A new, phenotypically distinct subpopulation of Regulatory Killer T ex-Th17 cells expressing CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi}ROR^{low}IL-17^{low}

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Th17 and regulatory T (Treg) cells are integral in maintaining immune homeostasis and Th17–Treg imbalance is associated with inflammatory immunosuppression in cancer. Here it is shown that in addition to ROR+Foxp3+ cells eTreg cells are a source of ex-Th17 CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi} (Regulatory Killer T – RKT) cells while the latest are much more suppressive. Moreover, we have identified a set of key cytokines that favor the generation and expansion 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.

Key words: Regulatory Killer T ex-Th17 cells, eTregs, CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi}ROR^{low}IL-17^{low} cells.

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 with regard to the ability of Treg cells to promote the development of tumors [3]. It was 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 were 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 antigenpresenting 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

hyperexpressed in ovarian cancer cell line SKOV-3 [9]. In its turn Smad6 is transcriptionally induced by the antiinflammatory cytokine TGF- β . On the one hand, the importance of the concentration of TGF- β was illustrated in selectively regulating Treg and Th17 development. Low concentrations of TGF- β favor Th17 differentiation by enhancing IL-23 receptor (IL-23R) expression, while high concentrations promote Treg differentiation by inhibit IL-23R up-regulation [10]. On the other hand, it was 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 low (≤ 30 pg/ml) or undetectable because of its short half-life [8, 11]. Importantly, most of all types of tumors secrete high amount of TGF- β . Whereas overexpression of HDAC11 was 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-12R β 1 receptor subunit and are characterized by overlapping effects on target cells. As shown before, IL-23 stimulation is not only crucial for attaining full effector function but also necessary for double expression of IL-17A and IFN- γ , induction of T-bet and subsequent deviation toward IFN- γ production [13]. Here, it was provided an insightful mechanism by which ex-Th17 CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi} cells are generated from eTreg cells and regulated by cytokines that favor the generation and expansion exTh17Foxp3^{low} cells.

Results

I conducted 3 experiments aimed at deriving eTreg cells using BALB/c mice (1st time) and 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 Dynobeads and IL-2 (Fig. 1). FACS analyses of the isolated population was at day 3 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 γ ⁺FOXP3⁺ T cells produced high level 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.

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.

Figure 3. CD4^{low}CD25^{hi}CD49^{hi}Foxp3^{hi} cells express low level of IL-17 and ROR.

We also sought 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-1 β , 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 differentiation and expansion of exTh17 Foxp3^{low} cells than do IL-12 and IL-1 β . By contrast, IL-6 stimulated IL-17-producing ROR+Foxp3+ T suppressive cells.

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 population of peripheral blood T cells with reduced CD4 and high CD25 expression (CD4^{low}CD25^{high}), that are able to 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 called Regulatory Killer T – RKT cells which fulfils 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-1 β 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 exTh17Foxp3^{low} (exTh17/Th1) cells. Based on these findings, it was predicted that cytokine milieu (low amounts of TGF- β and high amounts of IL-2, IL-12, IL-1 β 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-1 β 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 clearly 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-1 β and IL-23 that will be delivered locoregionally (intraperitoneally, intrahepatic artery etc) to decrease systemic toxicity.

Methods

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-GFP}+eTregcells using a FACS ARIA II instrument. Sorted 1,2x10⁵ eTreg cells were cultured in the presence of anti-CD3- and anti-CD28-coated (2,5 mcl) Dynabeads and IL-2 (100U/ml). In some cultures IL-12 (30ng/l), IL-1 β (30ng/ml), IL-6 (30ng/ml), IL-23 (30 ng/ml) were added. Cells were analysed with an 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 bovine serum [FBS]) at 37°C, and 5% CO₂, in 96-well round-bottom plates (Greiner, Frickenhausen, Germany).

