ERJ Express. Published on February 6, 2008 as doi: 10.1183/09031936.00093207

CTLA-4-mediated regulatory phenotype of T cells in tolerant lung recipients

Karine Botturi*, Yannick Lacoeuille*, Pascal Thomas°, Stéphanie Boniface*, Martine Reynaud-Gaubert*, Antoine Magnan*.

*UPRES EA 3287, Faculté de Médecine, Université de la Méditerranée, Marseille

° IFR Jean Roche, EA 2201, Marseille, France.

Corresponding Author:

Karine Botturi,

UPRES 3287, Pathologie Respiratoire Liée à l'Environnement,

Faculté de Médecine,

27, Bd Jean Moulin,

13005 Marseille, France.

Phone: 33 4 91 32 45 86

Fax: 33 4 91 32 42 26

E-mail: botturikarine@yahoo.fr

Running title: **CTLA-4-mediated tolerant TC phenotype in LTR**

Text word count: 3386 words

3 tables and 5 figures

Footnotes : This work was granted by the Association *Vaincre La Mucoviscidose*

Abstract:

Obliterative bronchiolitis (OB) is the major cause of long-term lung allograft loss resulting from an unclear immune process occurring in the absence of donor's immune cells. We hypothesized that interactions of autologous dendritic cell (DC) with T cells could differ in OB patients compared to healthy lung transplant recipients (LTR).

Monocyte-derived DC from 14 OB and 35 non OB LTR were cultured with autologous T cells. T regulatory (Treg) cells, co-receptors, cytokine production, DC phenotype and indoleamine 2,3 dioxygenase (IDO) expression were assessed by flow cytometry. Experiments were repeated in presence of *P. aeruginosa* or anti-coreceptor antibodies (Abs).

DC from non OB LTR up-regulated Treg cells, CTLA-4 and IL-10. By contrast CD28 and ICOS were down-regulated concomitantly to IL-13 and IL-4. Compared to OB, non OB DC displayed an immature phenotype with lower CD80 and CD83 and higher IDO levels of expression. Stimulation by *P. aeruginosa* did not abolish the tolerogenic effects of DC on non OB T cells. Finally, decreased Treg cells and IL-10 production were detected when adding anti-CTLA-4 Abs in non OB LTR.

We demonstrate that DC from non OB LTR induce a tolerant T cell phenotype witch is dependent on CTLA-4 engagement.

Abstract word count: 196 words

Key words: Obliterative bronchiolitis, CTLA-4, Dendritic cells, Lung transplantation, T regulatory cells

Introduction

Obliterative bronchiolitis (OB) is the manifestation of chronic lung allograft rejection occurring in 33% of patients at five years after surgery (1). Despite a clear decrease in the prevalence of the complication compared to earlier series, bronchiolitis obliterans syndrome (BOS) remains by far the most significant long term complication and the first cause of late postoperative death, accounting for 25-30% of all deaths occurring beyond the third year after surgery (1). In addition BOS also causes significant loss of health-related quality of life (2). At the histological level, OB is characterized at the early stage by the infiltration of the bronchiolar wall with lymphocytes, monocytes and histiocytes, followed by a fibrosis process, leading to obliteration of small airways (3). Although the understanding of risk factors associated with the occurrence of OB have increased in the recent years (4), immune characteristics distinguishing tolerant recipients from patients with BOS remain largely unknown (5, 6).

Acute rejection (AR) episodes are recognized as important risk factors for OB. AR mainly occurs within the first postoperative months, when donor's leucocytes and antigen presenting cells (APC) are still infiltrating the bronchi, so that a classical alloreactive immune response is incriminated. Dendritic cells (DC) are the most potent APC in the lung, interacting with T cells in association with major histocompatibility complex (MHC) class II molecules. T cells specifically recognize alloreactive MHC via T cell receptor (TCR). In addition to the cognate TCR- MHC recognition, engagement of co-receptors expressed on APC and T cells is required for the occurrence of a productive immune response. These co-stimulatory signals are mediated via CD80/CD86 molecules (B7-1/B7-2) on DC and CD28 or cytotoxic T lymphocyte antigen (CTLA)-4 receptors on T cells, leading to a positive or a negative regulation of T cell activation, respectively. A recent study on gene expression profile in bronchoalveolar lavage (BAL) showed that both CD28 and CTLA-4 co-receptors were up-regulated during AR (7).

By contrast, OB is an inflammatory process which occurs at distance of the procedure, when donor's cells are only cells locally renewed and proliferating, but not bone marrow cells (8, 9). Previous studies showed that an increased number of DC were present in chronically rejecting lung allografts compared with stable lung transplant recipients (LTR) (10, 11). Other authors have demonstrated that T cells were activated during chronic rejection, with deficient T regulatory (Treg) cell activation (12). Thus, OB could result from a DC/T cell interaction in which both DC and T cells are recipient's cells. This interaction could consist into an antigenic presentation in which the presented peptide, likely a donor-derived antigen, would bear the alloreactive specificity. We hypothesized that upon the nature of DC/T cell interaction in terms of T cell activation or tolerance induction, either OB or graft tolerance could occur respectively, whatever the peptide presented.

To test this hypothesis, we investigated the interactions of autologous monocyte-derived DC with T cells in LTR. We compared T cell activation in DC/T cell co-cultures between healthy lung transplant recipients (non OB) and recipients with bronchiolitis obliterans syndrome (BOS). As infection is a factor potentially associated with OB, this interaction was also tested in presence of microbial compounds. Lastly, we analysed the role of co-receptors in this process.

Material and methods

Study design

Blood samples were collected in consecutive LTR from July 2005 to April 2007. This project was approved by the Sud-Méditerannée II Ethic Committee and written informed consent was obtained from all the patients. To avoid bias related to perioperative complications, LTR transplanted for less than 6 months were not included. Microbiological examination was systematically performed in broncho-alveolar lavages (BAL), and samples with relevant infection (positive microbiology with clinical or radiological findings requiring specific antimicrobial therapy) were excluded from the study. In addition, samples taken within the two months following the treatment of infection or acute rejection were not considered.

BOS was diagnosed on a progressive bronchial obstruction according to guidelines (13). Details on follow-up, respiratory function and additional treatments of medication dosages are given in the supplementary data. Because BOS 0p recipients received an increase in immunosuppressive regimen, especially in steroids and to avoid bias linked to treatment, these recipients were excluded from the study.

DC differenciation and coculture with autologous T cells

PBMC were isolated from peripheral blood by Ficoll-Hypaque gradient centrifugation. Monocytes were purified from PBMC by adherence and differentiated into DC as previously described (14). Briefly, monocytes were cultured in 24-well plates (10^7cells/ml) for 24 hours in complete medium supplemented with 800U/ml GM-CSF and 500U/ml IL-4. Cells were then incubated with a combination of pro-inflammatory mediators for another 24 hours (1000U/ml TNF-α, 10ng/ml IL-1β, 10ng/ml IL-6, and 1μM PGE2). Autologous lymphocytes, recovered from non adherent cells, were kept in fresh medium during 48 hours.

