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A New, Phenotypically Distinct Subpopulation of Regulatory Killer T ex-Th17 Cells Expressing $CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi}$ $ROR^{low}IL\text{-}17^{low}$

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Abstract- Th17 and regulatory T (Treg) cells are integral in maintaining immune homeostasis and Th17– Treg imbalance has been associated with inflammatory immune suppression in cancer. Here it is shown that in addition to ROR+Foxp3+ cells eTreg (Effector Regulatory T Cells) cells are a source of ex-Th17 $CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi}$ (Regulatory Killer T – RKT) cells while the latest is much more suppressive. Moreover, we have identified a set of key cytokines that favor the generation and expansion of ex-Th17 $Foxp3^{low}$ cells. These findings should accelerate efforts to define the function of this new subset of Treg cells in the immune response to cancer.

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I. Significance Statement

In this work, the new subpopulation of ex-Th17 CD4 lowCD25hi CD49hi Foxp3hi cells has been described. Thus the relative concentration of IL-2, IL-12, IL-1β and IL-23 in the tumor microenvironment may be a critical factor for the generation of exTh17 RKT that will be converted into INF-γ- producing exTh17Foxp3low (exTh17/Th1) cells. Based on these findings, it had been predicted that cytokine milieu (low amounts of TGF-β and high quantity of IL-2, IL-12, IL-1β, and IL-23) in cancer favors the generation and expansion of exTh17Foxp3low cells, although further studies are needed to validate this concept. This knowledge should accelerate efforts to describe the new subpopulation ex-Th17 CD4lowCD25hiCD49hiFoxp3hi (RKT) cells in more detail and create new drugs for several immunogenic types of tumors.

II. Introduction

Treg cells consist of functionally diverse subsets of immune suppressive T cells that play a crucial role in the modulation of immune responses and the reduction of deleterious immune activation [1, 2]. Treg cells may participate in the progression of cancer, especially about the ability of Treg cells to promote the development of tumors [3]. It had been described that the levels of Intratumoral Treg cells correlating with better or worse outcomes depending on the tumor type [4, 5]. Recent studies indicate that human ovarian cancer cell line SKOV-3 could convert, in the presence of IL-2, Treg into Th17 cells. These results support the ability of the tumor microenvironment to regulate and expand IL-17-producing T-helper (Th17) cells. Similar results had been obtained upon stimulation of CD4+ T cells in the absence of tumors but in the presence of IL-1β/IL-6 and IL-2 [6]. Cytokine profile analysis revealed that ovarian tumor cells, tumor-derived fibroblasts, and antigen-presenting cells (APCs) secrete IL-1β/IL-6 [7]. IL-1β is a potent inducer of Th17 cell differentiation and expansion, whereas IL-6 is capable of expanding memory Th17 cells [8]. Gene profile analysis revealed that SMAD 6 and HDAC 11 are hyper expressed in ovarian cancer cell line SKOV-3 [9]. In its turn, Smad6 is transcriptionally induced by the anti-inflammatory cytokine TGF-β. On the one hand, the importance of the concentration of TGF-b had been illustrated in selectively regulating Treg and Th17development. Low concentrations of TGF-b favor Th17 differentiation by enhancing IL- 23 receptor (IL- 23 R) expression, while high amount promote Treg differentiation by inhibiting IL-23 R up-regulation [10]. On the other hand, it had been also described that ovarian tumor cells secreted a high amount of latent TGF-β (inactive form), but the level of active form of TGF-β was very reduced (≤30Pg/ml) or undetectable because of its short half-life [8, 11]. Importantly, most of all types of tumors secrete a high amount of TGF-β. Over expression of HDAC11 has been associated with inhibition of expression of the gene encoding IL-10 and higher IL-12 mRNA expression [12]. IL-12 and IL-23 shared the same IL-12Rβ1 receptor subunit and have been characterized by overlapping effects on target cells. As shown before, IL-23 stimulation is not only crucial for attaining full effect or function but also necessary for double expression of IL-17A and IFN-γ, induction of T-bet and subsequent deviation toward IFN-γproduction[13]. Here, it was provided an insightful mechanism by which $CD4^{low}$ $CD25^{hi}CD49^{hi}Foxp3^{hi}$cells are generated from Treg cells and regulated by cytokines ex-Th17 that favor the generation and expansion $exTh17Foxp3^{low}$ cells.

