permeabilization, fluorescence conjugated antibodies (IL17, IFN and Foxp3) were added into cells and analyzed by BD FACSarray flow cytometry. Flow data was analyzed with Flow Jo software and populations were all CD4 gated. (A) Expression of IL17 in stimulated cells was increased approximately 7 fold as compare to negative culture. Maximum IL17 expression detected in STIM IL6 HIGH condition and minimum expression observed in IL1 -free condition. STIM NEUT, STIM NEUT-free and STIM IL6-free conditions had similar expression percentage. (B) Expression of IFNg cytokine among CD4 gated T cells. According to culture conditions, IFNg expression was decreased except IL6 HIGH as compared to negative culture and STIM NEUT condition present minimum IFNg expression among other cultures. (C) IL17+Foxp3+ CD4 gated T cells were represented in Q10 gate. According to negative culture double positive cells were increased in all conditions, but most increase was detected in STIM IL6 HIGH condition whereas STIM NEUT culture had the minimum expression.

3.4. Activated Naive CD4 T Cells Differentiate into Th17 Phenotype

Cells were cultured with Th17 conditions started to became activated at first day and gradually increased and expression rate showed a maxima between 3rd and 6th days that finally on the 9th day reached to 57,4%. Activated cells started to express IL17 on day 3 and on 9th day 37,4% of them expressed IL17 expression that was 21,5% among all CD4+ T cells (Figure 3.4).