On day 2, monocyte-derived DC were then co-cultured with autologous lymphocytes at a ratio of 1/10 for 5 days in presence of IL-2 and IL-7. On day 7 cells were harvested for staining. In some experiments, we increased the ratio of DC on T cell from 1/10 to 4/10.

Bacterial stimulation

To assess the characteristics of DC/T cell interactions in presence of microbial compounds, lysed *Pseudomonas aeruginosa* was added to co-cultures. *Pseudomonas aeruginosa* was chosen as a source of recall antigens for the majority of patients displaying CF or bronchectasis. DC were incubated with 10 µl of *Pseudomonas aeruginosa* culture supernatant on day 2. After 12h of incubation, DC were co-cultured with autologous lymphocytes.

Co-receptor blockade

To determine the role of co-receptors in DC/T cell interactions, cultures were performed from day 2 with or without anti-CD28 (clone CD28.6, 10µg/ml), anti-ICOS (clone ISA-3, 20µg/ml) or anti-CTLA-4 monoclonal antibodies (Abs) (clone 14D3, 20µg/ml) (15). These Abs were purchased from ebioscience.

Flow Cytometry

Expression of T cell membrane antigens was assessed by adding specific Abs to cells at recommended concentrations (CD4-PECy5, CD25-FITC, *Beckman Coulter*-France; CD3-FITC *Dako*-Trappes; CD3- PECy5, CD69-PE, *Immunotools*-Germany; CD28-FITC, ICOS-PE, CTLA4-PE-Cy5, *BD Bioscience*-France*)*. Intracellular foxp3 expression by CD4+ T cells was measured after cell fixation and permeabilisation, by staining with intracellular anti-human Foxp3-PE antibodies (*ebiosocience* staining kit). T cell cytokine production was also measured after cell fixation and permeabilization, by staining T cells with intracellular Abs (IFNγ-FITC, IL-4-FITC, *BD Bioscience-France*; IL13-PE, IL-10-PE, *R&D system-France*)

after a 6 hours incubation in presence of monensin, as previously described (16). Expression of DC membrane antigens was studied (17) using specific Abs at recommended concentrations (CD83-PE, CD11c-PE, HLA-DR-PC5 *Beckman Coulter*-France; CD80-FITC *Immunotools*-Germany).

DC Indoleamine 2,3-dioxygenase (IDO) was also assessed by intracellular staining with a rabbit anti-human IDO Abs (*Chemicon*-France), counterstained by a FITC-labeled swine anti-rabbit secondary Abs (*Dako*-France).

Fluorescence was detected with a 15 mW argon ion laser on a three colors FACSCan® flow cytometer (*Becton Dickinson*, Franklin Lakes, NJ, USA). Standard acquisition and analysis software were obtained through Cellquest® Software (*Becton Dickinson*).

Statistical Analysis

Analysis was performed using the Statview® Software. Normal distributions of the variables were assessed by negativity of Kolmogorov-Smirnof's test. Comparisons between groups were determined using Student's *t*-tests. Paired Student's *t*-tests were used to compare T cell activation with and without DC, and DC maturation markers expression with or without *P. aeruginosa* or anti-co-receptors Abs. ANOVA was used to test differences between results obtained at various DC/T cell ratios. In case of significant ANOVA, Wilcoxon signed rank tests were used to compare each condition versus baseline. A p value < 0.05 was considered as statistically significant for all statistical tests. Results are given as mean ± standard error (SE).

Results

Patients

Characteristics of the patients are shown in Table I. Forty nine consecutive patients (24 males, 25 females) who underwent double lung (n=46) or single lung (n=3) transplantation in our institution were included in the study. The underlying diagnosis were cystic fibrosis (n=31), emphysema (n=8), bronchiectasis (n=2), pulmonary fibrosis (n=2), primary pulmonary hypertension (PPH, n=2), sarcoidosis (n=1), Langerhans cell histiocytosis (n=1), broncholithiasis (n=1) and Rendu-Osler disease (n=1). Thirty five were considered as healthy LTR (mean age: 39) and 14 displayed BOS (mean age: 34.9) at the end of the inclusion period. In our cohort, OB and non OB recipient groups showed no significant difference concerning donor/recipient CMV status or number of HLA mismatches.

DC from non OB LTR up-regulate T cell regulatory markers

The influence of DC on autologous T cell in healthy recipients was compared with OB patients in DC/T cell co-cultures. Treg population ($CD4+CD25^{high}+Foxp3+$) was evaluated after culture as shown in Fig 1A. In non OB recipients, DC induced an increase in Treg cells (Fig 1B) and IL-10 production (Fig 1B). In parallel, T cell CTLA-4 expression was increased by co-culture whereas ICOS and CD28 were down-regulated (Fig 1B and Table II). While T cell IFN-γ production was not affected by DC, Th2 cytokines significantly decreased (Fig 1B and Table II). In contrast in OB, neither co-receptor expression nor Th2 cytokines production varied in presence of DC (Fig 1B, Table II), while IFN-γ was up-regulated.

It thus appears that DC from non OB recipients induced a tolerant phenotype of autologous T cells while a pro-inflammatory phenotype is induced in OB recipients with up-regulation of IFN- γ production.

The T cell tolerant phenotype in healthy LTR is linked to the DC/T cell ratio

In order to assess whether DC are responsible for the phenotype of T cells, we increased the DC/T cell ratio from 1/10 to 4/10 in DC/T cell co-culture. In non OB recipients, increase in DC/T cell ratio induced a deactivation of T cells, reflected by a down-regulation of CD69 and CD28 expression, as well as IL-13 production (Fig 2). T cell activation reached a minimal level at a ratio of 4/10. T cell INF-γ production did not vary under DC stimulation. By contrast in OB recipients, the increase in the number of DC up-regulated cytokine production (IL-13, INF-γ) as well as CD69 expression (Fig 2). DC-induced modulation of T cell activation is thus dose-dependent.

DC from non OB recipients display an immature phenotype

The maturation stage of DC critically impacts the outcome of the immune response. We thus investigated DC markers related to DC maturation: CD11c, CD80, CD83 and HLA-DR. As compared to DC from OB recipients, DC from non OB subjects displayed a reduced expression of CD83 and CD80 (Fig 3A), while levels of CD11c and MHC-II were not different.

One mechanism by which APC may induce a tolerant T cell response is through the expression of IDO. According to a regulatory phenotype induced upon DC stimulation in non OB recipients, OB DC expressed lower levels of intracellular IDO than non OB patients (Fig 3B).

Thus compared to OB, non OB DC exhibit an immature phenotype, despite use of identical maturating cell culture conditions in both groups.