III. Results

I conducted three experiments aimed at deriving Treg cells using BALB/c mice (1^{st} time) and

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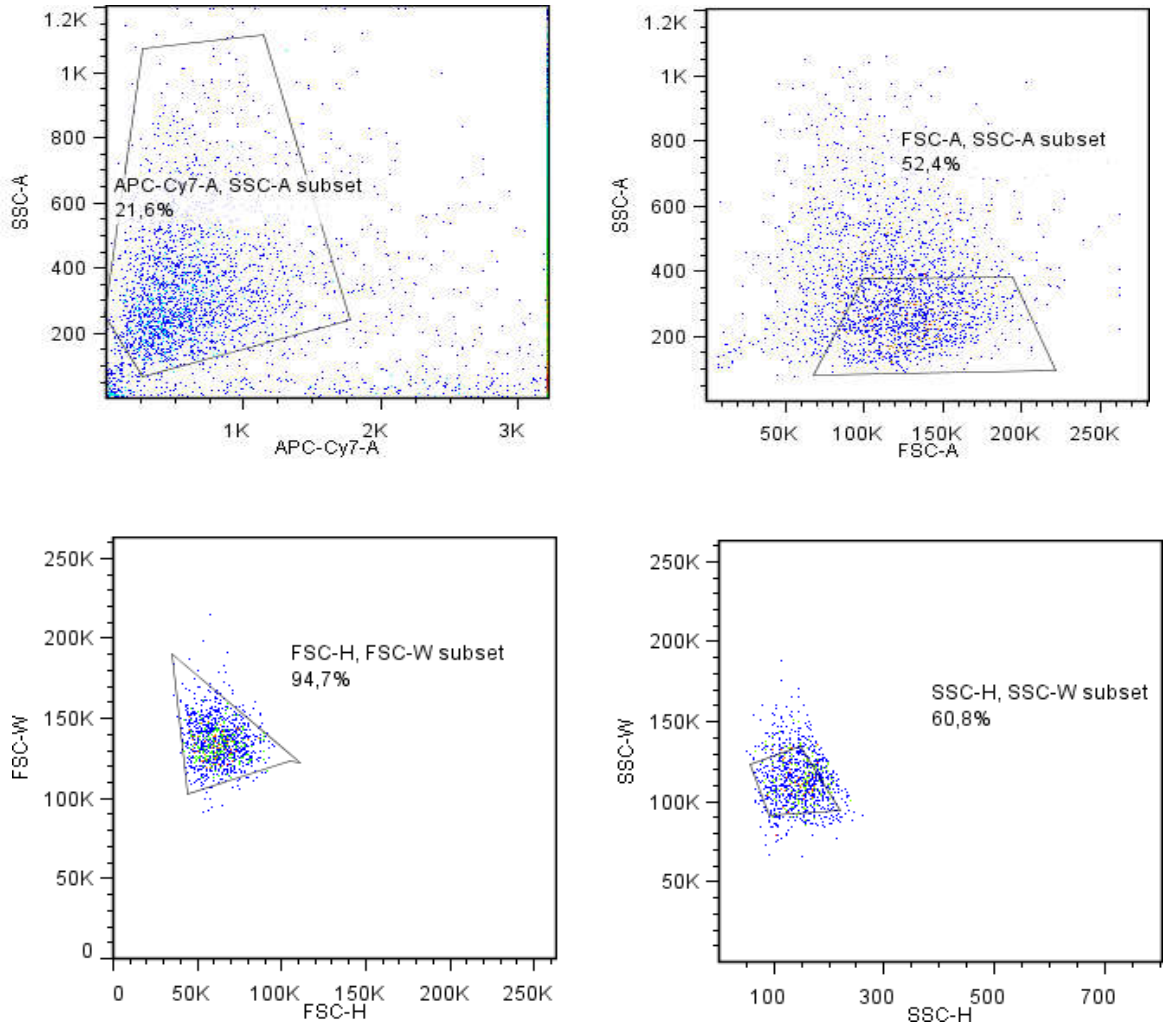
Foxp3-GFP-DTR (2 times), CD4+CD25+FOXP3$^{DTR-GFP}$ cells were isolated from lymph nodes and spleen by flow cytometry cell sorting to high purity and stimulated with anti-CD3/CD28 coated Dyno-beads and IL-2 (Fig. 1). FACS analyses of the isolated population has been at day three after stimulation. As shown in Figure 2, ex-Th17 CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi}cells were clearly detectable in populations from the purified CD4+CD25+ T-cell fractions after in vitro expansion. Staining with anti-IL-17 antibody revealed that ex-Th17 CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi}cells secreted low level of IL-17, although ROR+FOXP3+ T cells produced elevatedlevel of IL-17 Further experiments revealed that freshly isolated CD4^{low}CD25^{hi}T cells were strongly positive for CD49b and Foxp3 molecules and weakly positive for ROR (Fig. 3).