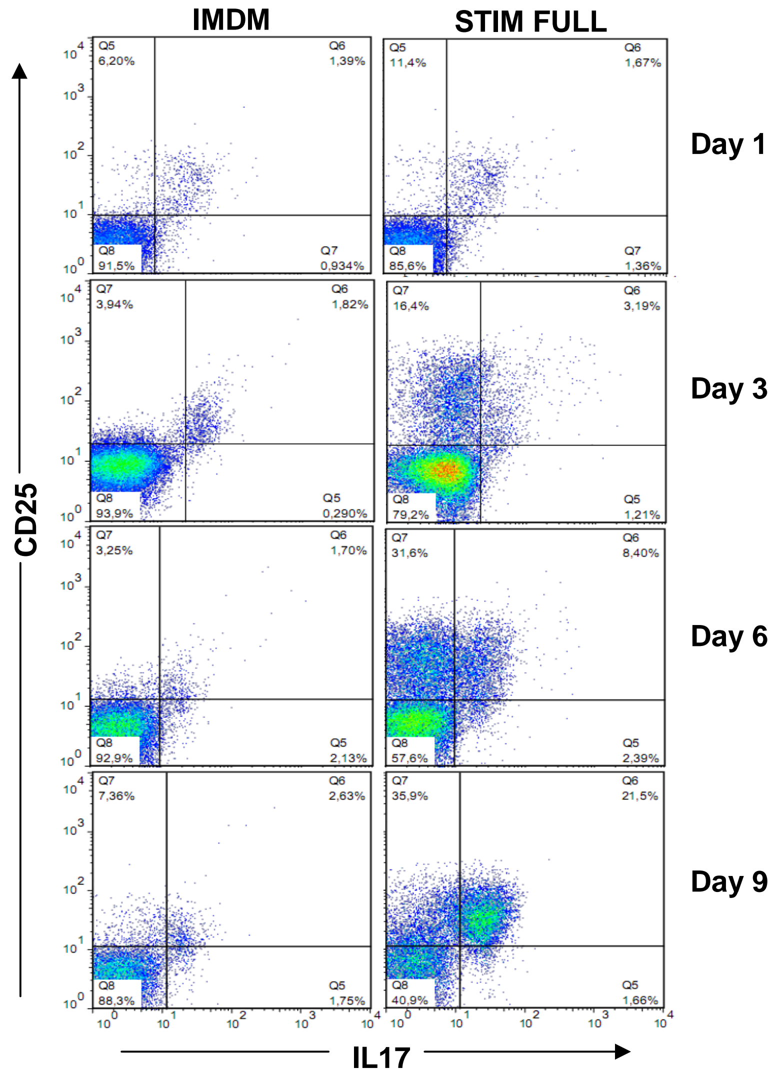

Figure 3.4. Activated T cells become IL17 producing cells in Th17 culture conditions. Naive CD4+ T cells were cultured in medium and STIM FULL condition and on the 5th day culture was changed with fresh medium supplemented with IL23 and anti-IFN and anti-IL4. Golgi Stop was added to the cultures 4h before intracellular immunofluorescence staining. After 4h incubation cells were washed and cell surface stained with CD4 and CD25 antibodies. After fixation and permeabilization, fluorescence conjugated IL17 antibody was added into cells and analyzed by BD FACSarray flow cytometry. Flow data was analyzed with Flow Jo software and populations were all CD4 gated. CD25 expression started to increase after 1st day in stimulated sample and after cells became activated they started to secrete IL17 cytokine. Finally, most of the cells were CD25+IL17+ on 9th day.

3.5. Expression Kinetics of Th Subset Specific Markers' During Th17 Differentiation

According to these results, we decided Stim Full condition as optimum Th17 culture because of maximum IFN inhibition and minimum Foxp3 expression to obtain most pure Th17 cells. Then we further analyzed molecules that may affect Th17

differentiation and how they were changed during polarization. Firstly, we evaluated the expression of Th17 specific molecules include IL17, RORC2 and CCR6. IL17 expression started to increase in early time points, especially after 6th day, and reaches to 19,6% on day 9 (Figure 3.5A). Th17 master regulatory transcription factor RORC2 expression was increased until 6th day rapidly (14,7%) and then it stayed stable (Figure 3.5B).

Chemokine receptor 6 expression did not change till 3rd day, but its expression was increased rapidly from 3rd to 9th day and reached to plateu (17,8%) (Figure 3.5C). Later, negative regulators of Th17 phenotype, including IFN and Foxp3 were measured. IFN was expressed immediately until 6th day –also its maximum (3,2%)- but then it was decreased between 6th and 9th days (2,4%) and became stable (Figure 3.5D). Increasement of T-bet was observed between 3rd and 6th day but then it was gradually decreased and finally came to same level with negative culture (Figure 3.5E). Foxp3 expression was induced faster than IFN , even on 1st day (data not shown), but after day 6, Foxp3 level was decreased (6,48%)(Figure 3.5F).

