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Células T reguladoras en infecciones de vías aéreas superiores

TESIS QUE PRESENTA

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Quantitative analysis of different regulatory T cell subsets in pediatric patients with upper respiratory tract infections

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Regulatory T cells and upper respiratory tract infection.

Regulatory T cells and infection.

Abstract

Background: The possible cause of recurrent upper respiratory tract infections (URTI) in pediatric patients has not been determined. Regulatory T cells have a key role in the modulation of the immune response and inflammation, and an abnormal number or function of these cells may impair the immunocompetence. In this work, we decided to explore the possible involvement of regulatory T cells in pediatric patients with recurrent URTI.

Methods: We studied twenty patients with a high (5-8/year), and seventeen with a low (<4/year, control group) number of URTI. Their age ranged from 16 to 36 months at the time of study, and all they were otherwise healthy, with no evidence of immunodeficiency. Peripheral blood was obtained, and the absolute number and percent of different T regulatory cells subsets were determined by immunofluorescence and flow cytometry.

Results: The levels (absolute number) of different subsets of induced regulatory T cells (Tr1, Tr1-like, and Th3 cells) were higher in the group of patients with a high number of URTI compared to control group ($p < 0.05$). In addition, there was a significant positive correlation between the levels of Tr1, Tr1-like, and Th3 cells and the number of episodes of URTI ($r = 0.48$, 0.61, and 0.62, respectively, $p < 0.05$ in all cases). In contrast, the levels of CD4+Foxp3+ natural T regulatory cells, and CD8+ T suppressor lymphocytes were similar in the two groups studied.

Conclusions: An enhanced level of different regulatory cells may have a role in the increased risk for recurrent URTI in otherwise healthy infants. Alternatively, it is also feasible that repeated episodes of URTI may induce an increase in the levels induced regulatory T cells. It would be interesting to

further study the possible function of regulatory T cells in patients with recurrent URTI.

Introduction

Upper respiratory tract infections (URTI) are the most common cause of visits to pediatrician clinics.[1]. The etiology of most of these episodes is viral, most of them are self-limited and are not associated significant morbidy and mortality.[2]. However, these infections have a great importance because they lead to parental anxiety and work absenteeism[3]. It has been established that normal children may have up to four to five episodes of URTI per year[4] and this does not imply the presence of immunodeficiency. However, it is evident that some otherwise healthy children have a higher frequency of URTI, with no apparent cause, and that this condition spontaneously disappear. In an early report, we found that apparently healthy children with a high frequency of URTI exhibit increased levels of CD8+ lymphocytes[5]. However, no additional studies on immunological parameters have been reported in this type of pediatric patients.

Two main subpopulations of regulatory T cells has been described, natural (or constitutive) and inducible (or adaptive) regulatory T lymphocytes, which likely have complementary and overlapping functions in the control of immune response[6,7]. CD4+ CD25+ natural regulatory T cells were first described by Sakaguchi et. al.[8]. These cells are generated in the thymus, during the process of negative selection and comprise from 5 to 10% of peripheral CD4+ T cells in healthy subjects[9-11]. This natural regulatory T cells are anergic[12], because they do not proliferate in response to antigenic stimulation in vitro[13]. These cells are able to inhibit the proliferation and the cytokine synthesis by effector lymphocytes through direct cell-cell contact[10]. Other subsets of regulatory T cells are those characterized by the synthesis of IL-10 or TGF-β-

secreting regulatory T cells, which are known as Tr1/Tr1-like cells (CD4+ CD25- IL-10+), and Th3 lymphocytes (CD4+ CD25- TGF-β+)[14,15]. Tr1 cells are generated in the periphery, seem to be antigen-specific, and are derived from conventional CD4+ CD25- naïve precursor upon exposure to antigen under conditions of limiting costimulation, and in the presence of IL-10[14,15]. Th3 cells were described initially by Weiner and co-workers[15,16] as CD4+ T cells induced by oral antigens in the mesenteric lymph nodes, which synthesize TGF-β and variable levels of IL-4 and IL-10[14-16].

Finally, there is another population of regulatory T cells, the suppressor or Ts lymphocytes, which are CD8+, and do not bear the costimulatory molecule CD28[17,18]. These regulatory cells mainly exert their effect through the induction of tolerogenic dendritic cells[17].

The mechanisms used by the host to control infection include the synthesis of pro-inflammatory cytokines and chemokines, the recruitment of inflammatory cells to the site of infection, and activation of different leukocyte subsets, including T and B lymphocytes and natural killer (NK) cells [19,20]. All these phenomena are under the control of different homeostatic mechanisms, including the T regulatory cells[19-21]. It has been suggested that the different outcomes of infection may be strongly influenced by the ratio of regulatory T cells to effector T lymphocytes[19,23]. This point is supported by a report by Mendez S. et al., showing that the activity of regulatory T cells plays a critical role in the reactivation of leishmaniasis at the primary site of disease induced by superinfection[24]. Thus, the equilibrium between effector and regulatory T cells modulates the efficiency of recall immune responses and infection outcome[19,25]. In addition, the critical role of T regulatory cells in disease progression has also

been described in parasitic and viral infections[23,26,27]. All these data indicate that although T regulatory cells exert an important homeostatic role in the control of both, the inflammatory response and the strength of the immune response, an excessive function of these cells may favor the appearance of infectious process. In this work, we have explored the status of different subsets of T regulatory cells in pediatric patients with recurrent URTI, with no evidence of immunodeficiency.