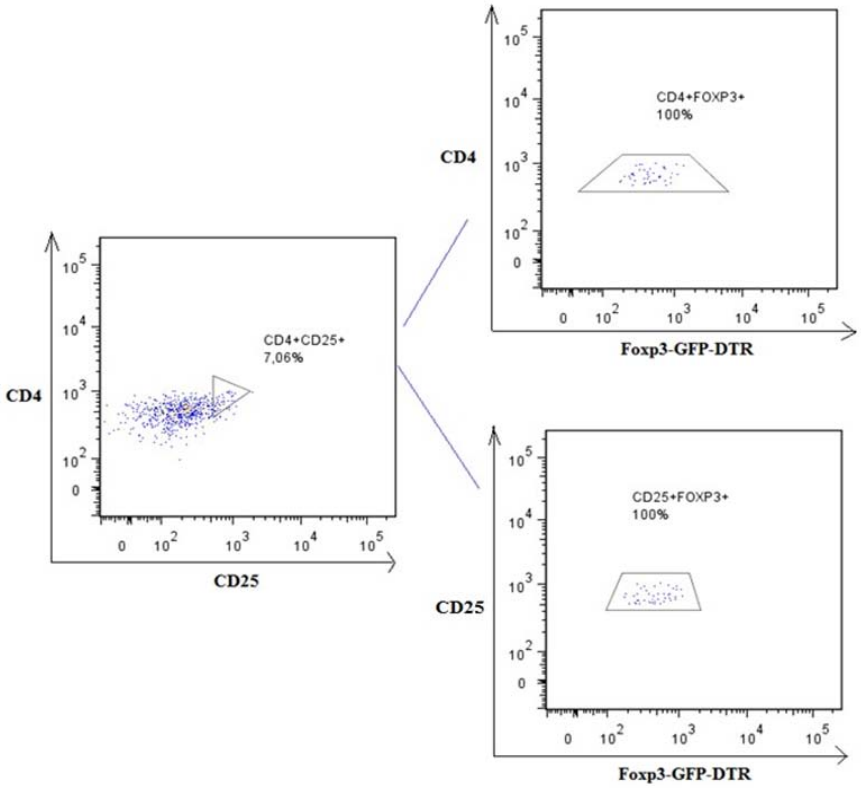
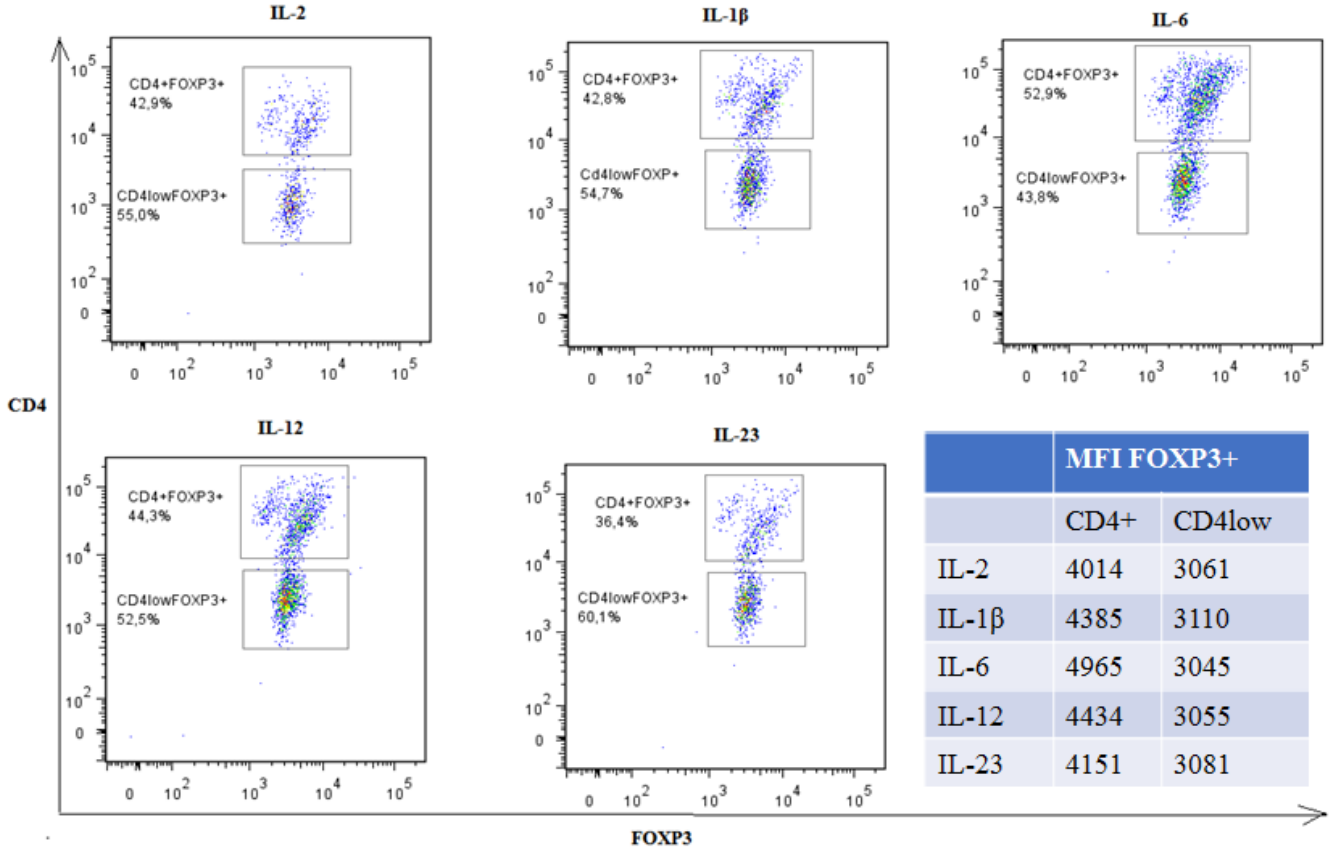
