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α E β 7 expression on CD8+ T-cells in COPD BAL fluid and on TGF- β stimulated T-cells *in vitro*

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Running title: α E β 7 expressing CD8+ T-cells in COPD

Abstract

The airway inflammation in patients with COPD shows increased numbers of CD8+ T-cells. Hitherto, few studies have shown any functional data indicating a role for these cells in the pathogenesis of COPD. This paper focuses on a subset of CD8+ T-cells present in human lung, the intra epithelial lymphocytes expressing the integrin α E β 7, and their presence in bronchoalveolar lavage fluid from COPD patients. In this study we demonstrate that 64-89% of the CD8+ T-cells in bronchoalveolar lavage fluid from COPD patients are positive for CD103, the alpha-subunit of α E β 7. We also present an *in vitro* system in which it is possible to differentiate peripheral T-cells into a phenotype resembling the one found in bronchoalveolar lavage fluid, i.e. CD8+ CD103+. In this *in vitro* system we demonstrate that, in addition to TGF- β ₁, cell-to-cell interaction between the T-cell and an antigen-presenting cell, here represented by the monocyte, is crucial for a rapid, high and sustained expression of CD103. The signal provided by the monocytes, is shown to be mediated through LFA-1 on the T-cell. Furthermore, differentiation of CD8+ T-cells by TGF- β ₁ and monocytes results in down regulation of INF- γ , TNF- α and GM-CSF production. IL-8 production is however retained in the α E β 7 expressing cells. We see this work as an initiation on the quest for a functional characterization of one the different types of CD8+ T cells present in COPD. In the longer perspective we hope this can lead to an increased understanding of how these cells can contribute to the disease pathology.

Abbreviations:

BAL – bronchoalveolar lavage

BrdU - bromodeoxyuridine

CD - cluster of differentiation

GM-CSF – granulocyte/macrophage colony-stimulating factor

IEL - intra epithelial lymphocytes

IL – interleukin

PBMC – peripheral blood mononuclear cell

TGF- β - transforming growth factor beta

TNF- α - tumour necrosis factor alpha

MESH terms:

Pulmonary Disease, Chronic Obstructive

CD8-Positive T-Lymphocytes

Bronchoalveolar Lavage Fluid

Transforming Growth Factor beta

Flow Cytometry

Introduction

Chronic obstructive pulmonary disease, COPD, is characterized by chronic inflammatory changes in the airways leading to sputum production and structural changes of the bronchioles and the lung parenchyma with chronic bronchitis and emphysema as a consequence. Neutrophils, macrophages and CD8+ T-cells are some of the cells believed to be of importance in this inflammation ^[1]. The numbers of macrophages and CD8+ T-cells in the bronchial mucosa and peripheral lung tissue correlate with a decline in lung function, indicating a role for these cells in the pathogenesis of COPD ^[2-6]. Studies of tissue samples from smokers and COPD patients have shown increased numbers of CD8+ T-cells in comparison to healthy control subjects, yet few studies have described the function of these cells. It is still not known how they migrate into the lung, if they are proliferative, whether they are cytotoxic effector cells, to what extent they are producing proinflammatory cytokines or if they represent quiescent anergic cells.

One characteristic feature of CD8+ T-cells is their potential to eradicate infected cells. COPD patients suffer from frequent exacerbations triggered by virus or bacteria and leading to an accelerated decline in lung function ^[7]. The first line of defence against these infectious agents is the intra epithelial lymphocytes, IELs. Many of these IELs in the bronchial mucosa as well as in the intestinal mucosa express the α E(CD103) β 7 integrin that recognizes E-cadherin on the epithelial cells ^[8]. In the intestinal mucosa over 90% of the IELs express α E β 7^[9], while in the bronchial mucosa about 35 % of the IELs are α E β 7+ ^[10]. The binding between α E β 7 and E-cadherin is important for the retention of IELs in the epithelium. α E β 7 has also been proposed to have a co-stimulatory effect on T-cells activated through the CD3 pathway, resulting in increased proliferation ^[11]. Intestinal T-cells expressing α E β 7 are believed to be involved in disorders like Crohn's disease and Sjögrens syndrome ^[12]. Less is known about α E β 7 bearing cells in respiratory diseases and whether they take part in the progression of the disease. However, studies on BAL fluids from patients with different respiratory diseases, such as idiopathic pulmonary fibrosis, asthma and sarcoidosis have shown that T-cells in BAL to a large, but varying, extent express CD103 ^[13,14].

