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Co-expression of membrane-bound TNF-alpha type 1 and 2 receptors differ in the subsets of immunocompetent cells

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ABSTRACT

The immunoregulatory cytokine tumor necrosis factor α plays crucial roles in the pathogenesis of a broad spectrum of disorders. However, its effect may depend on the expression and co-expression of receptors on the target cell. The aim of the present study was to evaluate the expression levels of type 1 and 2 tumor necrosis factor α receptors (TNFR1/2) on individual cell subsets from peripheral blood of healthy volunteers. Flow cytometry analysis was used to study whole populations as well as subsets (T regulatory cells, T memory cells, cytotoxic T cells, T helper cells). Significant differences in the co-expression of TNFR1/2 were seen within subsets and total pools. Further studies are necessary to explore the implications of the observed differences in the modulation of tumor necrosis factor α function in health and pathology.

1. Introduction

Receptors for immunoregulatory cytokines are attracting increasing attention as they are key molecules in the regulation of many biological processes. [1–3] Membrane-bound receptors ensure the appropriate activity of mediators by altering cell activities via different signaling pathways. Correlations with various pathogenetic processes have been demonstrated for a large number of cytokine receptors. For example, it has been demonstrated that even minimal expression of interferon-γ receptors in hepatocytes results in increases in tumor size, serum level of alpha-fetoprotein, and frequency of intra- and extrahepatic metastases. [4] Tumor necrosis factor (TNF) receptor type 1 plays a crucial role in the manifestation of the effects of TNF-α in cultured synovial fibroblasts from patients with rheumatoid arthritis [5]. Studies of numerous cytokines have demonstrated that the functional response of cells can depend on the level of receptor expression. For example, studies of the GM-CSF receptor revealed that addition of the GM-CSF cytokine to the culture only induced monocyte differentiation into dendritic cells when a threshold level of receptors was expressed. At lower levels of receptor expression the functional state of the cells did not change, even though the mediator had certain effects.[3] These results confirm that receptors of proinflammatory cytokines are involved in the pathogenesis of immune-mediated diseases, however, the extent of their participation remains unclear.

The immunoregulatory cytokine TNF-α plays a crucial role at all stages of pathogenesis in numerous autoimmune, [6–8] allergic, and infectious disorders. Therefore, therapeutics based on TNF-α inhibitors are widely used to treat a number of diseases [9]. The effects of TNF-α are only realized when sufficient numbers of type 1 and 2 receptors are expressed on cells, and variations in the ratios of the receptors may shift the balance between proapoptotic and proliferative signaling pathways [10]. During an active inflammatory process, variations in the expression levels of different types of TNF-α receptors are one of the molecular mechanisms which tune the effects of mediators. Changes in the receptor expression levels can be a result of pathological processes.

Earlier studies have demonstrated that the expression levels of type 1 and 2 receptors differ significantly between the main cell subsets in healthy volunteers. [11] Proinflammatory cytokine TNF-α plays a key role at different stages of most immune-mediated diseases, and so comprehensive evaluation of the TNF-α system allows highly accurate analysis of disease severity. [12] Changes in cytokine receptor machinery in pathology correlate with integral indicators of disease severity for rheumatoid arthritis and atopic eczema. These changes can therefore be used to build prognostic models of responses to therapy. No data are currently available regarding the co-expression of different TNF-α receptors for separate cell subsets actively involved in