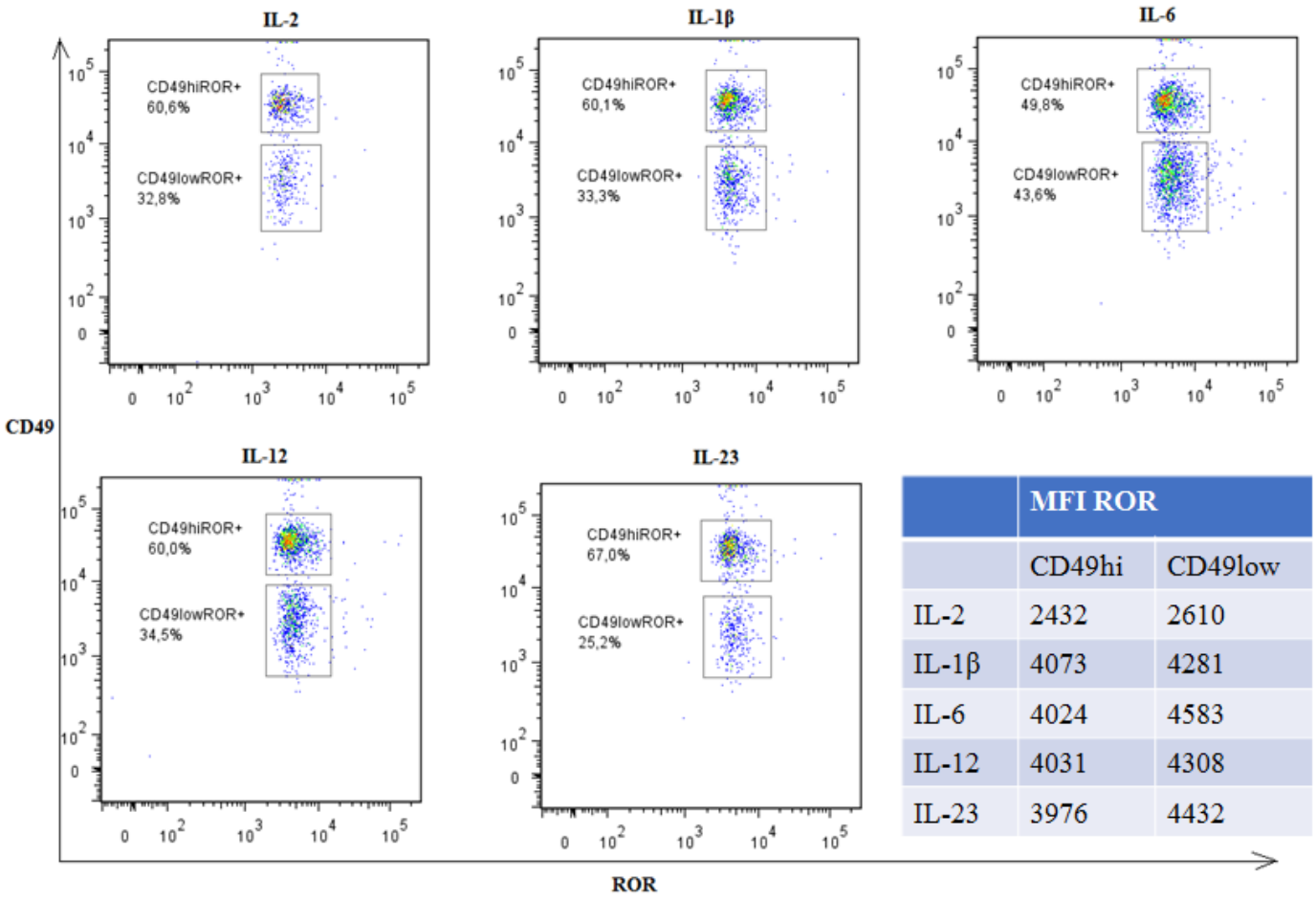