Materials and Methods.

Patients. All subjects included in this study were outpatients evaluated between January and December 2005. They were divided in two groups, according to the number of URTI during the previous year: patients with recurrent URTI, and controls. The inclusion criteria for the first group were: five to eight episodes of URTI in the last year, age between 16 and 36 months, normal weight and height, and no clinical evidence of immunodeficiency. In this group were included 20 patients, 10 female and 10 male. Patients with history of lower respiratory tract infections were excluded. The inclusion criteria for the control group were as follows: Four or less episodes of URTI during the previous year, age between 16 and 36 months and weight and height in normal parameters. Seventeen patients were included in this group, 9 female and 8 male. It is worth mentioning that the serum immunoglobulin levels fall into normal values in all cases in both groups, and that these levels were similar in children with recurrent URTI and controls (data not shown). A written informed consent was obtained from the parents of all patients before entry into the study. The Bioethical Committee of the Hospital Central Dr. Ignacio Morones Prieto approved this study.

Antibodies and Reagents. The following monoclonal antibodies (mAbs) were used: PerCyP labeled anti-human CD4, FITC tagged anti-human CD25, PE labeled anti-human CD152 (CTLA-4) and CD28, anti-human TGF-β biotin and PE-streptavidin. Isotype-matched controls were purchased from PharMingen (San Diego, CA). The percent of CD4+ lymphocytes synthesizing IL-10 was determined by using the appropriate commercial kit (Miltenyi), following the instructions of the manufacturer. In this assay, cells were first

incubated with a catch reagent (a bi-specific dimeric antibody) that binds to a cell surface antigen of leukocytes and that is also able to react with the IL-10 secreted by the cell. Then, cells were incubated for 60 min at 37 °C, washed and labeled with FITC-, and PE-tagged anti-CD4 and anti-IL-10 mAbs. Finally, double positive cells were detected with a FACSCalibur flow cytometer, and results were expressed as the percent of Tr1 lymphocytes.

Isolation of mononuclear cells (MNC). MNC were separated from peripheral venous blood by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich). Briefly, peripheral venous blood was diluted 1:2 with phosphate-buffered saline (PBS), carefully loaded onto Histopaque-1077, and centrifuged at 2500 rpm for 20 minutes at room temperature. Then, MNC were obtained, and washed two times with PBS. Cell viability was always greater than 98%, as determined by tripan blue dye exclusion.

Immunofluorescence and flow cytometry analysis. MNC immunostained with the indicated antibodies were washed and fixed. To quantify natural regulatory T cells, a double staining procedure using an FITC anti-CD25 and a PerCyP anti-CD4 mAb was performed. Ts cells were detected by using an FITC anti-CD8 and a PE anti-CD28 mAb, whereas Tr1/Tr1-like cells were identified by labeling with a combination of a PerCyP anti-CD4 mAb, and the detection of IL-10 synthesis by using the proper kit (Miltenyi). Finally, Th3 lymphocytes were analyzed with FITC anti-CD4, and TGF-β (TGF-β-biotin plus streptavidin-PE). Analysis of different cell subsets was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using the CellQuest software (Becton Dickinson). Results were expressed as the absolute number of positive cells/μl.

Statistical analysis. The differences in percentages an absolute numbers of regulatory T cells were analyzed by using the student's T test (parametric analysis) or the Mann-Whitney U test (non parametric analysis). Correlation between two variables was determined by using the Spearman sum rank test. Differences were considered significant when the p value was less than 0.05.

Results

Number of CD8+ lymphocytes is increased in patients with recurrent URTI. It is well known that the levels of blood lymphocytes are increased in different infectious diseases, including viral infections. Accordingly, we found an enhanced level of CD8+ T lymphocytes in the peripheral blood of patients with recurrent URTI compared with control group ($p<0.05$, Fig. 1A, and C). In contrast, no significant differences were found in the case of CD4+ T lymphocytes in both groups studied ($p>0.05$, Fig, 1B, and D).

Natural T regulatory cells in patients with recurrent URTI. Natural T regulatory cells have been studied in different conditions, including tuberculosis, infection by Epstein-Barr virus, and some parasitic diseases. Our results showed that patients with recurrent URTI had similar levels of $CD4+CD25^{bright}$ natural regulatory cells than control individuals ($p>0.05$, Fig. 2). These results were confirmed by flow cytometry analysis of the expression of the transcription factor Foxp3 by CD4+ T cells (data not shown).