Stimulation by *P. aeruginosa* modify DC maturation without affecting their tolerogenic effect

To verify whether the DC-induced tolerant phenotype of T cell was maintained or not in presence of microbial compounds (pathogen-associated molecular patterns and antigens), DC/T cell co-cultures were stimulated with 10µl of *P. aeruginosa* culture supernatant.

Stimulation of non OB recipient's DC by *P. aeruginosa* supernatant up-regulated DC expression of CD80 and decreased DC intracellular IDO production, with a down-regulation of CD83 (Fig 4A). By contrast, in OB DC, *P. aeruginosa* induced an increase in CD83 expression but without significant variation of IDO or other markers (data not shown). Thus, stimulation by *P. aeruginosa* modify DC maturation state both in non OB and OB recipients.

In these conditions, a slight up-regulation of T cell CD69 expression and INF-γ production was induced in OB recipients. Regarding co-receptors and Th2 cytokine production, no significant variation was found (fig 4B and Table III) In non OB recipients, adding DC in presence of microbial compounds still up-regulated IL-10 production, Treg cells and CTLA-4 expression, whereas CD28 and ICOS expression decreased (Fig 4B and Table III). Regarding Th2 and Th1 cytokines, no significant variation was found (Table III). Thus, even in the presence of *P. aeruginosa*, DC still induce a T cell tolerant phenotype in non OB recipients only.

The DC-induced tolerant phenotype of T cell in non OB recipients is dependent on CTLA-4.

To test if co-receptor engagement by B7 family molecules was responsible for the DC-induced tolerant profile found in non OB cells, we added anti-CD28, anti-ICOS or anti-CTLA-4 blocking Abs in DC/T cell co-cultures.

In non OB recipients, anti-CTLA-4 Abs decreased IL-10 production and Treg proportion (Fig 5A), whereas anti-CD28 or anti-ICOS Abs had no effect (data not shown).

Furthermore, anti-CTLA-4 Abs dramatically decreased DC CD80 and IDO expression, and increased CD83, HLA-DR and CD11c expression by DC from non OB recipients (Fig 5B).

By contrast in OB recipients, CTLA-4 blockade induced an increase in ICOS and CD69 expression (Fig 5A), without significant variation in DC maturation markers (data not shown).

Thus, anti-CTLA-4 Abs reversed the DC-induced T cell phenotype in non OB but not in OB recipients. These results indicate that B7-CTLA-4 interactions in DC/T cell co-cultures specifically mediate the tolerant phenotype of T cell induced by DC in non OB recipients only.

Discussion

In this study, we investigated the influence of autologous DC on T cell activation both in healthy non OB and OB recipients. DC from non OB recipients induced an up-regulation of IL-10 production and Treg cells. These Treg cells are responsible for tolerance induction in various models of solid organ transplantation (18). In blood LTR, it has been shown that Treg cells were decreased in BOS recipients compared to healthy recipients (12). Our findings are concordant with these results, suggesting that DC-induced Treg cells, seen in healthy LTR only, could have a protective role in the transplanted patients. According to a DC-induced tolerant phenotype of T cells, T cell CTLA-4 expression increased upon DC stimulation in non OB recipients. Indeed, CTLA-4 is expressed on activated T cells and as CD28, binds to CD80 and CD86 (19), but it is a regulatory molecule inhibiting T cell responses (20).

We examined *in vitro* influence of DC derived from peripheral monocytes on T cells. Although we did not study circulating DC nor pulmonary DC, we assume our results to be relevant in vivo. Indeed airways DC originate in peripheral blood monocytes from the bone marrow (6, 21). Monocyte-derived DC that we used for *in vitro* studies have similar costimulatory molecules (CD80, CD83) and HLA-DR expression to those from lung DCs (22). These molecules can be differentially expressed by myeloid DC and plasmacytoid DC, the two airways DC subsets able to induce distinct types of immune responses (23). Analysing DC from broncho-alveolar lavages or bronchial biopsies would confirm this analogy, but these cells are not enough represented to be studied properly with regard to their costimulatory capacities. Under conditions normally inducing mature DC, an immature phenotype was obtained in non OB recipients whereas in OB subjects the expected mature

phenotype was achieved. Indeed DC from non OB recipients significantly down regulated surface molecules involved in antigen presentation (CD80, CD83) and concomitantly over-expressed intracellular IDO, implicated in inhibition of T cell proliferation (24). This immature DC phenotype is likely responsible for the tolerant phenotype of T cells induced in co-cultures.

P. aeruginosa was used to challenge DC, both by stimulating toll like receptors (TLR) and by inducing a complete antigen presentation system, since 80 % of the studied patients, as CF or bronchiectasis patients had been previously infected by this pathogen. TLR stimulation by gram negative bacteria is expected to induce DC to differentiate in pro-inflammatory cells able to organize the DC-induced T cell activation towards a full anti-infectious response. However in our system, *P. aeruginosa* stimulation had little effect in non OB patients whereas it effectively increased the pro-inflammatory T cell phenotype in OB. This result indicates a robust pro-tolerant profile of DC in non OB recipients. It is noteworthy that no difference was seen in patients with pre-transplant P. aeruginosa colonization and other LTR. P. aeruginosa stimulation modified the DC phenotype in non OB recipients notably in increasing CD80 expression and reducing IDO production. However the tolerogenic effect of DC on T cells was not modified. This could be related to the fact that supernatant of P. aeruginosa culture and not live bacteria was used. It is also possible that the 7 days culture was too short to mimic the in vivo effect of P. aeruginosa. Our results also indicated that other factors than DC level of maturation were involved in the tolerogenic process. Indeed engagement of CTLA-4 proved to be pivotal.

The use of recombinant soluble form of human CTLA-4 previously demonstrated that CTLA-4/B7 system may regulate IDO expression in CD4+ and CD8+ T cells (25). Herein, anti-CTLA-4 induced in non OB recipients a concomitant decrease of Treg population and T cell IL-10 production, demonstrating that the DC-induced tolerant T cell phenotype observed in this group depends on CTLA-4 engagement (26, 27). It is noteworthy that in OB recipients, CTLA4 blockade increased T cell CD69 expression, showing that the receptor is functional

but incapable of inducing the tolerant phenotype in these patients. In addition, anti-CTLA-4 Abs specifically induced a decrease in DC IDO expression, and an increased expression of DC membrane antigens related to maturation. These results demonstrate that the B7/CTLA-4 axis works not only from DC to T cell but also from T cell to DC, in an amplification loop. Nevertheless, anti-CTLA-4 Abs influence neither Th1 nor Th2 cytokine production. In addition to CTLA-4, others co-receptors may be implicated in regulating T cell responses. Indeed, Munn D et al. showed that blocking CTLA-4 concomitantly to CD28 co-receptor induced a complete abrogation of IDO-mediated inhibition on T cells (28). In our experiments, anti-CD28 had no visible effect alone, but residual effect of DC on T cells treated by anti-CTLA-4 Abs can still be due to CD28. In addition, a very recent study established a critical role in the control of initiation and reversion of T cell anergy of a new molecular pathway involving B7-H1 on APC and its receptor on T cells PD-1 (Programmed Death 1) (29). It will be interesting to study the B7-H1/PD-1 axis in parallel to B7/CTLA-4 pathway in our system.