While CD103 expressing T-cells are common in mucosal tissue only a few percent of peripheral blood T-cells express CD103 ^[14]. Peripheral T-cells have previously been shown to up-regulate their CD103 expression when cultured *in vitro* in the presence of TGF- β . Stimulation of peripheral blood mononuclear cells (PBMCs) with TGF- β ₁ *in vitro*, results in an increased expression of CD103 on the T-cells ^[13]. This is in line with studies showing that

the production of TGF- β in the microenvironment within the epithelium *in vivo* is necessary to maintain the α E β 7 expression on IELs ^[15]. Interestingly, the airway epithelium of both smokers and COPD patients has been shown to express elevated levels of TGF- β ₁ ^[16,17]. Therefore, we hypothesize that the lung of COPD patients, withholding high levels of TGF- β and large numbers of macrophages, provides a good environment for differentiation of recruited CD8+ T-cells to become α E β 7 expressing cells. This in turn increases the possibility for the T-cells to interact with the epithelium through binding to E-cadherin. In this study an *in vitro* system was set up to differentiate peripheral CD8+ CD103- T-cells into CD103 expressing cells. The aim was to evaluate the interaction between the T-cells and monocytes leading to an enhanced expression of CD103. Using this system, activation, differentiation, proliferation and cytokine production of the T-cells were studied. Furthermore, the presence of α E β 7 expressing CD8+ T-cells in BAL from COPD patients and healthy subjects was evaluated using flowcytometry.

Materials and Methods

Recruitment of COPD Patients and Healthy Subjects

COPD patients and healthy subjects were recruited from the Department of Respiratory Medicine and Allergology at the University hospital in Lund. The COPD patients were 5 smokers or ex-smokers fulfilling the criteria for COPD according to GOLD, all in a stable phase without any sign of exacerbation ^[18]. An equal number of healthy subjects were also recruited. All patients and healthy subjects gave written informed consent to participate in the study, which was approved by the Lund University Ethics committee in Lund.

Handling of BAL and Blood Samples

The bronchoscopy was performed using a flexible bronchoscope, after local anaesthesia with lidocain. A bronchoalveolar lavage was performed in the lower lobe of the right lung. Saline was instilled with a wedged bronchoscope in portions of approximately 50 ml up to a total maximum volume of 150 ml and gentle suction was performed after each instillation. The recovered fluid was aspirated into a sterile tube and was kept on ice until further analysis. Blood samples from COPD patients were taken in EDTA tubes before performing the bronchoscopy and were kept in room temperature until further analysis.

Flowcytometric Analysis of BAL and Blood Cells

The BAL fluid was filtered through a nylon mesh and the cells were counted. The BAL fluid was then centrifuged (400xg, 10 min), the supernatant was decanted and the cell pellet was resolved in PBS- 1 % BSA. 300.000 cells were used in each sample. The cells were stained with antibodies against CD25, CD45R0, CD103 and CD8 (DAKO, Glostrup, Denmark) and then fixed in formaldehyde based fixation solution (DAKO Uti-Lyse, DAKO, Glostrup, Denmark). Blood cells were stained with the same antibodies as were used in the BAL staining. The cells were fixed and red blood cells were lysed using DAKO Uti-Lyse (DAKO, Glostrup, Denmark). Both BAL and blood cells were run on a Calibur flowcytometer from Becton Dickinson and the analysis was performed using CellQuestPro Software (BD Biosciences, San Jose, USA).

T-cell and Monocyte Extraction

Buffy Coats from healthy donors at Lund University hospital, Lund, Sweden, were used for purification of cells used in the *in vitro* system. PBMCs were extracted using Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) according to manufacturers recommendations. CD8⁺ T-cells and monocytes were isolated from PBMCs using the MACS magnetic bead system for positive selection of CD8 and CD14 respectively. The cells were collected using an Automacs all according to the manufactures instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the cells was tested by flowcytometry using antibodies against CD8, CD14 and CD16 (DAKO, Glostrup, Denmark).

Cultures of T-cells and Monocytes

Cells were cultured in 24 well plates with 1×10^6 cells per well, either 1×10^6 T-cells or with 0.5×10^6 T-cells together with 0.5×10^6 monocytes. Cells were cultured in RPMI 1640 with glutamax-1 and 25 MM HEPES (Invitrogen/Gibco, Paisley, UK) supplemented with 10% FBS and 1% penicillin-streptomycin (Invitrogen/Gibco, Paisley, UK) and stimulated with anti-CD28, IL-2 (BD Pharmingen, San Diego, USA), TGF- β_1 (R&D Systems, Minneapolis, USA) and plate bound anti-CD3 (BD Pharmingen, San Diego, USA) as outlined in Table 1. On day 3 and 6 the cells were restimulated and the monocytes were lost due to firm adherence to the plastic wells. Cells were harvested on day 0-3 and day 6-8, and CD103 expression, degree of activation (CD25) and proliferation (BrdU) was determined by flowcytometry.

Table 1 Stimulation of cell cultures

Stimuli	The addition of stimuli at restimulation		
	Day 0	Day 3	Day 6
TGF- β ₁ 0-10 ng/ml	+/-	+/-	+/-
α -CD28 5 μ g/ml	+	-	+
α -CD3 wells coated with 1 μ g/ml	+	-	+
IL-2 40ng/ml	-	+	+

Transwell Cultures

Transwell cell culture plates, with a pore size of 0.4 μ m (Costar Inc., Costar, USA), were used in cultures where T-cells and monocytes were co-cultured without cell-to-cell contact. The culture conditions were otherwise the same as outlined above.