Adaptive regulatory T cells are increased in patients with recurrent URTI. Whereas natural regulatory cells mainly recognize self-antigens, the immune repertoire of adaptive T regulatory cells is mainly directed against exogenous antigens, including those of infectious agents[19]. We have found enhanced levels of CD4+CD25- lymphocytes that synthesize IL-10, which mainly correspond to Tr1/Tr1-like cells ($p<0.05$, Fig. 3A). Likewise, we found a higher number of CD4+CD25- cells that synthesize TGF-β, which correspond to Th3 lymphocytes ($p<0.05$, Fig. 3B). In contrast, no significant differences were found in the case of suppressor T (CD8+CD28-) cells ($p>0.05$, Fig. 3C, and D),

although patients with recurrent URTI tended to exhibit higher values than controls.

Correlation between the number and the episodes of URTI and levels of regulatory T cells. We then determined the possible correlation between the levels of the different regulatory T cell subsets and the number of episodes of URTI. In this analysis were included both the patients with recurrent URTI, and the control infants, which according to inclusion criteria, had four or less episodes of URTI in the last twelve months. As shown in Fig. 4A, no significant correlation was found between the levels of natural regulatory T cells and the number of URTI ($r=0.17$, $p>0.05$). In contrast, the levels of adaptive regulatory T lymphocytes, both, Tr1/Tr1-like cells, and Th3 cells, showed a significant positive correlation with the number of URTI ($r=0.48$, $p<0.05$, and $r=0.62$, $p<0.01$, respectively, Fig. 4B and C).

Discussion

Regulatory T cells play a key role in immune homeostasis. The involvement of these cells in the pathogenesis of different autoimmune and infectious diseases has been clearly demonstrated [19,28]. In the case of infectious disease, it is evident the importance of a delicate balance between the generation of an effective immune response (that ensures the elimination of the infectious agent) and the avoidance of tissue damage by the effector mechanisms of immune cells. In this regard, it has been shown the importance of regulatory T cells in the tissue damage seen in different models of infectious disease[19-20]. On the other hand, it has been reported that an excessive proportion and/or function of regulatory cells results in the abrogation of an effective immune response, and a high risk for infection[19-21]. In this regard, it has been shown that regulatory T cells may prevent viral clearance, and that the removal of CD4+CD25+ T lymphocytes can help to resolve infection process[19,23]. Based on these data, we have hypothesized that otherwise healthy pediatric patients with recurrent URTI have increased levels of regulatory T lymphocytes, which may generate an increased risk for infection. Our data show that the levels of natural T regulatory cells are not significantly different in these patients compared to control group. Since it has been shown that CD4+CD25+ natural regulatory T cells mainly recognize self-antigens, this is not an unexpected finding. Thus, different data indicate that these cells appear to exert a more relevant role in the control of self reactivity, and therefore in the pathogenesis of autoimmune diseases. In this regard, we and others have recently reported that patients with systemic lupus erythematosus have a defective function of natural T regulatory cells, but normal levels of adaptive

regulatory lymphocytes[29]. We have also found a similar defect in patients with rheumatoid arthritis, and autoimmune thyroid disease[29,30].

In this work, we have found enhanced levels of the different adaptive regulatory T cell subsets in patients with recurrent URTI. These findings suggest that these enhanced levels might be causally associated with an increased risk for URTI. However, although this is a commendable possibility, it is also feasible that repeated episodes of URTI can induce an increase in the levels of adaptive regulatory T cells. In this regard, it has been recently reported that, in an animal model, the stimulation of through Toll-like receptors (specifically TLR4) favors the induction of IL-10-producing Tr1 lymphocytes[31]. It is of interest that these two possibilities are not mutually excluding, and it is feasible that some of these patients have basal enhanced levels of regulatory T cells, and that repeated URTI further enhance these levels, through the mechanism described above. In any case, we can conclude that, irrespective of the mechanism involved, there is a significant association between the levels of adaptive T regulatory cells in peripheral blood and the number of URTI. To further explore this association, it would be interesting to make a follow-up of these patients, performing repeated measurements of adaptive regulatory T cells, and a careful monitoring of URTI. In addition, it would be important to assess the inhibitory function of these cells through functional assays (i.e., inhibition of cell proliferation). However, the number of cells (and thus the volume of blood) necessary for this type of assays renders difficult to perform it in pediatric patients.

It is worth mentioning that all patients included in this study had normal levels of serum immunoglobulins, and that they did not show, aside of recurrent

URTI, clinical manifestations of immunodeficiency. In this regard, it is possible to speculate that enhanced levels of adaptive regulatory T cells may cause a diminution in the immunocompetence, and that therefore, in theses cases, these cells exert a undesirable effect. Fortunately, this condition seems to be transient, since in most of these patients the enhanced risk for URTI disappear when they grow up.