Whether results could have been biased by the use of different immunosuppression regimen in both groups is questionable. However, the tolerant profile of DC/T cell interactions found in non OB recipients was maintained when comparing non OB and OB recipients having the same immunosuppressive regimen (TAC+MMF+AZI) (data not shown). Furthermore, treatments given in OB or after acute rejection are more immunosuppressive and therefore expected to increase the T cell tolerant phenotype, thus diminishing the differences observed between the 2 groups rather than amplifying them.

As cytokines are major regulators of the immune system, polymorphisms in cytokine and cytokine receptor genes might influence susceptibility to rejection. Lacha J and coll demonstrated in kidney recipients, that IL-6 gene polymorphism was associated with worse 5-year outcome (30). Toll-like receptor (TLR)-4 has also been implicated in allograft rejection notably after cardiac (31) and renal transplantation. Indeed, renal transplant recipients with TLR-4 polymorphism dysplayed a lower risk of acute allograft rejection (32). In the same

view, our CTLA-4 results could be the consequence of polymorphism in CTLA-4 gene. Indeed, it was recently shown that a specific CTLA-4 haplotype (+49A/+6230G), which encodes for normal membrane-bound CTLA-4 expression but reduced soluble CTLA-4 production, was a co-dominant risk allele for acute rejection after clinical liver transplantation (33). HLA polymorphism can also be involved: recently, soluble HLA-G was showed to be associated to immunological heart and liver tolerance (34-36).

Taken together, our results provide new evidence for specific cross-talks between DC and T cell in LTR according to the presence of OB. The DC-induced tolerant T cell phenotype observed in non OB patients provides new insights into the pathophysiology of OB. As DC are the major cell type presenting antigens in lung and bronchi, we speculate that characteristics of DC/T cell interactions induced in non OB patients are responsible for long term tolerance. The signals leading to these characteristics are now to be studied. Importantly, if this characteristic DC/T cell interaction precludes the occurrence of OB, these co-cultures could be instrumental in the early diagnosis of chronic rejection. Finally, the use of autologous DC has been tested in some cancer therapy to induce a strong anti-tumoral effect (37). Our results suggest that in a similar approach, immature autologous DC, Treg cells, or a strategy aiming to stimulate the B7/CTLA4 axis could be used to treat or to prevent OB in LTR.

References

1. Trulock EP, Edwards LB, Taylor DO, Boucek MM, Keck BM, Hertz MI. Registry of the International Society for Heart and Lung Transplantation: twenty-third official adult lung and heart-lung transplantation report--2006. J Heart Lung Transplant 2006;25(8):880-92.

2. van Den Berg J, Geertsma A, van Der BW, Koeter GH, de BW, Postma DS, et al. Bronchiolitis obliterans syndrome after lung transplantation and health-related quality of life. Am J Respir Crit Care Med 2000;161(6):1937-41.

3. Yousem SA. Significance of clinically silent untreated mild acute cellular rejection in lung allograft recipients. Hum Pathol 1996;27(3):269-73.

4. Reynaud-Gaubert M. [Pathophysiology of obliterative bronchiolitis in lung transplants]. Rev Mal Respir 2003;20(2 Pt 1):224-32.

5. Jaramillo A, Fernandez FG, Kuo EY, Trulock EP, Patterson GA, Mohanakumar T. Immune mechanisms in the pathogenesis of bronchiolitis obliterans syndrome after lung transplantation. Pediatr Transplant 2005;9(1):84-93.

6. McDyer JF. Human and murine obliterative bronchiolitis in transplant. Proc Am Thorac Soc 2007;4(1):37-43.

7. Gimino VJ, Lande JD, Berryman TR, King RA, Hertz MI. Gene expression profiling of bronchoalveolar lavage cells in acute lung rejection. Am J Respir Crit Care Med 2003;168(10):1237-42.

8. Kelly K, Hertz MI. Obliterative bronchiolitis. Clin Chest Med 1997;18(2):319-38.

9. Merad M, Collin M, Bromberg J. Dendritic cell homeostasis and trafficking in transplantation. Trends Immunol 2007;28(8):353-9.

10. Yousem SA, Ray L, Paradis IL, Dauber JA, Griffith BP. Potential role of dendritic cells in bronchiolitis obliterans in heart-lung transplantation. Ann Thorac Surg 1990;49(3):424-8.

11. Leonard CT, Soccal PM, Singer L, Berry GJ, Theodore J, Holt PG, et al. Dendritic cells and macrophages in lung allografts: A role in chronic rejection? Am J Respir Crit Care Med 2000;161(4 Pt 1):1349-54.

12. Meloni F, Vitulo P, Bianco AM, Paschetto E, Morosini M, Cascina A, et al. Regulatory CD4+CD25+ T cells in the peripheral blood of lung transplant recipients: correlation with transplant outcome. Transplantation 2004;77(5):762-6.

13. Estenne M, Maurer JR, Boehler A, Egan JJ, Frost A, Hertz M, et al. Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. J Heart Lung Transplant 2002;21(3):297-310.

14. Dauer M, Obermaier B, Herten J, Haerle C, Pohl K, Rothenfusser S, et al. Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. J Immunol 2003;170(8):4069-76.

15. Vandenborre K, Delabie J, Boogaerts MA, De Vos R, Lorre K, De Wolf-Peeters C, et al. Human CTLA-4 is expressed in situ on T lymphocytes in germinal centers, in cutaneous graft-versus-host disease, and in Hodgkin's disease. Am J Pathol 1998;152(4):963-73.

16. Mamessier E, Nieves A, Vervloet D, Magnan A. Diesel exhaust particles enhance T-cell activation in severe asthmatics. Allergy 2006;61(5):581-8.

17. Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nat Med 2004;10(5):475-80.

18. Akl A, Luo S, Wood KJ. Induction of transplantation tolerance-the potential of regulatory T cells. Transpl Immunol 2005;14(3-4):225-30.

19. Alegre ML, Frauwirth KA, Thompson CB. T-cell regulation by CD28 and CTLA-4. Nat Rev Immunol 2001;1(3):220-8.

20. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. Immunity 1994;1(5):405-13.

21. McWilliam AS, Nelson DJ, Holt PG. The biology of airway dendritic cells. Immunol Cell Biol 1995;73(5):405-13.

22. Cochand L, Isler P, Songeon F, Nicod LP. Human lung dendritic cells have an immature phenotype with efficient mannose receptors. Am J Respir Cell Mol Biol 1999;21(5):547-54.