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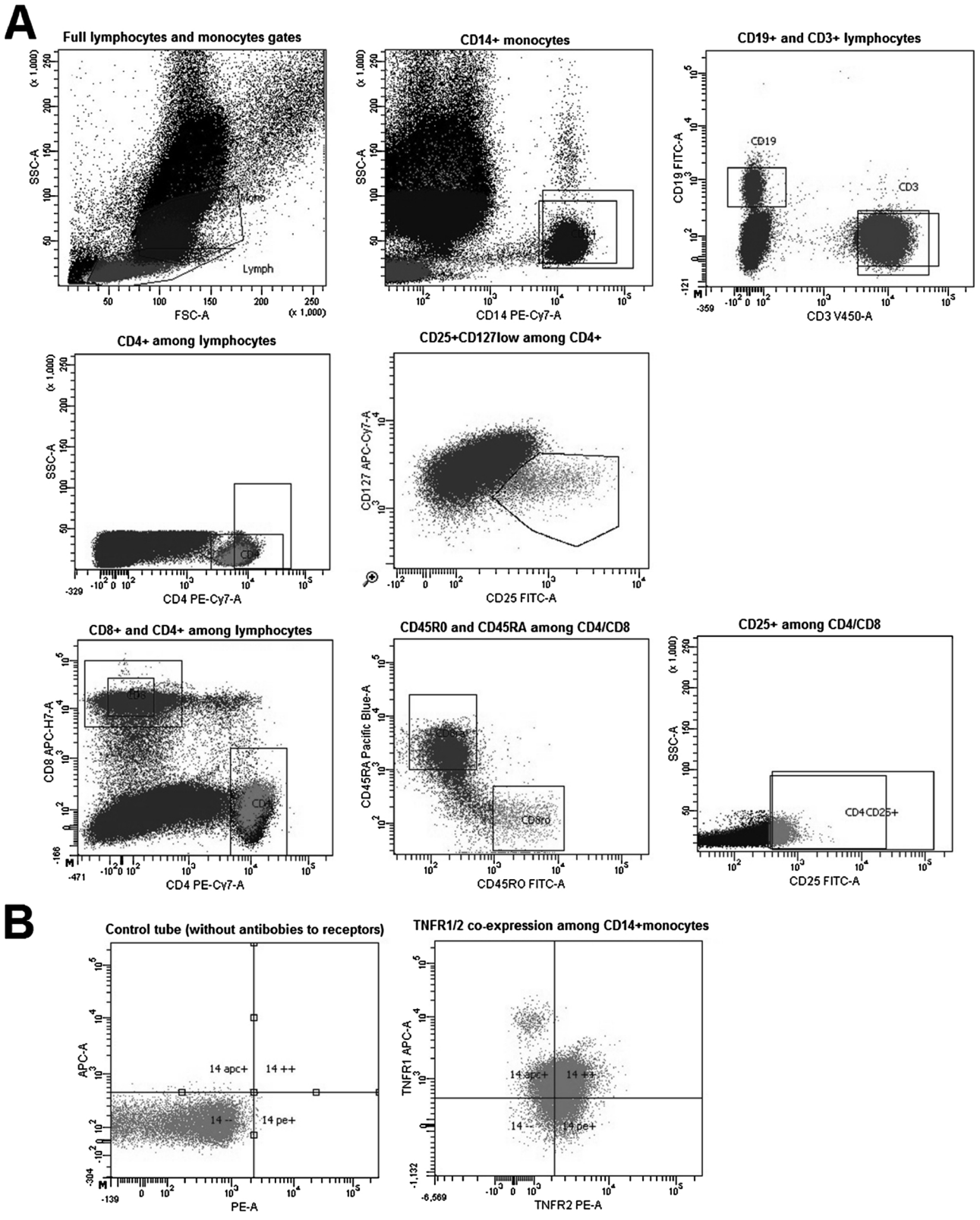

Fig. 1. (A) Gating strategy (B) Example of TNFR1/2 cells identification among subset.

pathological processes. These subsets, such as regulatory T cells, memory T cells, and T helper cells, can also change their functional activity significantly in pathology due to the response of the immune system to therapy. The regulation of receptor expression on certain subsets of target cells can therefore be used to modify the immune response, with significant therapeutic potential.