In summary, our data indicate that patients with recurrent URTI show enhanced levels of adaptive regulatory T cells in their peripheral blood, and that there is a significant relationship between these levels and the number of infectious episodes. We can speculate that these data may suggest a causal association between these two variables.

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References

1. Pitrez PM, Pitrez JL. Acute upper respiratory tract infections: outpatient diagnosis and treatment. *J Pediatr (Rio J).* 2003; 79(1):S77-86.

2. Hemming VG. Viral Respiratory diseases in children: Classification, etiology, epidemiology and risk factors. *J Pediatr.* 1994; 124:S13-S16.

3. Manjarrez ME, Rosete DP, Rincon M, et al. Comparative viral frequency in Mexican children under 5 years of age with and without upper respiratory symptoms. *J Med Microbiol.* 2003; 52:579-583.

4. Donald GA. Epidemiology and Prevention of Pediatric Viral Respiratory Infections in Health-Care Institutions. *Emerg Infect Dis.* 2001; 7:249-253.

5. Leigh MW, Carson JL, Denny FW. Pathogenesis of Respiratory Infections Due to Influenza Virus: Implications for Developing Countries. *Rev Infect Dis.* 1991; 13:S501-S508.

6. O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med.* 2004; 10:801-805.

7. Thompson C, Powrie F. Regulatory T cells. *Curr Opin Pharmacol.* 2004; 4:408-414.

8. Sakaguchi S, Sakaguchi N, Asano M, et al. Immunologic Self-Tolerance Maintained by Activated T cells Expressing IL-2 Receptor α-Chains (CD25). *J. Immunol.* 1995; 155:1151-1164.

9. Shevach EM. Regulatory/Suppressor T cells in Health and Disease. *Arthritis Rheum* 2004; 50:2721-2724.

10. Fehérvari Z, Sakaguchi S. Development and function of $CD25^+$ $CD4^+$ regulatory T cells. *Curr Opin Immunol.* 2004; 16:203-208

11. Maloy K, Powrie F. Regulatory T cells in the control of immune pathology. *Nat Immunol.* 2001; 2:816-822,

12. Piccirillo CA, Shevach EM. Naturally-ocurring $CD4^+$ $CD25^+$ immunoregulatory T cells: central players in the arena of peripheral tolerance. *Semin Immunol.* 2004; 16:81-88.

13. Suciu-Foca N, Manavalan JS, Cortesini R. Generation and function of antigen-specific suppressor and regulatory T cells. *Transpl Immunol.* 2003; 11:235-244.

14. Roncarolo MG, Bacchetta R, Bordignon C, et al. Type 1 T regulatory cells. *Immunol Rev.* 2001; 182:68-79.

15. Weiner HL. Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells. *Immunol Rev.* 2001; 182:207-214.

16. Bach JF. Regulatory T cells under scrutiny. *Nat Rev Immunol.* 2003; 3:189-198.

17. Cortesini R, LeMaoult J, Ciubotariu R, et al. $CD8^+$ $CD28^-$ T suppressor cells and the induction of antigen-specific, antigen-presenting cell-mediated suppression of Th reactivity. *Immunol Rev.* 2001; 182:201-206.

18. Brimmes J, Allez M, Dotan I, et al. Defects in $CD8^+$ Regulatory T Cells in the Lamina Propia of Patients with Inflammatory Bowel Disease. *J Immunol.* 2005; 174:5814-5822.

19. Mills KH. Regulatory T cells: Friend or foe in immunity to infection? *Nat Rev Immunol.* 2004; 4:841-855.

20. Belkaid Y, Rouse BT. Natural Regulatory T cells in infectious disease. *Nat. Immunol.* 2005; 6:353-360.

21. Kamradt T, Göggel R, Erb K. Induction, exacerbation and inhibition of allergic and autoimmune diseases by infection. *Trends Immunol.* 2005; 26:260-267.

22. Reed SG. TGF-β in infections and infectious diseases. *Microbes Infect.* 1999; 1:1313-1325.

23. Rouse BT, Sarangi PP, Uvas S. Regulatory T cells in virus infections. *Immunol Rev.* 2006; 212:272-286.

24. Mendez S, Reckling S, Piccirillo C, et al. Role for $CD4^+$ $CD25^+$ regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity. *J. Exp. Med.* 2004; 200:201-210.

25. Jiang H, Chess L. Regulation of Immune Responses by T Cells. *N Engl J Med.* 2006; 354:1166-1176.

26. Suvas, S, Kumaraguru U, Pack C, et al. $CD4^+$ $CD25^+$ T cells regulate virus-specific primary and memory $CD8^+$ T cell responses. *J. Exp Med.* 2003; 198:889-901.

27. Hisaeda H. Escape of malaria parasites from host immunity requires $CD4^+$ $CD25^+$ regulatory T cells. *Nature Med.* 2004; 10:29-30.