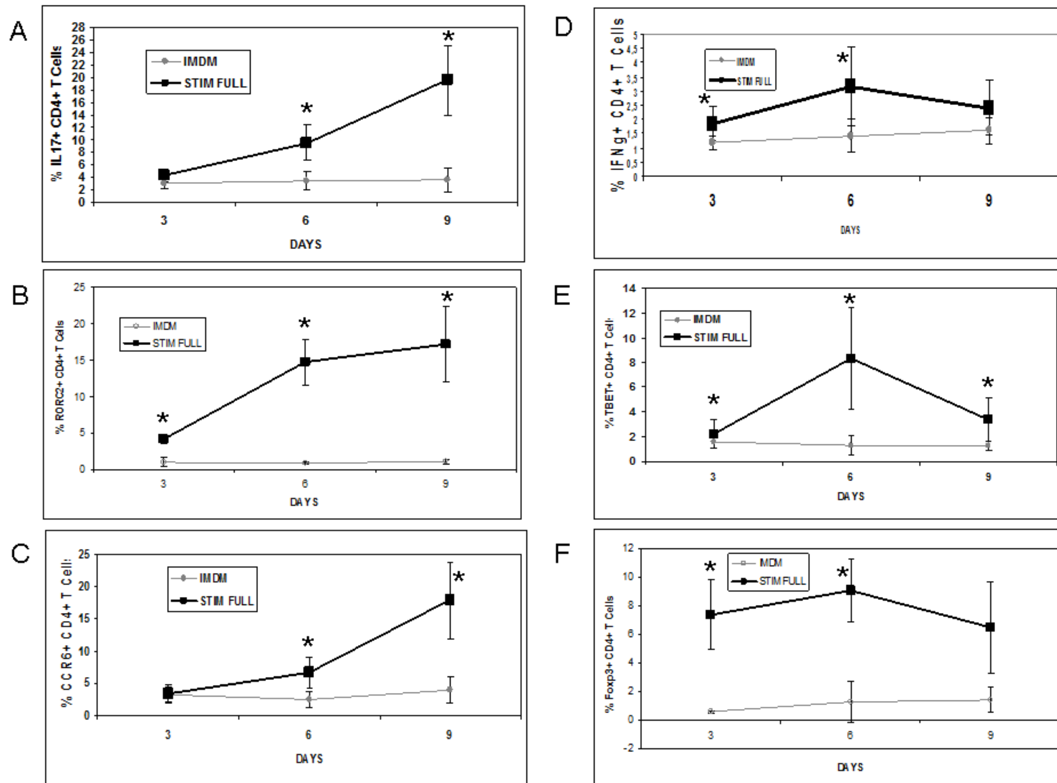

Figure 3.5. Expression kinetics of Th subset specific markers' during Th17 differentiation. Naïve CD4+ T cells were cultured in medium and STIM FULL condition and on 5th day culture was changed with fresh medium supplemented with IL23 and anti-IFN and anti-IL4. Golgi Stop was added to the cultures 4h before intracellular immunofluorescence staining. After 4h incubation cells were washed and cell surface stained with CD4 and CCR6 antibodies. After fixation and permeabilization, fluorescence conjugated antibodies (IL17, RORC2, IFN , T-bet and Foxp3) were added into cells and analyzed by BD FACSarray flow cytometry. Flow data was analyzed with Flow Jo software and populations were all CD4 gated. (A) IL17 expression starts in early time points but especially after 6th day, expression rate increasment upregulated and on 9th day IL17 expression reach to 19,6%. (B) Expression of RORC2 increased until 6th day and reached to its maximal on day 9. (C) Expression of CCR6 did not change until 3rd day and then started to increase and after day 9. (D) IFN expression increased until 6th day and then started to decrease gradually and finally reached to its beginning level. (E) Increasement of T-bet was observed between 3rd and 6th day but then it was gradually decreased and finally came to same level with negative culture. (F) Expression of Foxp3 immediately increased in early days but after 6th day expression was keenly decreased and became stable. Error bars represent standard deviation and "*" indicates $p<0,05$.

3.6. Characterization of Th17 Cells

Cells that were cultured with Th17 condition were analyzed on day 9 to characterize the subset. Stimulated cells expressed 19,4% IL17, 12,5% RORC2, 27,8 CCR6 and 3,17% T-bet. We observed 10,4 fold increase of IL17, 14,1 fold increase of RORC2 and 5,7 fold increase of CCR6 detected as compared to un-stimulated culture (Figure 3.6A). Later on, we analyzed cells whether they were pure Th17 or not. IL17

expressing cells were not co-expressing IFN and Foxp3. There was only limited double positive expression that IL17+IFN + cells were 1,63% and IL17+Foxp3+ cells were 0,3% (Figure 3.6B and C).