Evaluation of LFA-1 – ICAM-1 Interactions

To block LFA-1 signalling, a blocking antibody against CD11a (lymphocyte functional antigen-1, LFA-1) (Immunotech, Marseille, France) and an IgG1 isotype control (Serotec, Oxford, UK) were used. T-cells and monocytes were preincubated with 10 μ g/ml of the blocking antibody or the isotype control prior to setting up the co-cultures using the same conditions as outlined above. In other experiments, as indicated in figure legends and results, recombinant ICAM-1 (R&D Systems, Minneapolis, USA) was coated to 48 well plates (50 μ g/ml). All other culture conditions in these experiments were kept as in previous cell cultures outlined above.

Flowcytometric Analysis of in vitro Cultured Cells

Cells were harvested and washed in PBS-1%BSA. The cells were subsequently labelled with antibodies against CD8, CD103 and CD25 (DAKO, Glostrup, Denmark), and analysed on a FACS Calibur flowcytometer from Becton Dickinson.

BrdU Proliferation Assay

Proliferation of the cells was measured as degree of incorporated bromodeoxyuridine (BrdU). BrdU flow kit was purchased from BD Pharmingen, (San Diego, USA), and used according to the manufactures instructions. In brief, cells were incubated with BrdU for 2 hours. They were then washed and stained for surface markers (CD103 PE and CD8 RPE-Cy5) before they were fixed, permeabilised and DNase treated. Thereafter the cells were stained with a FITC labelled antibody against BrdU and analysed on the flowcytometer.

Hoechst DNA Quantification Assay

Hoechst 33342 (Sigma B-2261) was used in a DNA quantification assay where Hoechst solution was added to the cells, incorporated into the DNA and fluorescence intensity was measured on a fluorometer. Briefly, the cells were spun down in their wells, the supernatant was decanted and the cells were resuspended in Hoechst solution (Hoechst dissolved in PBS, 4 μ g/ml). The plate was incubated in the dark for 30 min in room temperature. The fluorescence intensity in each well was then measured on a fluorometer and compared to the DNA-standard measured at the same time.

Luminex Cytokine Assay

For analysis of multiple cytokines the Luminex system was used. Beads and antibodies against IL-4, IL-8, IL-10, TNF- α , INF- γ , GM-CSF were used together with a Fluorescence MAP Base kit from R&D Systems, (Minneapolis, USA). The assay was performed according to the manufactures instructions and read on a Luminex 100 from Luminex Corporation, (Texas, USA).

Statistical Analysis

Data on BAL recovery and CD103 expression on BAL and blood cells are presented with median and range. Data in the Figures are presented as median values and quartiles. Statistical analysis for kinetic experiments was done using Kruskal-Wallis test and if significant effect was shown, ad hoc analysis using the Mann-Whitney U test were made. A correction of the p-values in the Kruskal-Wallis test was done according to Bonferroni. For statistical analysis of non-kinetic experiments the Mann-Whitney U test were made. A p-value of < 0.05 was considered statistically significant.

Results

Patient Characteristics

The COPD patients were between 48 and 61 years old, with a smoking history of 25-60 pack years and FEV₁ values ranging between 50 and 80 percent of predicted. Patients using inhaled corticosteroids were excluded from the study. The healthy subjects were 5 non-smokers in the ages between 48 and 56 with FEV₁ values between 108 and 139 percent of predicted. Characteristics for each individual are given in Table 2.

Recovery of BAL Fluid

The median instilled volume of saline was 110 ml (Range: 110-140 ml) in COPD patients and median recovery of BAL fluid was 48 ml (Range: 27-55 ml) corresponding to 45% (Range: 19% to 50%) of the instilled amount of fluid while for healthy subjects the median instilled volume was 110 (Range: 110-130 ml) and median recovery was 60 ml (Range: 50-80 ml) corresponding to 55% (Range: 46-62%). The total number of cells in BAL from COPD patients was on average 18×10^6 (Range: $1-30 \times 10^6$) and in healthy subjects 5×10^6 (Range: $3-15 \times 10^6$).

CD103+CD8+ Cells in Blood and BAL

In blood from COPD patients 2% (Range: 2-2%) of the CD8+ T-cells expressed CD103. The expression on CD8+ T-cells in Buffy coats from healthy donors was 3% (Range: 3-5%). In BAL from COPD patients and healthy subjects 70% (Range: 64-89%) and 59% (Range: 37-85%) respectively of the CD8+ T-cells expressed CD103, hence, there was no significant difference between the two groups (Table 2). Figure 1 illustrates the different appearance of the flowcytometric analysis of blood and BAL CD8+ T-cells. Very few of the BAL CD8+ T-cells were positive for CD25, 2% (Range: 0-2%) in COPD and 2% (Range: 1-2%) in healthy subjects whereas CD45R0 expression was high in all subjects, 88% (Range: 80-98%) in COPD and 93% (Range: 91-98%) in healthy subjects. There were no significant differences between the groups for these markers. CD8+ T-cells constituted on average 1% of the BAL cells from the COPD patients. This differed significantly ($p < 0.05$) from the healthy subjects where 4% of the cells were CD8+.