28. Shevach EM. Regulatory T Cells in Autoimmunity. *Annu Rev Immunol.* 2000; 18:423-449.

29. Alvarado-Sánchez B, Hernández-Castro B, Portales-Pérez D, Baranda L, Layseca-Espinosa E, Abud-Mendoza C, Cubillas-Tejada AC, González-Amaro R. Regulatory T cells in patientes with systemic lupus erythematosus. *J Autoimmun.* 2006; 27:110-118.

30. Marazuela M, García-Lopez MA, Figueroa-Vega N, de la Fuente H, Alvarado-Sanchez B, Monsiváis-Urenda A, Sánchez-Madrid F, González-

Amaro R. Regulatory T cells in human autoimmune thyroid disease. *J Clin Endocrinol Metab*. 2006; 91:3639-3646.

31. M den Haan JM, Kraal G, Bevan MJ. Lipopolysaccharide Induces IL-10 Regulatory CD4+ T Cells That Suppress the CD8+ T Cell Response. *J Immunol*. 2007; 178:5429-5433.

Figure Legends

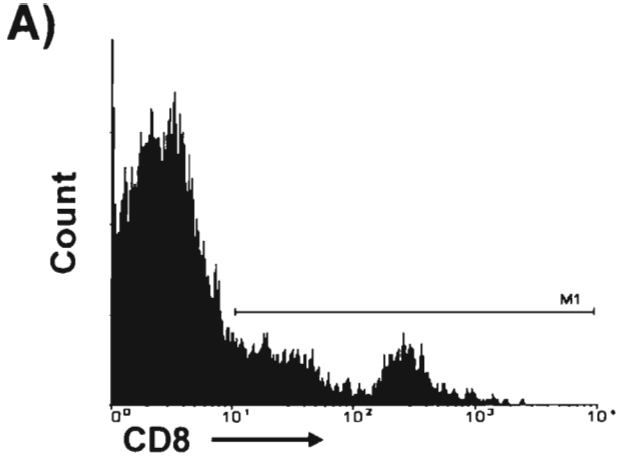
Fig. 1. Quantification of CD4+, and CD8+ T lymphocytes in patients with recurrent URTI and controls. Peripheral blood samples were obtained from patients with recurrent URTI (n=20), and controls group (n=17), and the number of CD4+, and CD8+ T cells was determined by flow cytometry analysis, as indicated in Materials and Methods. Representative histograms are shown in A), and B), and the values of CD8+, and CD4+ T lymphocytes in C), and D). n.s., not significant.

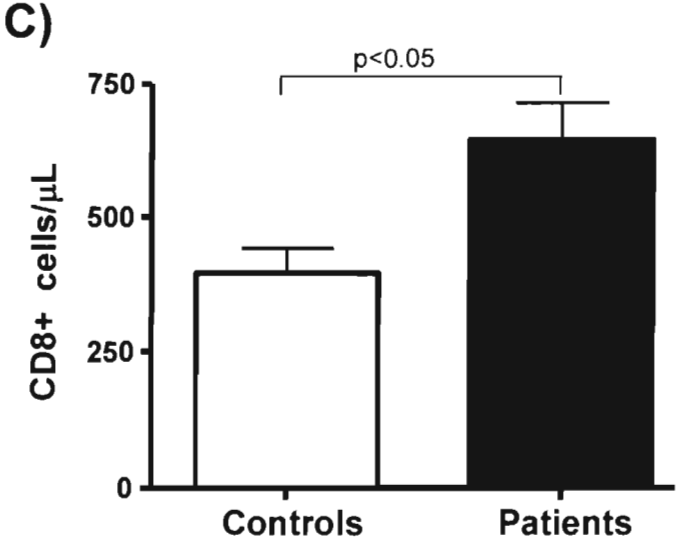
Fig. 2. Quantification of natural regulatory T cells in patients with recurrent URTI and controls. Peripheral blood samples were obtained from patients with recurrent URTI (n=20), and healthy controls (n=17), and the number of CD4+, $CD25^{bright}$ T cells was determined by flow cytometry analysis, as indicated in Materials and Methods. A representative histogram is shown in A), and the values of natural regulatory T cells in B). n.s., not significant.

Fig. 3. Quantification of adaptive regulatory T cells in patients with recurrent URTI and controls. Peripheral blood samples were obtained from patients with recurrent URTI (n=20), and healthy controls (n=17), and the number of CD4+, IL-10+ (Tr1/Tr1-like cells), CD4+, TGF-β+ (Th3 cells), and CD8+, CD28- (T suppressor cells) was determined by flow cytometry analysis, as indicated in Materials and Methods. Values of Tr1/Tr1-like cells, and Th3 lymphocytes are shown in A), and B). A representative histogram, and values of T suppressor cells are shown in C), and D), n.s., non-significant

Fig. 4. Correlation between the number of episodes of URTI, and levels of T regulatory cells in children with recurrent URTI, and healthy controls. The levels of regulatory T cells were determined in the peripheral blood from patients with

recurrent URTI (n=20), and healthy controls (n=17), as stated in Materials and Methods, and the relationship between these values and the number of episodes of URTI was determined. Values of Spearman correlation coefficient (r), and their statistical significance (p), are shown.