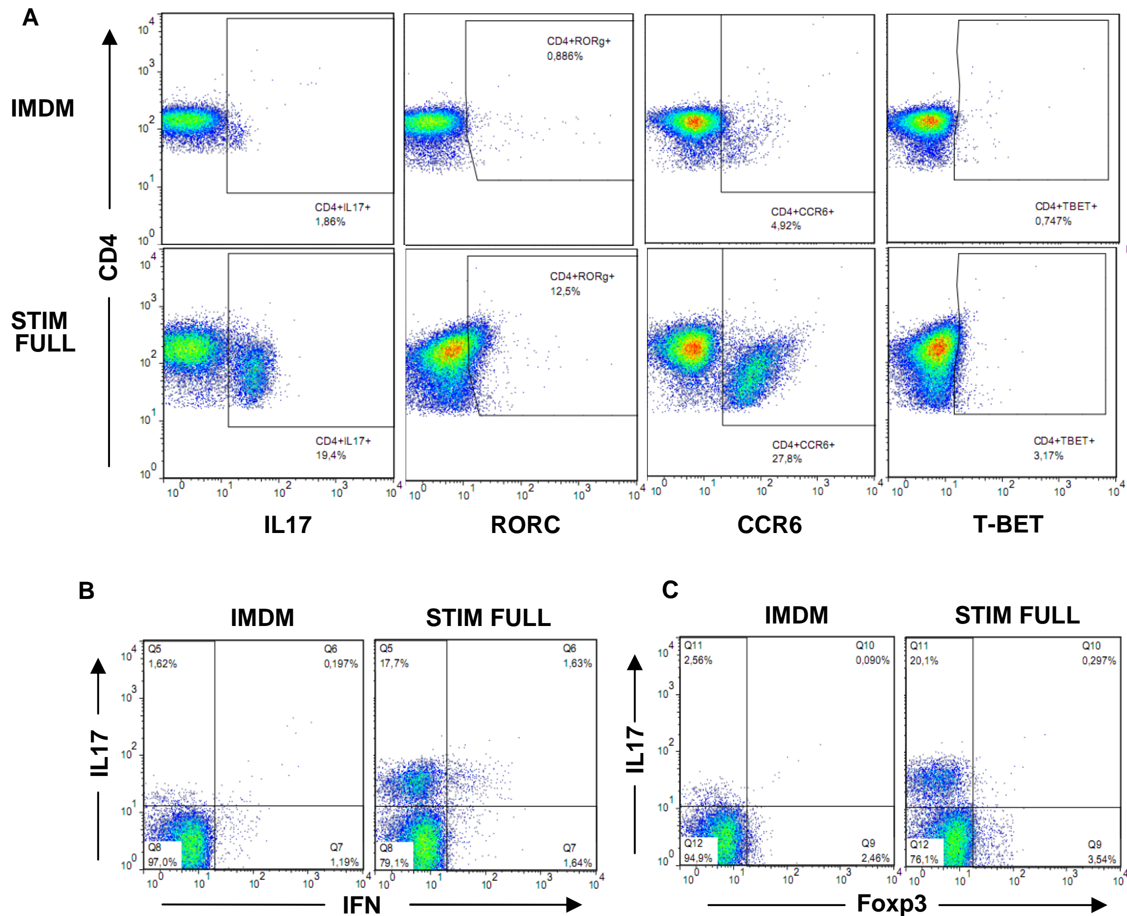

Figure 3.6. Characterization of Th17 cells. Naïve CD4+ T cells were cultured in medium and STIM FULL condition and on 5th day culture was changed with fresh medium supplemented with IL23 and anti-IFN and anti-IL4. Golgi Stop was added to the cultures 4h before intracellular immunofluorescence staining in 9th day. After 4h incubation cells were washed and cell surface stained with CD4 and CCR6 antibodies. After fixation and permeabilization, fluorescence conjugated antibodies (IL17, RORC2, T-bet, IFN , Foxp3) were added into cells and analyzed by BD FACSarray flow cytometry. Flow cytometry data was analyzed with Flow Jo software and populations were all CD4 gated. (A) On 9th day stimulated cells express IL17 cytokine and CCR6 abundantly. These cells also express Th17 master regulator RORC2 but not Th1 related transcription factor T-bet. (B) In the same time point cells were also analyzed as if they were IL17/IFN (C) IL17/Foxp3 double positive cells, but most of them were not co-expressing these molecules.

CHAPTER 4

DISCUSSION

Th17 cells are seems to be important according to observations about their roles in immune disorders and some cancer types so it is highly crucial to understand molecular aspects of Th17 and its differentiation. However recent publications showed that although culture conditions and molecules take role in differentiation were mostly identified in mouse, human Th17 culture conditions and regulatory molecules are not clear yet. Because of incomplete, irregular culture conditions and also limited protein level research in the literature, we aimed to optimize best culture conditions in *in vitro* human Th17 cells and figure out cytokines' and transcription factors' expression in protein level by monitoring them during differentiation.

In this study we reached to maximum IL17 expression among human studies by optimizing a new culture condition. In this aspect, we cultured naïve CD4+ T cells with various combinations that are mentioned in method section. We used IMDM as a medium because a recent publication suggests that higher tryptophan derivatives induce more Th17 cells as compared to RPMI (Veldhoen et al., 2009). Culture conditions were designed based on information in the literature (Acosta-Rodriguez et al., 2007a, Veldhoen et al., 2009, Zhou et al., 2008) and to assess effects of cytokines we design cytokine free combinations as described in methods. Our results indicate that although IL6 high culture condition gives maximum IL17 expression, it gives high levels of IFN and Foxp3 expression. Hence, this condition also favors Th1 and Treg phenotype differentiation. On the other hand, it is obviously seen that IL1 is the most necessary cytokine among others because of the decrease of IL17 expression in IL1 free cultures. IL6 free cultures have slightly decreased IL17 expression, but IL6 seems to have inhibitory effect on IFN and Foxp3 expression. In order to prevent unwanted IFN expression we used neutralizing antibodies (anti-IFN and anti-IL4). In neutralizing free condition we detect similar IL17 expression as compared to "Stim full" culture, but we detect higher levels of IFN expression. "Stim Full" culture condition gives minimum IFN and Foxp3 expression and high IL17 expression (Figure 3.3) According to these observations we suggest maximum pure Th17 phenotype (no expression of IFN and

Foxp3) was produced in Stim Full culture condition so we continue further investigation using this condition.