Table 2 Characteristics of COPD patients and Healthy subjects

Individual	Age	Sex	FEV ₁ % of predicted	% CD103+ of CD8+ cells in BAL
COPD patients				
1	59	f	58	73
2	60	f	50	64
3	61	f	80	89
4	48	m	77	70
5	52	m	72	65
Median	59		72	70
Healthy Subjects				
6	56	f	110	57
7	48	f	108	37
8	58	f	139	59
9	52	f	137	85
10	52	m	113	69
Median	52		113	59

CD103 Expression on in vitro Cultured CD8+ T-cells is Under the Influence of TGF- β ₁ and Monocyte Interactions

Monocytes and CD8+ T-cells, in buffy coats from healthy donors, were purified using the MACS system to a purity of at least 91% and 95 % respectively. CD8+ T-cells were cultured in anti-CD3 coated wells in the presence or absence of monocytes, with or without TGF- β ₁ (10 ng/ml) and with anti-CD28 and IL-2 supplemented to the medium. In the presence of monocytes TGF- β ₁ induced high levels, 95% (Range: 90-98%), of CD103 expression on the T-cells. In the absence of TGF- β ₁ 15% (Range: 7-20%) of the T-cells expressed CD103 at the end of the culture. Supplementary experiments with increasing doses of TGF- β ₁ (0.01-10 ng/ml) showed a dose-response effect. Even in the absence of monocytes the CD103 expression was up-regulated by TGF- β ₁ however not to the same extent 67%, (Range: 43-85%) and with slower kinetics. In cultures of CD8+ T-cells without monocytes and TGF- β ₁ 8% (Range: 5-16%) of the cells expressed CD103 at day 8 of culture (Figure 2A). In contrast to the CD103 expression, the general activation marker CD25 was not affected by the addition of TGF- β ₁ to T-cells grown in the presence of monocytes. However in the absence of

monocytes, TGF- β ₁ stimulated T-cells showed a significant decrease in expression of CD25 on day 6-8. (Figure 2B).

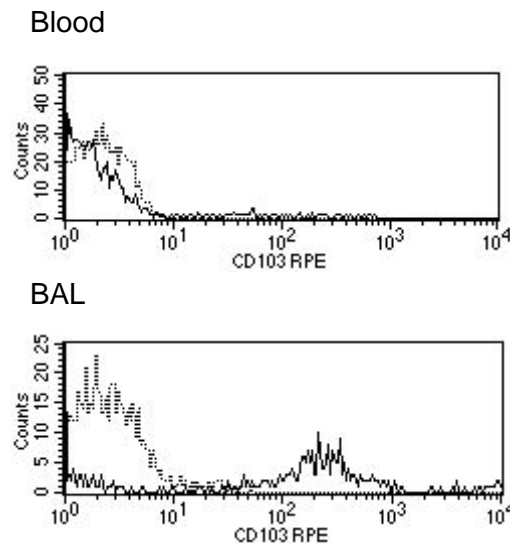


Fig. 1 Flowcytometric analysis of CD103 expression on CD8⁺ T-cells in blood and BAL. The dotted line represents background staining using an isotype matched control antibody. Solid line represents intensity of CD103 expression. While only a few percent of the peripheral blood CD8⁺ T-cells express CD103 the majority of the BAL CD8⁺ T-cells does.

Monocyte Derived Co-stimulation Requires Cell-to-Cell Interaction

To investigate whether increased expression of CD103 in the presence of monocytes was dependent on cell-to-cell interaction T-cells and monocytes were co-cultured in Transwell systems. This enabled the two cell types to share soluble factors without cell-to-cell contact. The CD103 expression on T-cells cultured in the Transwell system did not significantly differ from the CD103 expression on the T-cells grown in the complete absence of monocytes (Figure 2C).

The Role of LFA-1—ICAM-1 on CD103 Expression

To investigate the role of LFA-1 – ICAM-1 interactions in the T-cell:monocyte interaction, TGF- β ₁ stimulated CD8⁺ T-cells and monocytes were grown in the presence of either a blocking antibody against LFA-1 or an IgG1 isotype control. The effect of blocking LFA-1 interactions resulted in a marked reduction of CD103 expression. At day 3 in co-culture 94% (Range: 77-96%, n=4) of the T-cells grown in the presence of TGF- β ₁ and an IgG1 isotype control expressed CD103. Blocking with an antibody against LFA-1 significantly lowered the