The results of this study offer insight into cytokine network function. This information paves the way to develop new approaches for the diagnosis and prognosis of immunopathological conditions by revealing deviations from normal values. Therefore, the **objective of this study** was to evaluate the expression levels of type 1 and 2 TNF-α receptors on individual cell subsets from peripheral blood of healthy volunteers. The

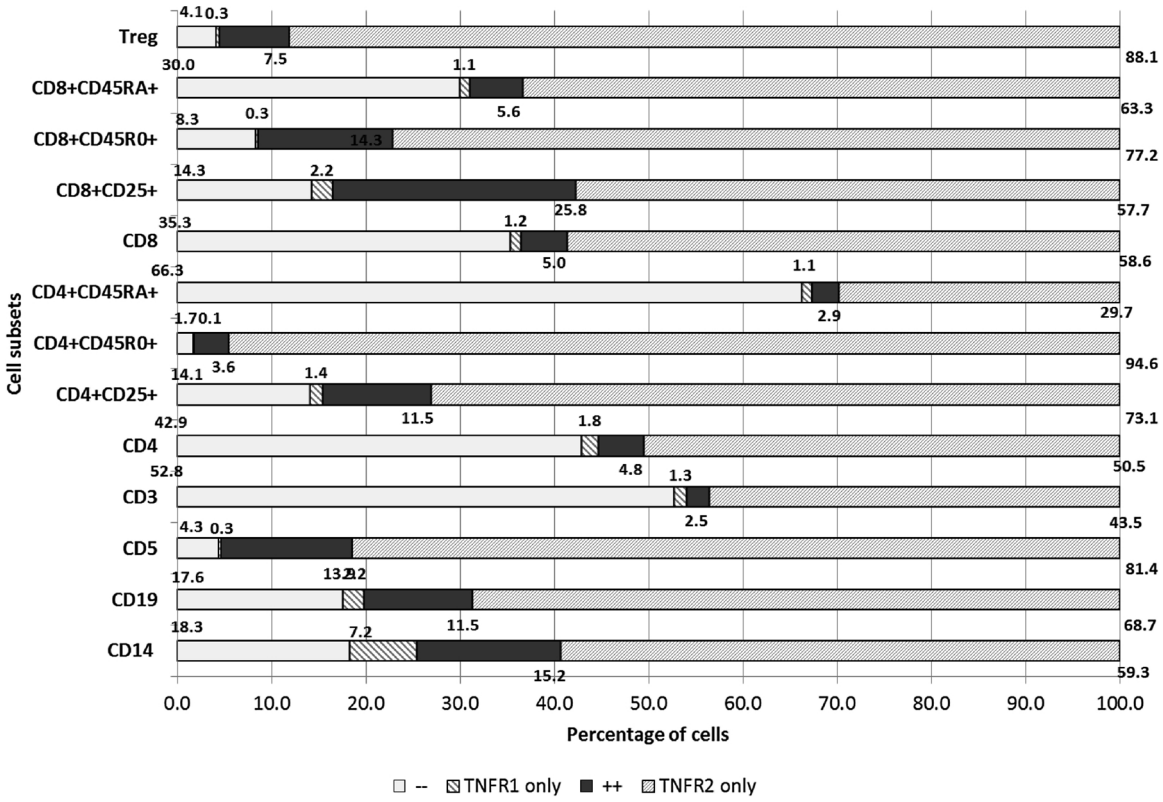

Fig. 2. The percentage of cells with different variants of co-expression of TNFα receptors types 1 and 2 in the main populations studied. The data are shown as the median values subjected to total-sum normalization.

normal values and the differences of subsets of cells actively involved in immunoregulatory processes were assessed.

2. Methods

2.1. Samples

Mononuclear cells (MNCs) were isolated from the peripheral blood of healthy donors and used to assess the levels of expression and co-expression of type 1 and 2 TNF-α receptors (blood samples were obtained from the Blood Collection Station no. 1 "Novosibirsk Clinical Blood Center.")

The study involved 46 healthy volunteers aged 18–77 years (median age 36.5, interquartile range 30–54 years) including 16 (34.8%) males and 30 (65.2%) females.

Venous blood was collected from the median cubital vein under sterile conditions into vacuum tubes (6 ml per tube) containing K3-EDTA anticoagulant (tri-potassium salt of EDTA, Vacuette K3-EDTA, Greiner Bio-One GmbH, Austria).