A)
Count
M1
CD8


C)
p<0.05
750
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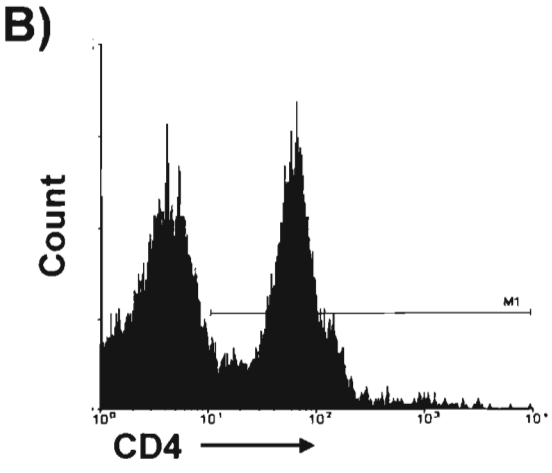
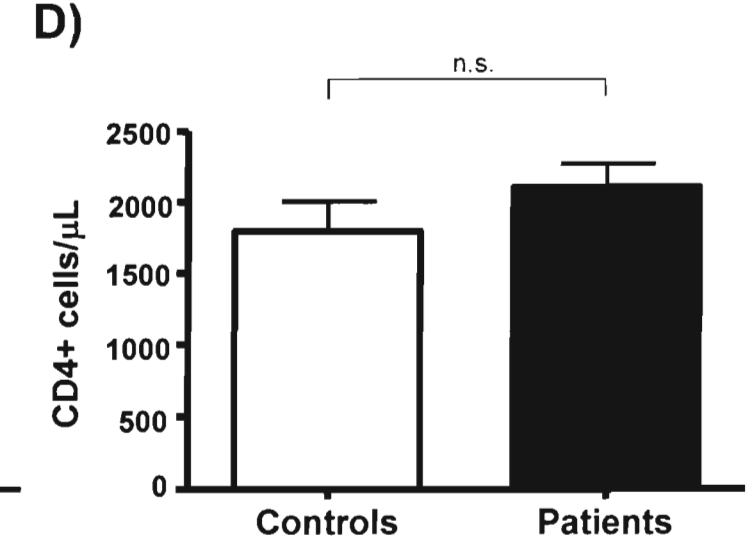
250
0
CD8+ cells/µL
Controls
Patients


B)
Count
M1
CD4


D)
n.s.
2500
2000
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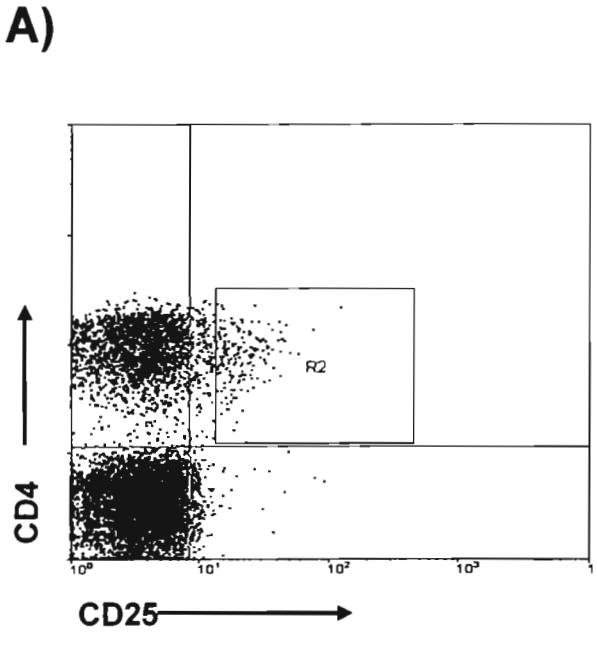
0
CD4+ cells/µL
Controls
Patients