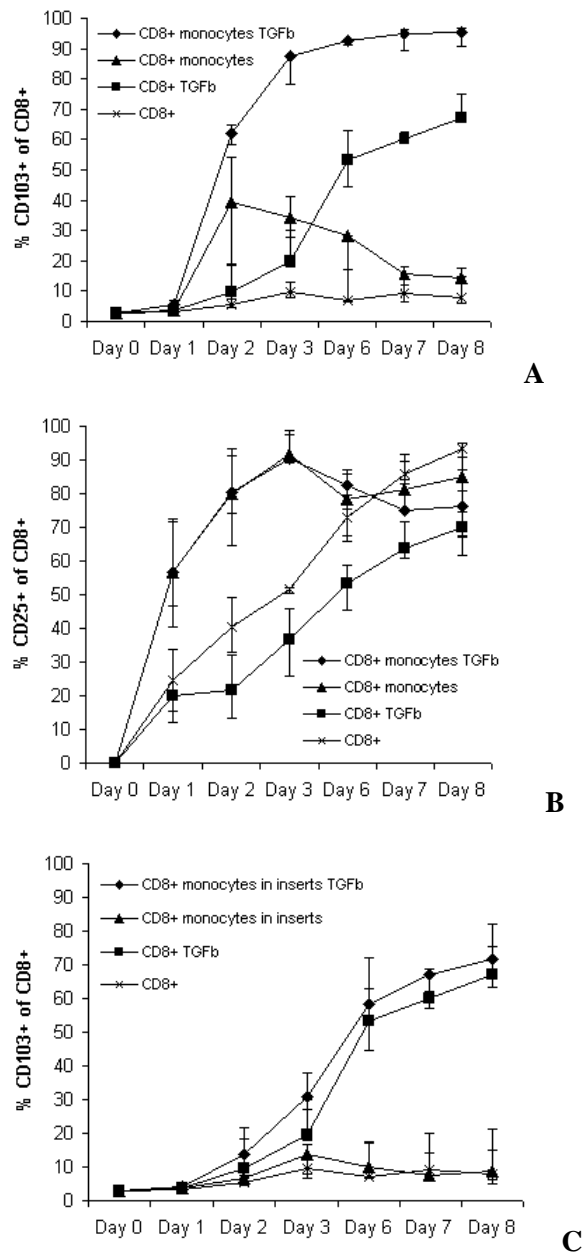


Fig. 2A, B, C Peripheral CD8⁺ T-cells were cultured alone or together with monocytes in the presence or absence of TGF- β ₁. Cultures in Transwell systems, allowing the two cell types to share medium without being able of cell-to-cell contact, were also performed. The expression of CD103 and CD25 on the CD8⁺ T-cells was measured by flowcytometry. Stable expression of CD103 required TGF- β ₁ and was significantly enhanced by the presence of monocytes $p=0.01$ (A). This was in contrast to general activation (CD25 expression) that was not affected by the addition of TGF- β ₁ when monocytes were present. However, in the absence of monocytes TGF- β ₁ significantly decreased the CD25 expression on day 6-8 $p<0.01$ (B). Soluble factors from monocytes growing in transwell inserts, sharing culture medium with the T-cells, did not significantly increase the CD103 expression on the T-cells (C). Data expressed as median values and quartiles, $n=6$.

expression ($p < 0.05$), resulting in 31% (Range: 24-47%, $n=4$) of the cells expressing CD103 (Figure 3A). To investigate whether the LFA-1 signalling was required directly on the T-cells, or if this was an effect mediated via the monocytes, pure T-cell cultures were supplemented with immobilized ICAM-1 and the level of CD103 induction in response to $\text{TGF-}\beta_1$ was recorded. In the cultures where CD8⁺ T-cells were grown together with immobilized ICAM-1 74% (Range: 71-76%, $n=2$) of the cells expressed CD103 while T-cells grown in the absence of ICAM-1 showed a low expression of CD103, 5% (Range: 4-6%, $n=2$) (Figure 3B).

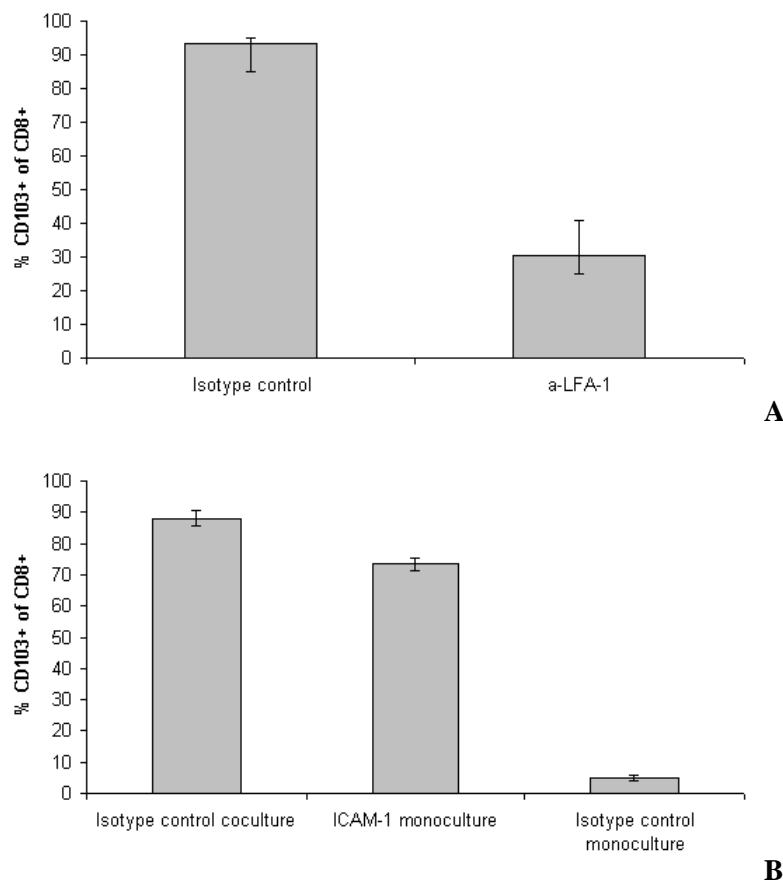


Fig. 3A, B Adding anti-LFA1 to the cells in T-cell/monocyte cocultures significantly lowered the expression of CD103 on the CD8⁺ T-cells, $p < 0.05$ (A). Supplementing pure T-cell cultures with immobilized ICAM-1 increased the CD103 expression to a level corresponding more like the one seen in cocultures of monocytes and T-cells than to the one seen in pure T-cell cultures (B). Data expressed as median values and quartiles, $n=4$ in Figure 3A and $n=2$ in Figure 3B.