23. Masten BJ, Olson GK, Tarleton CA, Rund C, Schuyler M, Mehran R, et al. Characterization of myeloid and plasmacytoid dendritic cells in human lung. J Immunol 2006;177(11):7784-93.

24. Hwu P, Du MX, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. J Immunol 2000;164(7):3596-9.

25. Boasso A, Herbeuval JP, Hardy AW, Winkler C, Shearer GM. Regulation of indoleamine 2,3-dioxygenase and tryptophanyl-tRNA-synthetase by CTLA-4-Fc in human CD4+ T cells. Blood 2005;105(4):1574-81.

26. Eagar TN, Karandikar NJ, Bluestone JA, Miller SD. The role of CTLA-4 in induction and maintenance of peripheral T cell tolerance. Eur J Immunol 2002;32(4):972-81.

27. Vandenborre K, Van Gool SW, Kasran A, Ceuppens JL, Boogaerts MA, Vandenberghe P. Interaction of CTLA-4 (CD152) with CD80 or CD86 inhibits human T-cell activation. Immunology 1999;98(3):413-21.

28. Munn DH, Sharma MD, Mellor AL. Ligation of B7-1/B7-2 by human CD4+ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. J Immunol 2004;172(7):4100-10.

29. Tsushima F, Yao S, Shin T, Flies A, Flies S, Xu H, et al. Interaction between B7-H1 and PD-1 determines initiation and reversal of T-cell anergy. Blood 2007.

30. Lacha J, Hribova P, Kotsch K, Brabcova I, Bartosova K, Volk HD, et al. Effect of cytokines and chemokines (TGF-beta, TNF-alpha, IL-6, IL-10, MCP-1, RANTES) gene polymorphisms in kidney recipients on posttransplantation outcome: influence of donor-recipient match. Transplant Proc 2005;37(2):764-6.

31. Methe H, Zimmer E, Grimm C, Nabauer M, Koglin J. Evidence for a role of toll-like receptor 4 in development of chronic allograft rejection after cardiac transplantation. Transplantation 2004;78(9):1324-31.

32. Ducloux D, Deschamps M, Yannaraki M, Ferrand C, Bamoulid J, Saas P, et al. Relevance of Toll-like receptor-4 polymorphisms in renal transplantation. Kidney Int 2005;67(6):2454-61.

33. Perkins JD. The influence of CTLA-4 gene polymorphisms on liver transplant outcomes. Liver Transpl 2006;12(10):1552-3.

34. Luque J, Torres MI, Aumente MD, Lozano JM, Garcia-Jurado G, Gonzalez R, et al. sHLA-G levels in the monitoring of immunosuppressive therapy and rejection following heart transplantation. Transpl Immunol 2006;17(1):70-3.

35. Basturk B, Karakayali F, Emiroglu R, Sozer O, Haberal A, Bal D, et al. Human leukocyte antigen-G, a new parameter in the follow-up of liver transplantation. Transplant Proc 2006;38(2):571-4.

36. Le Rond S, Azema C, Krawice-Radanne I, Durrbach A, Guettier C, Carosella ED, et al. Evidence to support the role of HLA-G5 in allograft acceptance through induction of immunosuppressive/ regulatory T cells. J Immunol 2006;176(5):3266-76.

37. de Vries IJ, Lesterhuis WJ, Scharenborg NM, Engelen LP, Ruiter DJ, Gerritsen MJ, et al. Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. Clin Cancer Res 2003;9(14):5091-100.

Legends of the figures :

Figure 1: T cell phenotype and cytokine production in DC/T cell co-culture

T regulatory cells (CD4+CD25+Foxp3+) where assessed by flow cytometry and determined among CD4+ T cells and distinguished from the whole CD4+CD25+ T cell population on their CD25 high fluorescence intensity and Foxp3 expression. R2 corresponds to $CD4+CD25^{medium}+$ cells and R3 to $CD4+CD25^{Hi}+$ cells; R2+R3 representing the all CD4+CD25+ cells (A). IL-13, IL-4 or IL-10 producing T cells (TC) as well as Treg cells ($CD4+CD25+^{Hi}Foxp3+$), ICOS and CTLA-4 expressing TC were assessed in non OB (○, n=30) and OB recipients (●, n=14) in presence or absence of monocyte-derived denditic cells (DC). Results were expressed as percentage of total TC and compared in each recipients group in the presence or absence of DC. OB = obliterative bronchiolitis. - : mean of one group. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$ (B).