Figure 1: Sorting strategy and the purities of resultant population CD4+CD25+Foxp3+T cells

IL-2 – 100U/ml



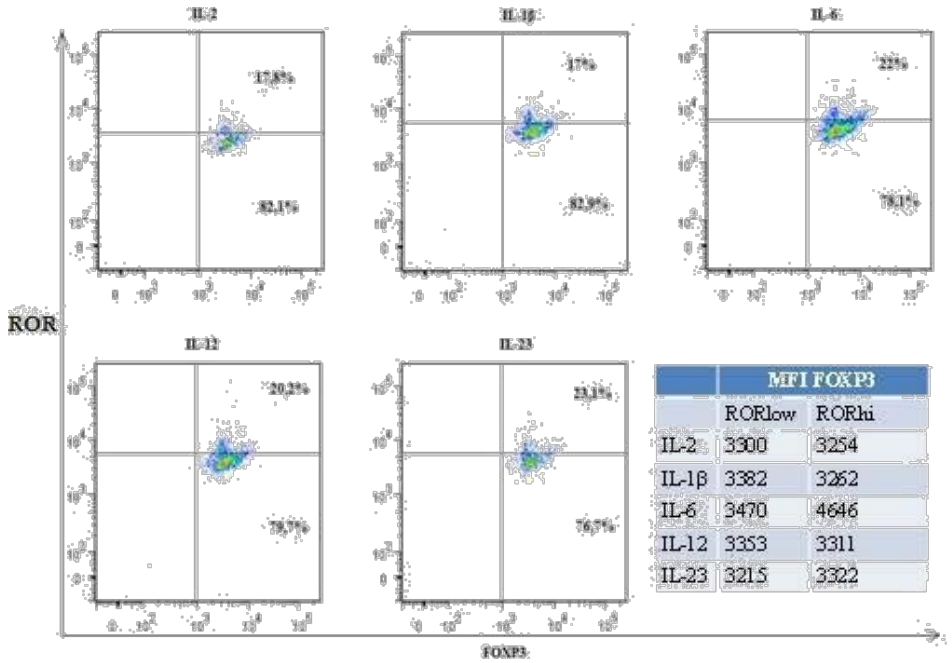
	MFI FOXP3+	
	CD4+	CD4low
IL-2	4014	3061
IL-1β	4385	3110
IL-6	4965	3045
IL-12	4434	3055
IL-23	4151	3081