TGF- β_1 Mediated Effects on Proliferation

Proliferation of the T cells was measured as degree of incorporated BrdU. Pure T-cell cultures and co-cultures with T-cells and monocytes grown in the presence or absence of TGF- β_1 were examined. No significant effect on proliferation was recorded in response to TGF- β_1 when CD8⁺ T-cells were cultured together with monocytes except from at day 3 where there was a transient decrease in proliferation of the TGF- β_1 stimulated cells ($p < 0.01$, $n = 9$). In the absence of monocytes, there was no significant decrease in proliferation in response to TGF- β_1 (Figure 4A and B).

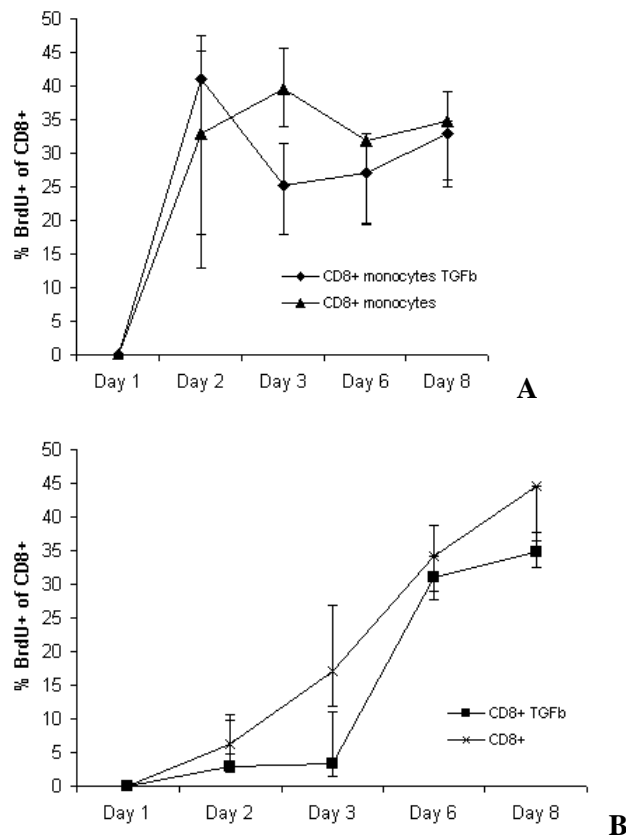


Fig. 4 A, B Proliferation of T-cells in the cell cultures was measured as degree of incorporated BrdU during 2 hours. A significant decrease in proliferation due to the stimulation of TGF- β_1 was seen only on day 3 in the T-cell-monocyte co-cultures ($p < 0.01$) (A). No change in proliferation due to the addition of TGF- β_1 was seen in the pure T-cell cultures (B). Data expressed as median values and quartiles, $n = 9$ ($n = 8$ on day 3) in Figure 4A and $n = 4$ ($n = 3$ on day 3) in Figure 4B.

Production of Several Cytokines was Altered by the Presence of TGF- β ₁

The production of secreted cytokines from T-cells that had been in co-culture with monocytes was measured. The cells were restimulated at day 6 and the cell culture medium was harvested 48 hours thereafter. Luminex cytokine assays were performed on the cell culture medium and the cell cultures analysed for DNA content using the Hoechst-assay. The cytokine levels were adjusted to the DNA content of the corresponding culture and data presented as pg cytokine/ μ g DNA content. TGF- β ₁ significantly suppressed the expression of TNF- α ($p < 0.01$, $n = 8$), GM-CSF ($p < 0.01$, $n = 8$) and INF- γ ($p < 0.01$, $n = 6$) while the expression of IL-8 was retained (Figure 5A and B). The effect of TGF- β ₁ was clearly dose dependent in a range between 0.01ng/ml to 10 ng/ml with maximum inhibition reached at 1 ng/ml as illustrated for TNF- α in Figure 6. IL-10 was low in all cultures, and as IL-8 unaffected by TGF- β ₁ (Figure 5A). No IL-4 could be detected.