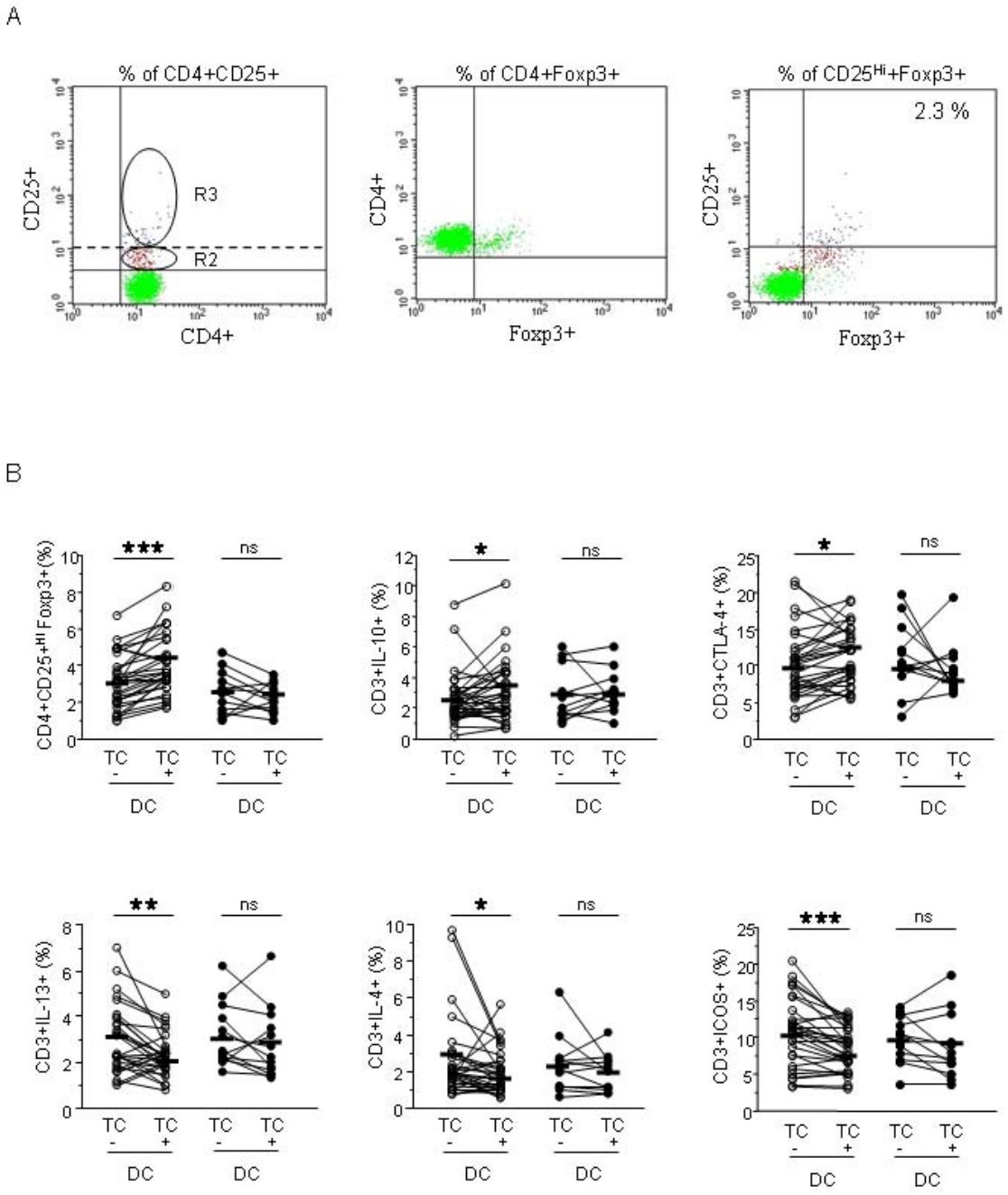

Figure 1

Figure 2: T cell phenotype when DC/T cell ratio increase

CD69, CD28 expression and IL-13 or IFN-γ producing T cells were assessed by flow cytometry in DC/T cell co-culture while increasing DC/T cell ratio from 1/10 to 4/10. Results were given for non OB (○, n=16) and OB recipients (●, n=10) and compared in each group

with baseline. OB = obliterative bronchiolitis ; TC = T cell ; DC = dendritic cell. * = p<0.05; ** = p<0.01; *** = p<0.001

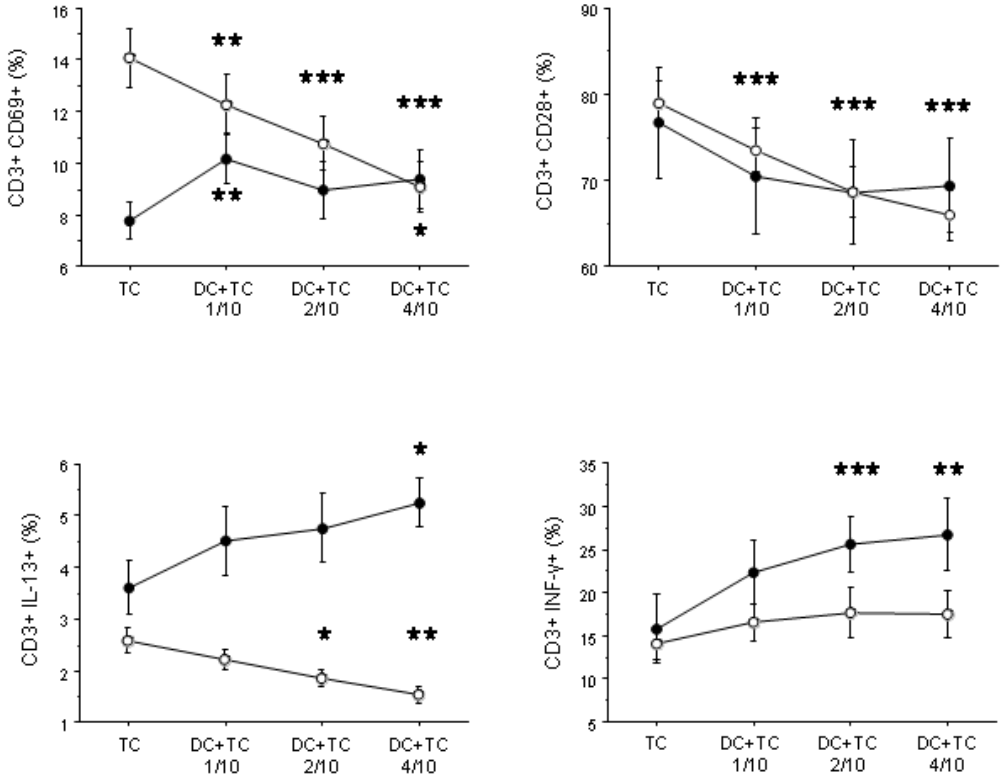

Figure 2

Figure 3: DC maturation markers and IDO expression

CD80, CD83, HLA-DR, CD11c (A) and IDO expression by DC. - : mean of one group (B) were assessed in DC/T cell co-culture by flow cytometry. Results were given as percentage of total DC. Results were compared between non OB (○, n=28) and OB recipients (●, n=14). DC = dendritic cell ; OB = obliterative bronchiolitis. * = p<0.05; ** = p<0.01; *** = p<0.001

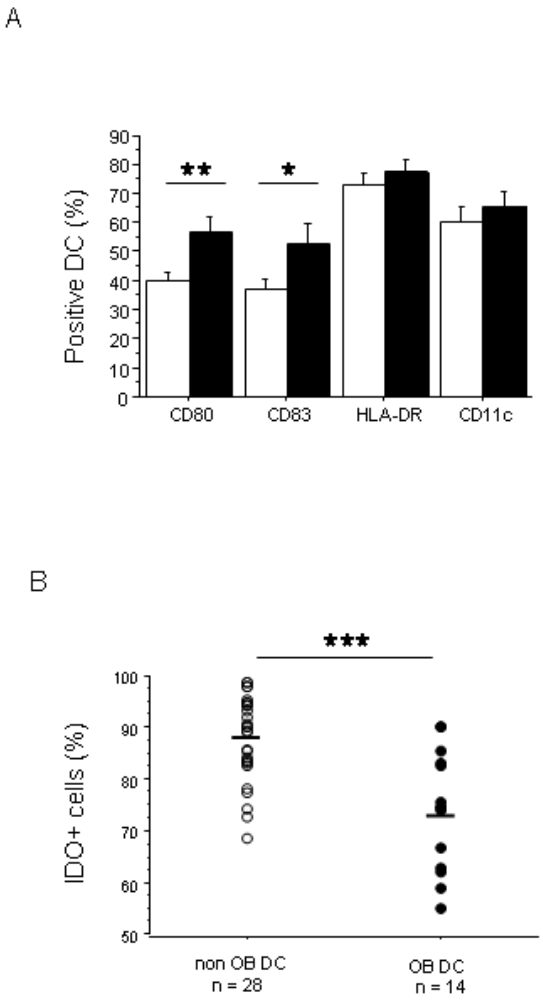

Figure 3

Figure 4: T cell and DC phenotype in the presence of *P. aerugisosa*

DC/T cell co-cultures where performed in the presence of *P. aeruginosa* culture supernatant. CD80, CD83 and IDO expression by non OB DC (n=16) were assessed by flow cytometry in DC/T cell co-cultures. Results were compared in the presence or absence of *P. aeruginosa* (*P.a*) (A). Treg cells, ICOS and CTLA-4 expressing T cells or IL-10 producing T cells were also assessed in T cells from non OB (○, n=20) and OB recipients (●, n=10). Results were compared in the presence or absence of DC (B). OB = obliterative bronchiolitis, TC = T cell; DC = dendritic cell . - : mean of one group. - : mean of one group. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