Fig. 1


A)
B)
R2
CD4
CD25
10^0
10^1
10^2
10^3
CD4+CD25bright cells/µL
100
75
50
25
0

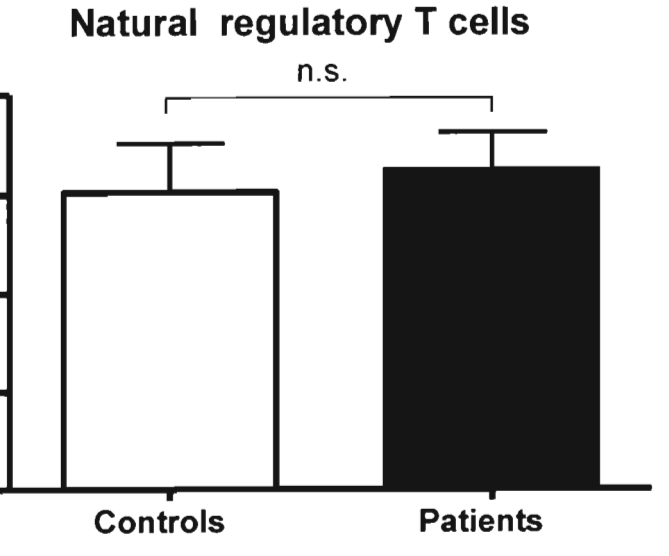
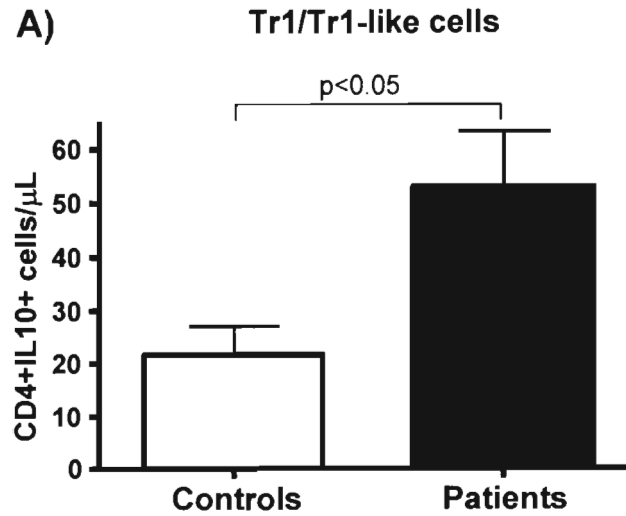
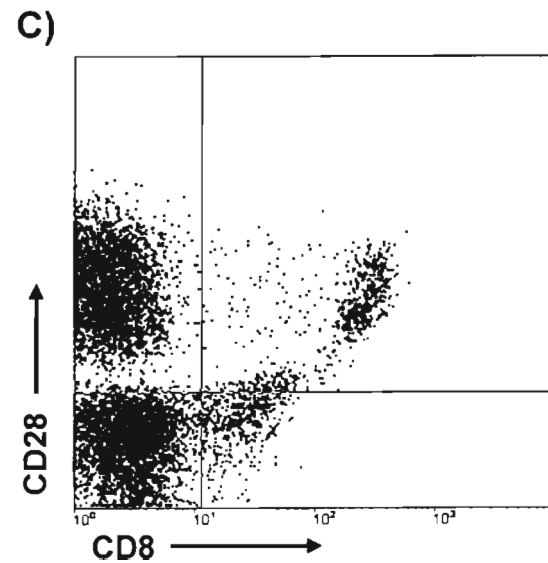
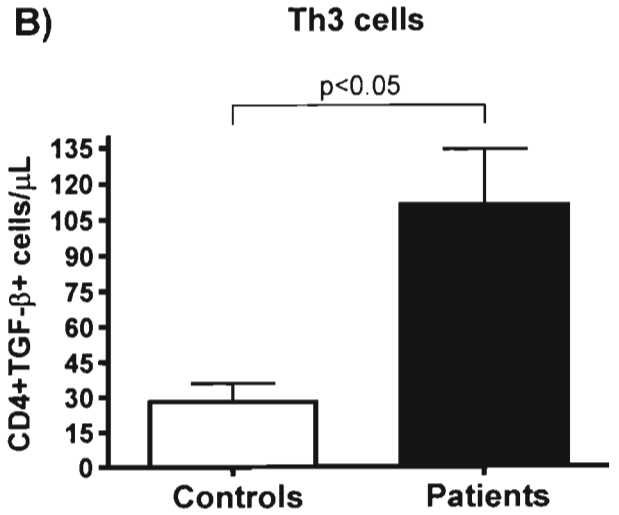


Fig. 2


A)
Tr1/Tr1-like cells
p<0.05
CD4+IL10+ cells/µL
60
50
40
30
20
10
0
Controls
Patients


C)
CD28
CD8
10⁰
10¹
10²
10³


B)
Th3 cells
p<0.05
CD4+TGF-β+ cells/μL
135
120
105
90
75
60
45
30
15
0
Controls
Patients