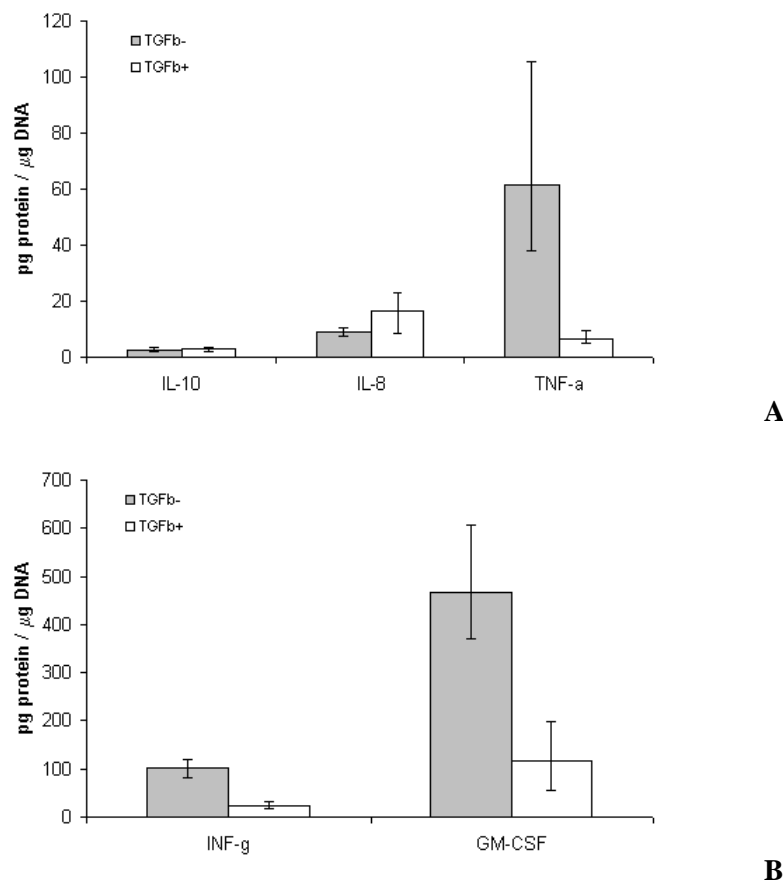


Fig. 5 A, B Peripheral CD8⁺ T-cells were, for three days, cultured together with monocytes, in the presence (TGFb⁺) or absence (TGFb⁻) of TGF- β ₁ (10 ng/ml). The T-cells were then restimulated and put on new culture plates resulting in pure T-cell cultures. The T-cell cultures were restimulated a second time on day 6. On day 8 Luminex cytokine assays were run on the cell culture medium and the cytokine levels were adjusted to the DNA

content of the corresponding culture. The expression of TNF- α , INF- γ and GM-CSF was significantly down regulated by TGF- β ₁ ($p < 0.01$) while IL-10 and IL-8 was not. Data presented as median values and quartiles, $n=4$ (IL-10), $n=8$ (IL-8, TNF- α , GM-CSF), $n=6$ (INF- γ).

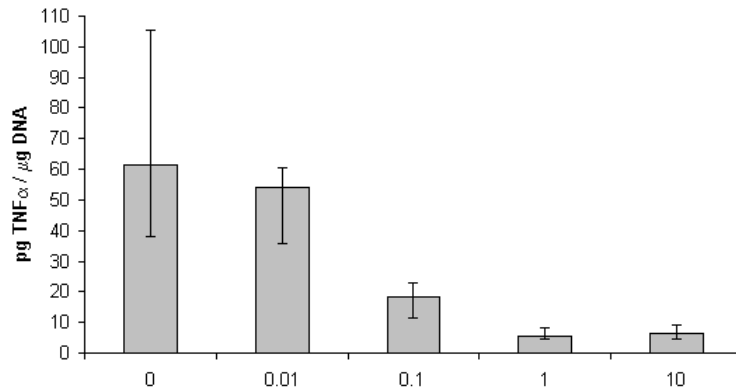


Fig. 6 CD8+ T-cells were cultured in 0-10 ng/ml of TGF- β ₁. The expression of TNF- α in the cell cultures was influenced by TGF- β ₁ in a dose-dependent manner. Data presented as median values and quartiles, $n=6$. The graph is representative also for INF- γ and GM-CSF.

Discussion

This study shows that CD8+ T-cells upregulate α E β 7 after stimulation with TGF- β and that the expression is further induced and sustained by cell-to-cell contact with monocytes. The signal between the monocyte and the T-cell seems to be running through LFA-1 on the T-cell. These TGF- β stimulated cocultures of CD8+ T-cells and monocytes show retained IL-8 production and a down regulation of secreted GM-CSF, TNF- α and INF- γ in comparison to non TGF- β stimulated cultures.

In the interaction between T-cells and mucosal epithelial cells the binding between α E β 7 and E-cadherin has been implied to play a role in the homing and retention of intraepithelial lymphocytes, but also for establishing a firm adhesion required to mediate T-cell cytotoxicity [15,19]. In blood, only a very limited number of CD8+ T-cells express α E β 7, whereas the majority of intraepithelial lymphocytes express this molecule. In this study, flowcytometric analysis of CD8+ T-cells in BAL from COPD patients and healthy subjects showed that the majority of the CD8+ T-cells expressed CD103 and that the expression was within the same range in the two different groups. Since this is the first study made on CD8+ T-cells in BAL from COPD patients there are no former references to compare the results with. The results from the healthy subjects, though, correspond well with studies that compare other respiratory

diseases to healthy subjects^[13,14]. To our knowledge, so far nothing has been published upon CD103 expression in COPD tissue. The occurrence of CD103 expressing cells in tissue could very well vary between COPD and healthy individuals. An analogous situation is the lack of significant difference in our data between the total numbers of CD8⁺ cells per ml of BAL fluid from COPD patients vs. healthy subjects, in spite of overwhelming evidence of an increase of CD8⁺ cells in tissue from COPD patients^[3,6]. The difference in percentage of CD8⁺ T-cells that we found in our study is most probably due to increased numbers of alveolar macrophages (data not shown) in BAL from COPD patients. The number and percentage of CD8⁺ T-cells in BAL from healthy subjects correspond well with other studies^[14]. Almost all of the CD8⁺ BAL cells in this study were CD45R0⁺ CD25⁻. This implies that the cells have previously been activated and thus represent a memory phenotype of cells, but that they most probably are not in an active state at the time of bronchoscopy.