Blood samples were prepared using BD FACS Lysing Solution (catalog number 349202, BD, USA) according to the manufacturer's instructions.

2.2. Flow cytometry

Phenotypic characteristics of MNCs were evaluated using flow cytometry (FACSVerse™ cytofluorometer, BD Biosciences, USA) using the following monoclonal antibodies: anti-human CD3 V421, anti-human CD19 FITC, anti-human CD8 APC/Cy7, anti-human CD5-APC-Cy7, anti-human CD14 Pe/Cy7, anti-human CD25 FITC, anti-human CD127 (IL-7Rα) APC/Cy7, anti-human CD45RO FITC, anti-human CD45RA Pacific Blue, anti-human CD4 Pe/Cy7, anti-human TNF RI-PE, anti-human TNF RII-PE, anti-human TNF RI-APC, anti-human TNF RII-APC (R&D Systems, USA). For the calculations, we used a compensation matrix constructed by cytometer program using fluorescence minus two controls. Data analysis and calculation of fluorescence intensity were conducted using BD FacsDiva software (BD Biosciences, USA).

A BD QuantiBRITE PE kit (BD Biosciences, USA) containing four fractions of lyophilized beads with different levels of phycoerythrin (PE), was used to plot a calibration curve. The curve was used to convert the fluorescence intensities of the cells expressing a marker to the absolute number of receptors.

We used a modified protocol to assess the density of specific receptors on the cell surface. [11] The relationship between the logarithm of the number of PE molecules and the logarithm of fluorescence intensity was graphed using the results of analysis of the beads according to the manufacturer's instructions. The mathematical and linear functions were obtained by curve fitting. The resulting function was used to derive a formula to convert observed fluorescence intensity values to the number of PE molecules for each of the subsets studied. The mean number of receptors per cell was calculated using a procedure described in the literature. [11] Simultaneous evaluation of the numbers of type 1 and 2 TNF-α receptors was carried out by labeling samples with antibodies to both type 1 (TNFR1) and type 2 (TNFR2) receptors. The number of type 1 receptors on double-positive cells and on $TNFR1^{+}TNFR2^{-}$ cells was determined for the sample labeled with anti-human TNF RI-PE and anti-human TNF RII-APC. The number of type 2 receptors on double-positive cells and on $TNFR1^{-}TNFR2^{+}$ cells was determined for the sample labeled with anti-human TNF RI-APC and anti-human TNF RII-PE. The percentage of fractions with different combinations of receptor co-expression was considered the mean of the two samples (Fig. 1).