Antibodies and reagents

Allophycocyanin (APC)- and Cy7- conjugated anti-CD4 (RM 4-5) mAb, phycoerythrin (PE) – and Cy7-conjugated anti-CD4(RM 4-5) mAb, peridinin chlorophyll protein complex (PerCP)- and Cy5.5-conjugated anti-CD25 (PC-61) mAb, phycoerythrin (PE) anti-ROR (Q31-378) mAb were 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 were purchased from Biolegend. Recombinant murine IL-6, IL-12, IL-23, IL-1 β were purchased from Sarstedt and Bioscience.

All results were received by the author personally in 01.2018 and reported on a scientific club in the laboratory on January 29, 2013, and this was the continuation of the author's dissertation work [16].

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References

1. Zhang et al. Analysis of CD81 Treg cells in patients with ovarian cancer: a possible mechanism for immune impairment Cellular & Molecular Immunology 2015 12, 580–591
2. Facciabene A, Motz GT, Coukos G. T-regulatory cells: key players in tumor immune escape and angiogenesis. Cancer Res 2012; 72: 2162–2171.
3. Viehl CT, Moore TT, Liyanage UK, Frey DM, Ehlers JP, Eberlein TJ et al. Depletion of CD41CD251 regulatory T cells promotes a tumorspecific immune response in pancreas cancer-bearing mice. Ann Surg Oncol 2006; 13: 1252–1258.
4. Liu S, Foulkes WD, Leung S, Gao D, Lau S, Kos Z et al. Prognostic significance of FOXP31 tumor infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration. Breast Cancer Res 2014; 16: 432.
5. Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D et al. Tumor-infiltrating FOXP31 T regulatory cells show strong prognostic significance in colorectal cancer. J Clin Oncol 2009; 27: 186–192.
6. Leveque et al. Interleukin 2-mediated Conversion of Ovarian Cancer-associated CD4+ Regulatory T Cells Into Proinflammatory Interleukin 17-producing Helper T Cells. J Immunother 2009;32:101–108
7. Nilsson MB, Langley RR, Fidler IJ. Interleukin-6, secreted by human ovarian carcinoma cells, is a potent proangiogenic cytokine. Cancer Res. 2005;65:10794–10800.
8. Miyahara et al. Generation and regulation of human CD4+IL-17-producing T cells in ovarian cancer. PNAS; October 7, 2008; vol. 105, no. 40; 15505–15510
9. http://amp.pharm.mssm.edu/Harmonizome/gene_set/SKOV3/CCLE+Cell+Line+Gene+Expression+Profiles
10. Zhou et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. [Nature](#). 2008 May 8;453(7192):236-40.
11. Carr TM, Adair SJ, Fink MJ, Hogan KT (2008) Immunological profiling of a panel of human ovarian cancer cell lines. Cancer Immunol Immunother 57:31–42.

12. Villagra et al. The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. NATURE IMMUNOLOGY. VOLUME 10 NUMBER 1 JANUARY 2009; pp. 92-100
13. Hirota et al. Fate mapping of IL-17-producing T cells in inflammatory responses. nature immunology VOLUME 12 NUMBER 3 MARCH 2011 pp. 255-260
14. Bryl et al. Human CD4^{low} CD25^{high} regulatory T cells indiscriminately kill autologous activated T cells. Immunology 2009, 128, e287–e295
15. Bezman et al. Molecular definition of the identity and activation of natural killer cells. Nature Immunology VOLUME 13 NUMBER 10 OCTOBER 2012 1000-1009
16. <http://www.ronc.ru/node/2246>

ROC

II-1

II-1p

II-6

II-12

II-23

F1 Score

IL 3

IL 14

IL 16

IL 13

IL 25

CD40

II-2

II-4

II-6

II-12

II-13

II-30

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Key findings		
	Analysis	Results
Fig. 1	10%	10%
Fig. 2	10%	10%
Fig. 3	10%	10%
Fig. 4	10%	10%
Fig. 5	10%	10%

H.2

H.3

H.4

H.17

H.12

H.23

FOXP3

IL-12

IL-13

IL-4

IL-12

IL-13

FOXP3

10

CD4

CD15