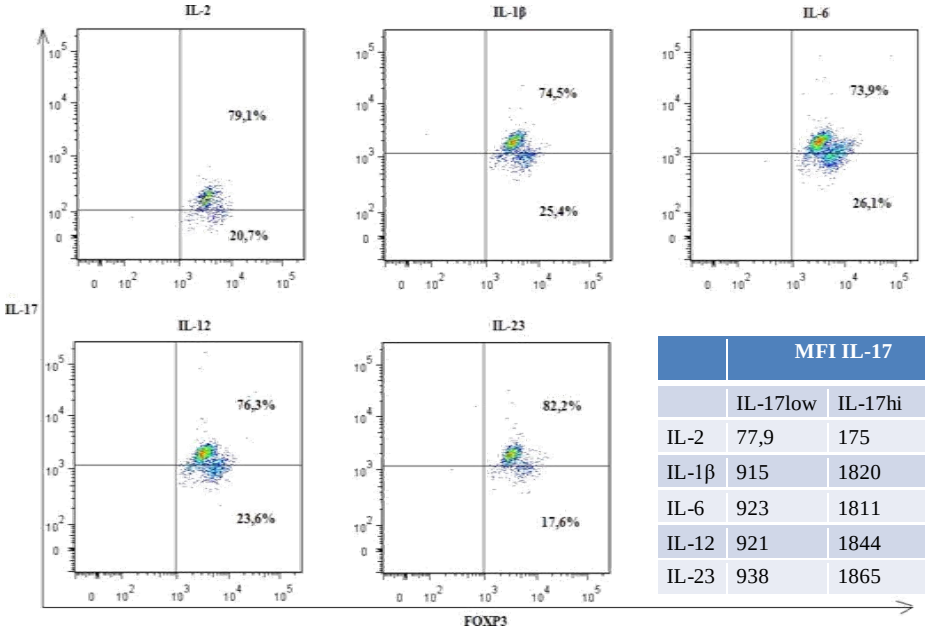
	MFI ROR	
	CD49hi	CD49low
IL-2	2432	2610
IL-1β	4073	4281
IL-6	4024	4583
IL-12	4031	4308
IL-23	3976	4432

Figure 2: CD4^{low}T cells express Foxp3 and CD49b

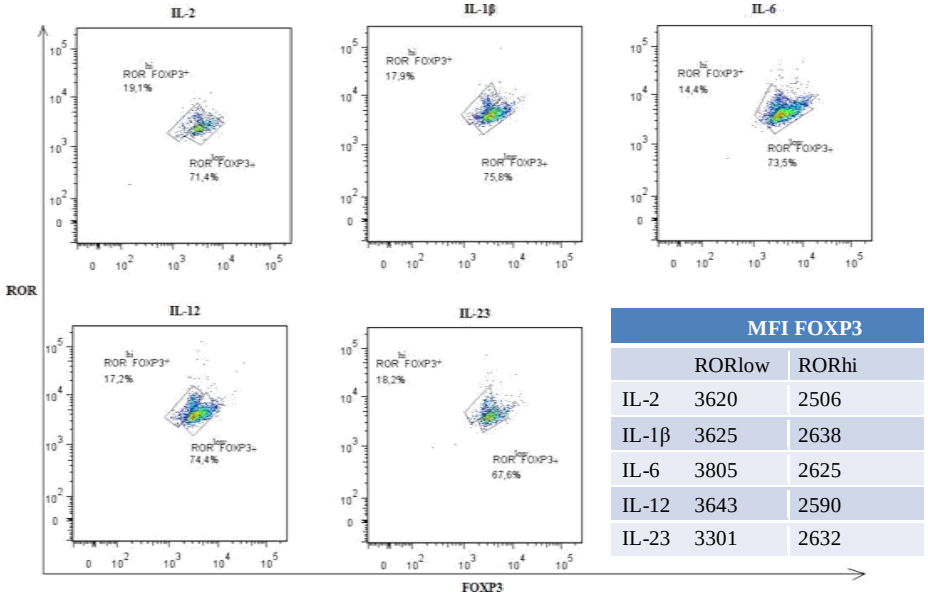
So I am the first who describe this subpopulation of ex-Th17 CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi} cells.