2.3. Statistics

Statistical analysis was performed using STATISTICA 7.0 software

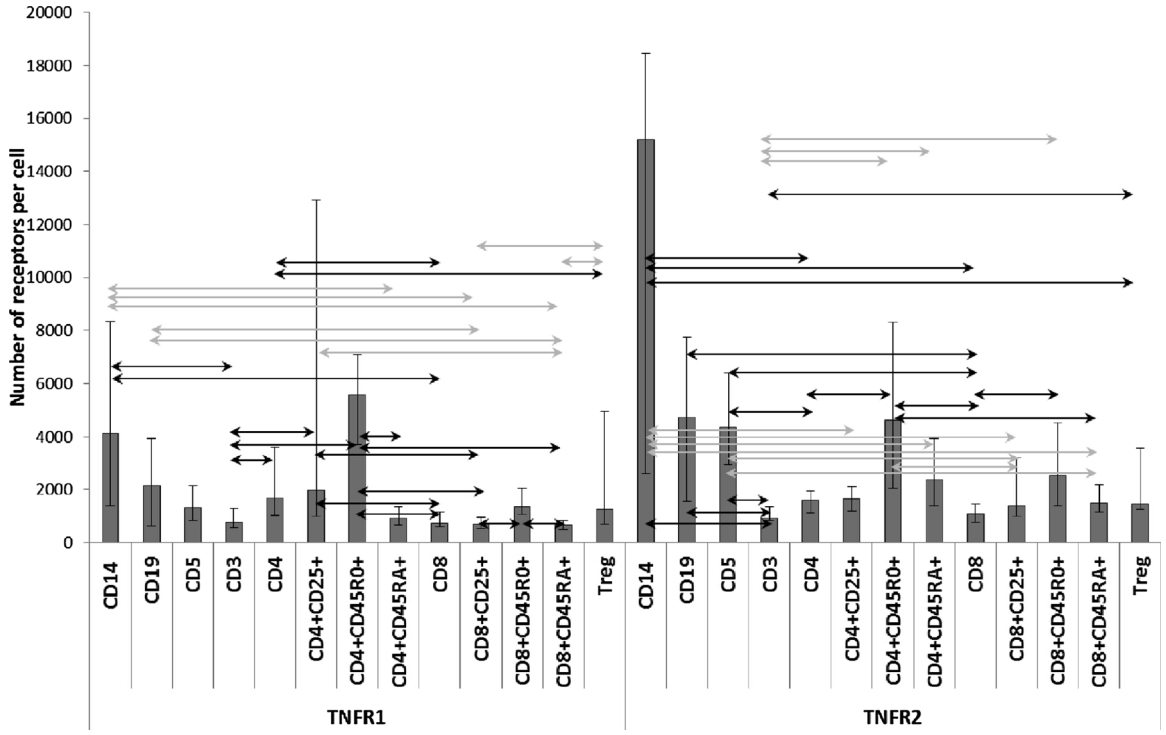

Fig. 3. The number of TNFα receptors types 1 and 2 expressed on cells in the main populations studied. The data are shown as the median values and the interquartile range. Black arrows indicated differences ($p < 0.05$) between main populations of cells and among subsets of one population. Grey arrows indicated all other significant differences ($p < 0.05$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(StatSoft, USA). The data are presented as the median and the interquartile range (IQR). Independent samples were compared and statistical significance calculated using the Kruskal-Wallis test by ranks with multiple comparison of the median values (when comparing identical indicators for different subsets and revealing the differences between the subgroups of study subjects). The correlations between parameters were determined using the Pearson's correlation coefficient (at $p < 0.05$). The statistical significance of changes was determined using the Wilcoxon test after the disease-modifying treatment had been corrected for in dependent samples.

3. Results

The main studied subsets of cells (T cells, B cells, and monocytes) differed significantly in terms of co-expression of type 1 and 2 TNF-α receptors (Figs. 2 and 3). The highest percentage of double-positive cells was seen in the activated cytotoxic T cells (25.8%), with somewhat lower percentages of double-positive cells seen in monocytes (15.2%), cytotoxic memory T cells (14.3%), CD5 + B cells (13.9%), and the total pool of B cells and activated T helper cells (11.5% each). For other subsets, the percentage of double-positive cells was below 10%.

All studied small subsets were characterized by very low percentages of cells expressing only the type 1 receptor, which was always at least twofold lower than the percentage of double-positive cells ($p < 0.001$ for all subsets except $CD4^+CD45RA^+$ cells ($p = 0.017$)). Regulatory T cells, memory cytotoxic T cells and $CD5^+$ B cells were characterized by an almost total absence of cells expressing only type 1 receptors (< 0.5%).

In contrast, the percentage of cells expressing only type 2 receptors was higher than 25% in all studied populations, and was at least twice as high as the percentage of double-positive cells in all cases ($p < 0.05$ for all subsets).

Almost all regulatory T cells expressed at least one type of receptor, which was a significant increase compared with the total pool of T cells (Table 1) ($p < 0.001$). The percentage of regulatory T cells carrying only the type 2 receptor was approximately twofold higher than that of the total pool of T cells ($p < 0.001$). Similar differences (a reduced percentage of double-negative cells, almost total absence of $TNFR1^+TNFR2^-$ cells, and an increased percentage of cells with type 2 receptors only) were observed for $CD5^+$ B cells compared with the total pool of B cells.