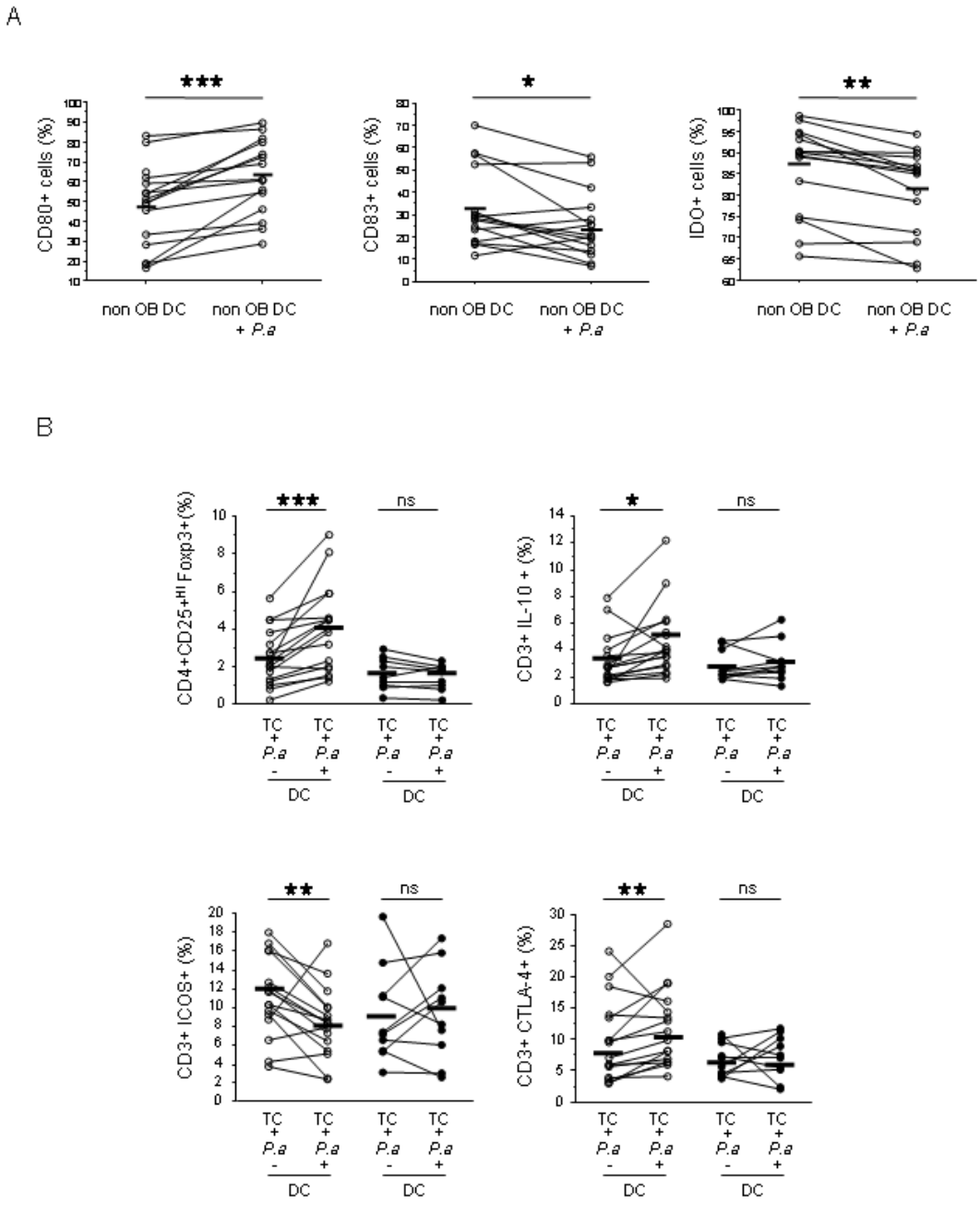

Figure 4

Figure 5: T cell phenotype and DC markers expression in the presence of anti-CTLA-4 Abs.

DC/T cell co-cultures where performed in the presence anti-CTLA-4 Abs. Treg cells, ICOS and CD69 expression, and IL-10 producing T cells were assessed by flow cytometry in non

OB (○, n=18) and OB recipients (●, n=10). Results were expressed as percentage of total T cells and compared in the presence or absence of DC and anti-CTLA-4 Abs (A). CD80 and IDO expression by non OB DC (○, n=16) were assessed in DC/T cell co-culture. Results were given as percentage of total DC and compared with or without anti-CTLA-4 Abs. **-** : mean of one group (B). OB = obliterative bronchiolitis, TC = T cell; DC = dendritic cell .***** = $p<0.05$; ****** = $p<0.01$; ******* = $p<0.001$