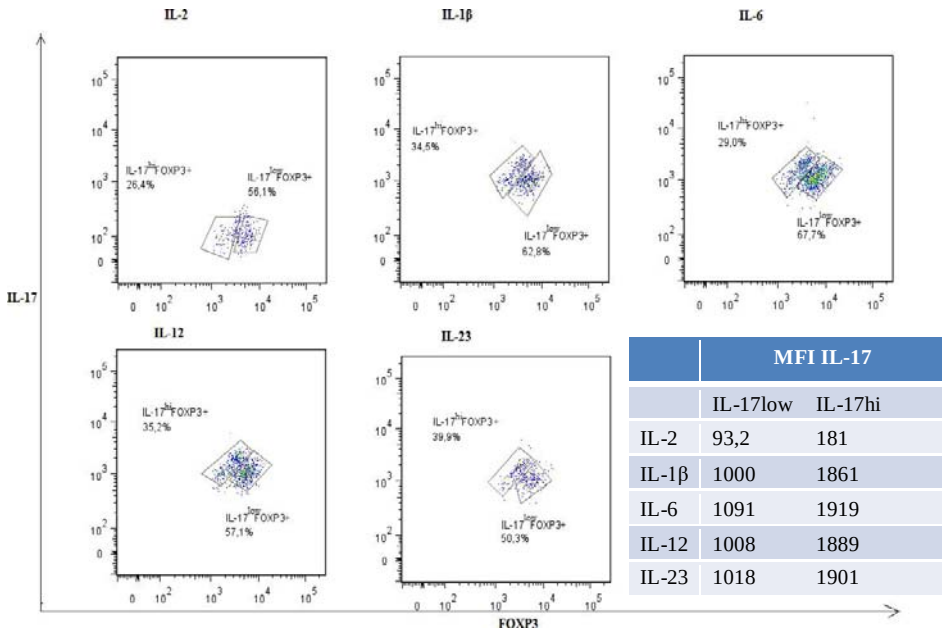
	MFI FOXP3	
	RORlow	RORhi
IL-2	3300	3254
IL-1β	3382	3262
IL-6	3470	4646
IL-12	3353	3311
IL-23	3215	3322



	MFI IL-17	
	IL-17low	IL-17hi
IL-2	77,9	175
IL-1β	915	1820
IL-6	923	1811
IL-12	921	1844
IL-23	938	1865



	MFI FOXP3	
	RORlow	RORhi
IL-2	3620	2506
IL-1β	3625	2638
IL-6	3805	2625
IL-12	3643	2590
IL-23	3301	2632



	MFI IL-17	
	IL-17low	IL-17hi
IL-2	93,2	181
IL-1β	1000	1861
IL-6	1091	1919
IL-12	1008	1889
IL-23	1018	1901

Figure 3: CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi}cellsexpress low level of IL-17 and ROR

We also decided to determine the role of key cytokines, as discussed earlier, in the generation and expansion of exTh17Foxp3^{low} cells.

In the next stage, we tested the effects of IL-12, IL-1β, IL-6, IL-23 on exTh17Foxp3^{low} differentiation and expansion by using Treg cells from Foxp3$^{DTR-GFP}$ mouse. IL-2-containing medium provided a baseline for comparison. Analysis of ex-Th17 CD4^{low}CD25^{hi}CD49^{hi} Foxp3^{hi} cells revealed that IL-23 plays a more prominent role in the differentiation and expansion of exTh-17Foxp3^{low} cells than do IL-12 and IL-1β. By contrast, IL-6 stimulated IL-17-producing ROR+Foxp3+T suppressive cells.

IV. Discussion

In this work, we have described the new subpopulation of ex-Th17 CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi} cells. Importantly, our data demonstrate the differentiation of eTreg cells to ROR+Foxp3+ cells and exTh17 RKT cells. Bryl et al. have previously reported the population of peripheral blood T cells with reduced CD4 and high CD25 expression (CD4^{low}CD25^{high}), that can non-specifically suppress the proliferation of autologous, previously polyclonally activated CD4+ lymphocytes and to kill them by direct contact. CD4^{low}CD25^{high} T cells expressed significant amounts of both intracellular perforin and granzyme B. At the same time common NK/NKT antigens, including CD16, CD56, CD94, CD158b, CD161 and invariant NKT (iNKT), - were not present on CD4^{low} T cells [14].