After that, we investigated activation and apoptosis status of cells in Stim Full condition in order to identify culturing time. We saw that stimulated cells were activated rapidly due to anti-CD3 and anti-CD28 molecules. Activated T cells became effector and started to express IL17 cytokine (Figure 3.4). We also controlled viability of the cells and we observed that after 9th day apoptosis rate (ANN+7AAD+) exceed 20% which made us to stop cultures (data not shown).

Later on, we investigated expression profiles of Th17 using Th1 and Treg related molecules to clarify their roles in Th17 differentiation. Firstly, we investigated Th17 related molecules IL17, RORC2 and CCR6. We detect expression of IL17 rapidly in early time points and it is obviously seen that increasement of RORC2 expression up-regulates IL17 expression which was consistent with the literature (Ivanov et al., 2006, Manel et al., 2008). Interestingly after 5th day, RORC2 expression was decreased but IL17 expression increasement continued. This made us to think another player may affect rather than RORC2 transcription factor. Also CCR6 which is thought to over-expressed in Th17 cells was measured and expression was induced after 3rd day and increasement continued until 9th day and became stable. Expression of this chemokine receptor has been described as associated with Th17 cell subset, but also there are indications that Treg cells also express this molecule too (Annunziato et al., 2007, Kleinewietfeld et al., 2005, Tran et al., 2009, Valmori et al., 2010, Zhou et al., 2008). It was observed that at the very first day of the culture, Foxp3 which is a Treg related transcription factor was up-regulated and remains in high concentration till 6th day of the culture. Then it was dramatically down-regulated. When we compare all these observations Th17 and Treg cells seems highly related because either CCR6 or Foxp3 expression may take role in Th17 differentiation according to their expression pattern (Figure 3.5C and F). Indeed Foxp3 inhibits IL17 expression by binding ROR t (Li and Greene, 2007, Zhou et al., 2008) but also TGF- which induces Foxp3 transcription factor works in a dose dependent manner that it can promote either Th17 or Treg cell lineage differentiation. High dose of TGF- induce Treg differentiation but low dose favor to Th17 subgroup (Zhou et al., 2008). According to literature low dose TGF- , inhibits Th1 polarization so indirectly take a positive role in Th17 differentiation. Meanwhile, based on our results we detected very low amount of population of

IL17/Foxp3 double producers which indicates that our TGF- dose is appropriate for Th17 differentiation culture.

However we also observed IFN (Figure 3.5C) and T-bet (Figure 3.5D) expression in a group of cells. These molecules expression was increased together at the same time points which were later decreased dramatically. Normally, we know that T-bet controls IFN expression so their co-expression was not surprising, but at late time points these cells disappear and we do not know whether they are dying or differentiating into Th17 phenotype.

Then we characterized cultured cells and interestingly we observed higher amounts of IL17 expression than RORC2 expression (Figure 3.6A) which means some IL17 producing cells do not co-express RORC2 master transcription factor. So it is possible that IL17 expression can also be regulated by another transcription factor rather than RORC2. Meanwhile we showed that these cells are purely Th17 cells because they do not express T-bet and also they are not co-expressing IL17/IFN and IL17/Foxp3 together (Figure 3.6B and C).

CHAPTER 5

CONCLUSION

Results on optimizing culture condition, reveals necessity of IL1 , underrated importance of TGF- , IL6 and their effects on Th17 cell priming on dose-dependent manner. Further the simultaneous cytokine profiles and transcription factor analysis point out the master regulators of the Th17 differentiation in a time-dependent manner which facilitates the incorporation between Th17 cell phenotype and the activity of these transcription factors. However there are still some points needed to be clarified for further analysis of Th17 cells to fully understand their role in disease pathologies and the maintenance of the immune system homeostasis. One of these points is the further verification and analysis of the minor cell population that do not express RORC2 while secreting IL17.

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