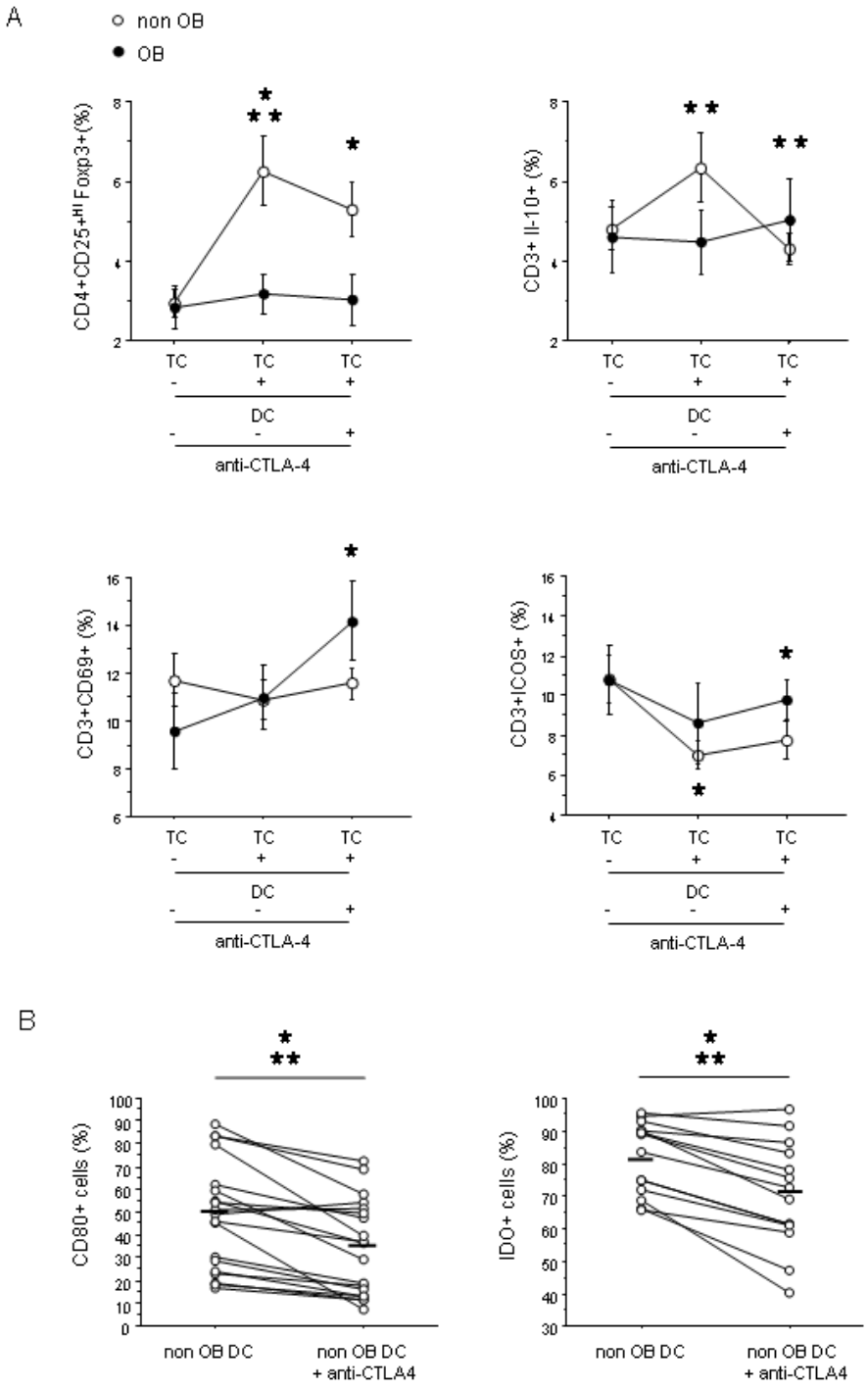
A
non OB
OB
CD4+CD25+HI Foxp3+(%)
CD3+ Il-10+ (%)
CD3+CD69+ (%)
CD3+ICOS+ (%)
TC
DC
anti-CTLA-4
B
CD80+ cells (%)
IDO+ cells (%)
non OB DC
non OB DC
+ anti-CTLA4

Figure 5

Table I. Patients characteristics (n=49). OB = obliterative bronchiolitis ; TP = transplantation ; FEV1= forced expiratory volume in 1 second ; TAC = tacrolimus ; AZA = azathioprine ; MMF = mycophenolate mofetyl ; CsA = cyclosporine ; AZI = azithromycin.

* Values are expressed as mean ± SE

	Non OB (n=35)	OB (n=14)
Age, year *	39 ± 2.01	34.86 ± 3.52
Gender		
Male	20	4
Female	15	10
Transplantation		
Single	3	0
Bilateral	32	14
Indication		
Cystic Fibrosis	23	8
Emphysema α1 anti-trypsin	7	1
Bronchiectasis	1	1
Fibrosis	1	1
Primary pulmonary hypertension	0	2
Others	2	1
% pred FEV1 *	84.29 ± 3.22	38.07 ± 3.53
Months from TP *	190.57 ± 143.42	88.28 ± 15.54
HLA-A, HLA-B, HLA-DR mismatch		
0-2	0	0
3-4	2	1
5-6	33	13
CMV mismatch (donor +/ recipient -)	3	2
Treatment (when sampled)		
TAC, AZA	7	0
CsA, AZA	10	0
TAC, MMF	5	0
CsA, MMF	1	0
TAC, AZA, AZI	2	4
TAC, MMF, AZI	10	10
P. aeruginosa colonisation status		
Pre-transplant colonisation	24	8
Post-transplant re-colonisation	19	8

Table II. Effect of DC on T cell surface antigens and IFN-γ expression in non OB and OB recipients. T cells (TC) from non obliterative bronchiolitis (non OB) and OB recipients were cultured or not in the presence of autologous monocyte-derived dendritic cells (+DC, -DC). T cell phenotype was assessed by flow cytometry. Results are expressed as mean ± SE of total T cell. Statistics are comparing within each group (non OB or OB), IFN-γ and receptors expression in the presence or absence of DC. ***** = $p<0.05$; ****** = $p<0.01$; **** *** = $p<0.001$

	non OB TC – DC	non OB TC + DC	OB TC – DC	OB TC + DC
	n=30	n=30	n=14	n=14
CD3+IFN-γ+	11.65 ± 1.41	11.89 ± 1.51	14.52 ± 2.54	18.11 ± 2.51*****
CD3+CD69+	16.78 ± 1.36	14.05 ± 1.13******	14.59 ± 1.93	15.27 ± 2.39
CD3+CD28+	81.14 ± 2.17	75.94 ± 2.35*******	80.35 ± 4.72	75.07 ± 4.48*****

Table III. Effect of DC on T cell surface antigens and cytokines expression in the presence of *P. aeruginosa,* in non OB and OB recipients. Dendritic cells from non obliterative bronchiolitis (non OB) and OB recipients were stimulated with 10 µl of *Pseudomonas aeruginosa* culture supernatant and then co-cultured with autologous T cells (TC). T cell phenotype with or without DC (+DC, -DC) was assessed by flow cytometry. Results are expressed as mean ± SE of total T cell. Statistics are comparing within each group (non OB or OB), cytokines and receptors expression in the presence or absence of DC. ***** = $p<0.05$; ****** = $p<0.01$; ******* = $p<0.001$

	non OB TC – DC	non OB TC + DC	OB TC – DC	OB TC+ DC
	n=20	n=20	n=10	n=10
CD3+IL-13+	4.12 ± 0.71	4.46 ± 0.71	3.80 ± 0.71	4.14 ± 0.66
CD3+IL-4+	3.01 ± 0.41	2.69 ± 0.28	2.82 ±0.64	2.74 ± 0.58
CD3+IFN-γ+	10.03 ± 1.42	12.47 ± 1.64	16.21 ± 3.96	20.87 ± 4.96*****
CD3+CD69+	11.43 ± 1.16	10.56 ± 0.96	13.73 ± 2.23	16.21 ± 2.65*****
CD3+CD28+	82.85 ±2.17	76.07 ± 3.61******	82.16 ± 3.42	82.34 ± 3.65