The aim of the *in vitro* system in this study was to investigate the requirements for CD103 expression and to characterize the potential functions for this cell type. Using this system where purified CD8⁺ T-cells were stimulated via anti-CD3 and anti-CD28 in the presence or absence of autologous monocytes, we confirmed that the CD103 expression is dependent on TGF- β . Despite the fact that TGF- β is generally considered to inhibit T-cell activation and proliferation, the overall proliferative response to anti-CD3/anti-CD28 stimulation in this system was not markedly decreased by the addition of up to 10 ng/ml of TGF- β_1 . Another main finding was that co-stimulatory signals provided by the monocytes further increased the expression of CD103 and that this effect could not be mediated by monocyte derived soluble factors but was dependent on cell-to-cell contact. Using a blocking antibody against LFA-1 we were able to reverse the co-stimulatory effect of the monocytes. This indicates that the signalling between the T-cells and the monocytes acts through LFA-1, signalling either through the monocyte or the T-cell. In order to clarify for which cell the effect of LFA-1 costimulation was required, pure CD8⁺ T-cells were cultured in ICAM-1 coated wells in the presence of TGF- β . In such cultures the expression of CD103 rose to 74% on day 3. This should be compared to experiments where CD8⁺ T-cells were cultured in the presence of TGF- β_1 but in the absence of monocytes where only a few percent of the cells expressed CD103 on day 3 in culture. That immobilized ICAM-1 has the potential to stimulate the CD8⁺ T-cells like this suggests that LFA-1 signalling of the T-cell is required, and that this is not a secondary effect mediated via the monocytes. Blocking experiments using antibodies against ICAM-1 were also set up but no difference in CD103 expression could be detected (data not shown). This may reflect the redundancy in the integrin signalling system where ICAM-1 as well as ICAM-2 could mediate the crosslinking of LFA-1 on the T-cell.

The function of CD103 expressing cells was investigated in terms of the cytokine production profile of TGF- β ₁ stimulated CD103+ vs. cells stimulated in the absence of TGF- β ₁, CD103- cells. The concentration of TNF- α , INF- γ and GM-CSF synthesized was inversely correlated to the concentration of TGF- β ₁ added to the cultures. This was in contrast to the IL-8 production that was not down regulated, but instead showed a tendency of an increased expression. The cytokine profile of the CD103+ cells per se, thus seem less pro-inflammatory than that of regular CD8+ T cells. However, the retained production of IL-8 is interesting, since this cytokine has the potential of recruiting pro-inflammatory cells such as neutrophils to the area. Neutrophils are known to produce large amounts of proteases, such as neutrophil elastase. This protease could shift the protease/protease inhibitor imbalance already known to exist in COPD and lead to damage of the epithelium and further inflammation ^[1].

This *in vitro* system provides a helpful tool when studying the CD103 expressing cells and their function. As shown in this study BAL CD8+ T-cells from COPD patients do to a large extent express CD103 indicating a need to gain better understanding of these cells in order to understand their potential role in the pathogenesis of COPD. Also for cells in sputum of COPD patients 25-60% of CD8+ T-cells express CD103 ^[20]. The number of T-cells to be extracted from BAL, in the intention to be used in functional assays, is still very limited. Therefore, even though the relevance of an *in vitro* system always can be questioned, a system in which high numbers of CD8+ T-cells can be differentiated into CD103 expressing cells is valuable. It can be used for studying the α E β 7 expressing T-cells further and also as a hypothesis-generating tool for further studies of CD8+ T-cells in COPD. These *in vitro* studies could include the use of different additional stimuli with relevance to COPD such as cigarette smoke extract (CSE). The cytolytic capacity of these cells also has to be investigated, to evaluate their potential role in the tissue destruction or protection in COPD. The increased numbers of both CD8+ T-cells and monocytes/macrophages in the lungs of COPD patients ^[1,2] in combination with the increased expression of TGF- β and its correlation to the number of intraepithelial macrophages ^[16,17] merits a closer study of this particular intra epithelial T-cell population. In fact, it still has to be established whether the presence of these cells is beneficial or detrimental to the disease. Given the fact that one important feature of mucosal lymphocytes is their ability to defend the epithelium from infectious agents, such as virus, a reduction in their capacity to do so, due to for example harmful substances in cigarette smoke, would increase the risk of having reoccurring infections. Since COPD patients often have exacerbations triggered by airway infections^[7] a strong and well

functioning defence is of importance. One could postulate that smokers with reduced capacity to fight infections would be the ones developing the most severe symptoms of COPD.

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