Naïve T cells (CD4RA and CD8RA) differed significantly in their co-expression of TNF-α receptors. Whereas about 70% of cytotoxic T cells were found to express at least one type of receptor, and the median receptor numbers on positive cells were 667 (IQR 492-822) (type 1 receptor) and 1497 (IQR 1160 – 2161) (type 2 receptor), the percentage of T helper cells expressing at least one receptor was found to be significantly lower (40.3%, $p < 0.01$). However, the median number of receptors on T helper cells was approximately 1.5-fold higher, at 918 (IQR 642-1351) and 2373 (IQR 1394-3904) for type 1 and type 2 receptors respectively ($p < 0.001$ in both cases).

Table 1
Expression of receptors to TNFα on main subsets.

Subset	Mean (± SE) percentage of cells expressing at least one type of receptors to TNFα
CD14	77.6 ± 3.2
CD19	77.8 ± 4
CD5	94.3 ± 1.4
CD3	51.1 ± 3.9
CD4	61.5 ± 3.6
CD4 + CD25 +	84.8 ± 2.5
CD4 + CD45R0 +	93.9 ± 1.7
CD4 + CD45RA +	40.3 ± 3.3
CD8	68.5 ± 3.4
CD8 + CD25 +	83.5 ± 2.9
CD8 + CD45R0 +	89.8 ± 1.8
CD8 + CD45RA +	71.7 ± 2.9
Treg	94.4 ± 0.9

4. Discussion

Earlier studies [11] have demonstrated that the expressions of type 1 and 2 TNF-α receptors on T cells, B cells, and monocytes differ significantly both in terms of the percentage of positive cells and the number of receptors. Within populations such as these, significant heterogeneity in receptor expression can be observed. We therefore decided to study the co-expression of TNF-α type 1 and 2 receptors in the most important lymphocyte subsets. Furthermore, we calculated the mean number of receptors on the cells for each co-expression variant. The following populations were selected: the total monocyte pool, the total B-cell pool, and the total T-cell pool. The following subsets were also studied: cytotoxic T cells ($CD8^+$), T helper cells ($CD4^+$), activated $CD8^+$ cells, activated $CD4^+$ cells, memory T cell subsets ($CD45RO^+$), and naïve T cells ($CD45RA^+$) among cytotoxic and T helper cells; regulatory T cells ($CD4^+CD25^{high}CD127^{low}$); and B1 cells ($CD45^+CD19^+CD5^+$).

Although the level of cells expressing only the type 1 receptor was minimal in all subsets studied, almost all subsets shared the following feature: the densities of both receptors on double-positive cells was higher than the densities of the corresponding receptors on the cells carrying only one type of receptor. In other words, co-expression of TNF-α type 1 and 2 receptors has a mutually potentiating effect.

The co-expression of TNF-α receptors differed between populations, and the percentage of double-negative cytotoxic and helper memory T cells was low. Although the co-expression varied significantly, both subsets were characterized by high densities of type 1 and 2 receptors. Therefore, memory T cells subsets have strong potentials for responding to TNF-α cytokine.

The extremely high variability observed in some populations (such as $CD14^+$ or $CD4^+CD25^+$) may be due to the heterogeneity of these populations and the fact that within them there are separate groups of cells that differ in function and activity.

The subsets investigated in this study showed significant differences in the receptor densities on the cell surface. Although variation in the expression levels between different functional cell types was expected, it had not been previously proven that the number of receptors normally carried by these cells varies significantly.

5. Conclusion

The main T-cell subsets vary in terms of their co-expression of type 1 and 2 TNF-α receptors, which may cause different levels and types of cell response to the cytokine. The presence of both types of receptors on the cell surface is associated with increased densities of both receptors. In order to prove practical significance of the differences, indicators of co-expression in patients with different nosological entities of disease need to be compared with those in healthy individuals.

Statement of equal author contribution

SS, AA, JZ, AK, and JL contributed equally to this study.

Conflict of interest

The authors declare no conflict of interest.

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