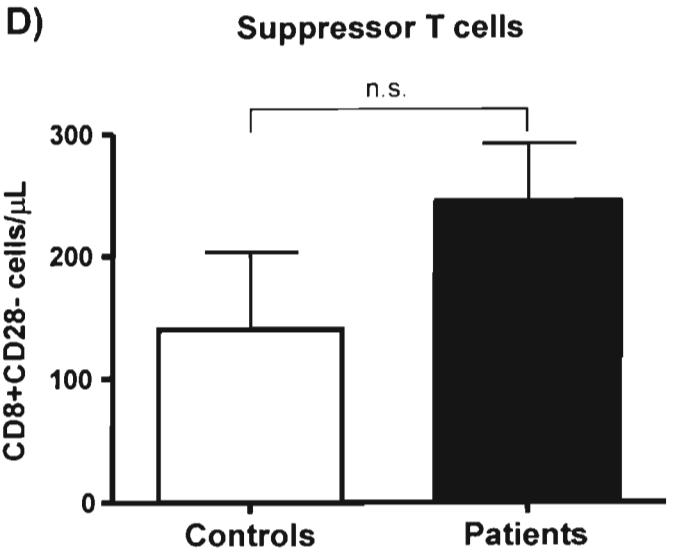
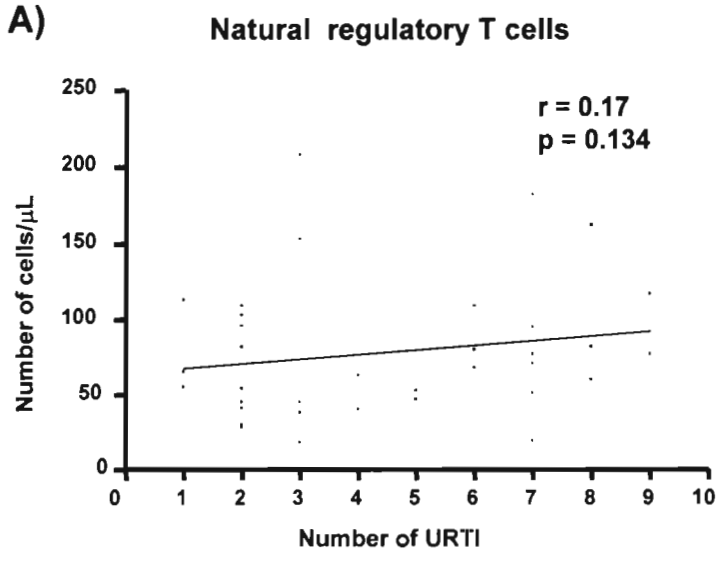
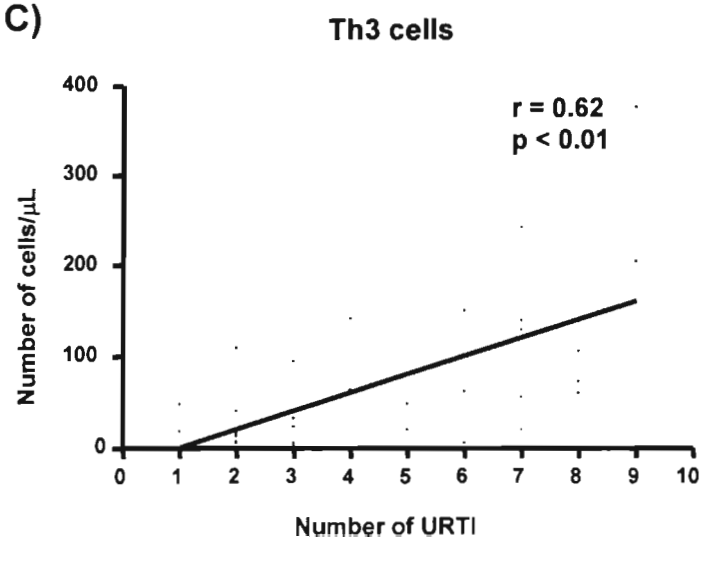

D)
Suppressor T cells
n.s.
CD8+CD28- cells/μL
300
200
100
0
Controls
Patients

Fig. 3


A)
Natural regulatory T cells
r = 0.17
p = 0.134
Number of cells/µL
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10
Number of URTI


C)
Th3 cells
r = 0.62
p < 0.01
Number of cells/µL
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
Number of URTI

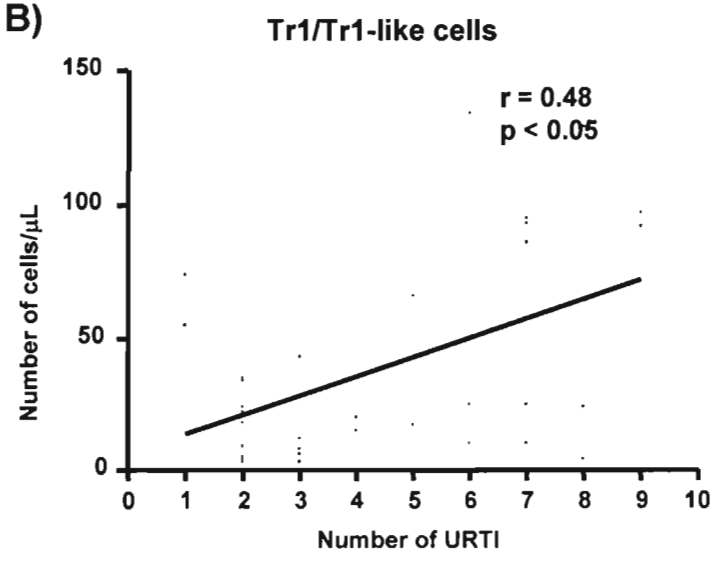

B)
Tr1/Tr1-like cells
r = 0.48
p < 0.05
Number of cells/µL
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
Number of URTI

Fig. 4