Also using whole-genome microarray data sets of the Immunological Genome Project, it was demonstrated a closer transcriptional relationship between NK cells and T cells than between any other leukocytes, distinguished by their shared expression of genes encoding molecules with similar signaling functions, including NT cells and Treg [15]. In terms of common expression of Zap70 and Prscq and potential expression of perforin and granzyme B we concluded that the definition of a CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi}cells phenotype is enough to unambiguously detect and study the regulatory function of new subpopulation. It's called Regulatory Killer T – RKT cells which fulfil the current phenotypic criteria identifying the exTh17 RKT cells by simultaneously expressing low amounts of ROR and IL-17A.

Thus, the relative concentration of IL-2, IL-12, IL-1β, and IL-23 in the tumor microenvironment may be a critical factor for the generation of exTh17 RKT that will have been converted into INF-γ-producing exTh17Foxp3^{low} (exTh17/Th1) cells. Based on these findings, it had been predicted that cytokine milieu (low amounts of TGF-β and high quantity of IL-2, IL-12, IL-1β and IL-23) in cancer favors the generation and expansion of exTh17Foxp3^{low} cells, although further studies are needed to validate this concept.

In terms of several types of tumors secrete some cytokines, for example colorectal cancer express high level of IL-23, ovarian cancer – IL-12 (I am planning to prove it) the combination of IL-2, IL-12, IL-1β, and IL-23 in different ways enhanced the differentiation of exTh17Foxp3^{low} (exTh17/Th1) cells from eTreg cells while retaining their ability to expand ROR+Foxp3+ T cells. IL-23 as a critical factor driving exTh17Foxp3^{low} cell expansion. Our findings support the emerging concept that tumor environmental factors drive the generation and expansion of exTh17Foxp3^{low} cells. This knowledge should accelerate efforts to describe the new

subpopulation ex-Th17 $CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi}$ (RKT) cells in more detail and create several drugs for several immunogenic types of tumors (melanoma, ovarian cancer, renal cancer, colorectal cancer) on the basis of IL-2, IL-12, IL-1β and IL-23 that will be delivered locoregionally (intraperitoneally, intrahepatic artery etc) to decrease systemic toxicity.

V. Methods

a) Cell culture

CD4+Tcells that were isolated from FDG mouse by negative selection with mouse CD4+Isolation Kit and were further separated into $CD4+CD25+FOXP3^{DTR\text{-}GFP}$+eTregcells using a FACS ARIA II instrument. Sorted 1,2x10^5 eTreg cells were cultured in the presence of anti-CD3- and anti-CD28-coated (2,5 mcl) Dyna-beads and IL-2 (100U/ml). In some cultures IL-12 (30ng/l), IL-1β (30ng/ml), IL-6 (30ng/ml), IL-23 (30 ng/ml) were added. Cells were analyzed with a FACS Canto instrument 3 days later.

Cells were cultured in culture medium (RPMI-1640 supplemented with 100 U/mL penicillin, 100 g/mL streptomycin, 5 mM 2-mercaptoethanol, 0.05% and 10% fetal bovie serum [FBS]) at 37°C, and 5% CO2, in 96-well round-bottom plates (Greiner, Frickenhausen, Germany).

b) Antibodies and reagents

Allophycocyanin (APC)- and Cy7- conjugated anti-CD4 (RM 4-5) mAb, phycoerythrin (PE) – and Cy7-conjugated ant-CD4(RM 4-5) mAb, peridinin chlorophyll protein complex (PerCP)-andCy5.5-conjugated anti-CD25 (PC-61) mAb, phycoerythrin (PE) anti-ROR (Q31-378) mAbhad been purchased from Bioscience. Brilliant Violet 421(BV421) – conjugated anti-IL-17 (TC-11-18H 10.1) mAb, Alexa Fluor 647-conjugated anti-CD49b (DX5), LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit had been purchased from Biolegend. Recombinant murine IL-6, IL-12, IL-23, IL-1βhad been purchased from Sarstedt and Bioscience.

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The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

All data used to support the findings of this study are included within the article. The research data were performed as part of the employment (Immunology Frontier Research Center, Osaka University).

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