Cellular Immunology 269 (2011) 128–134

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Neutrophil elastase treated dendritic cells promote the generation of $CD4^+FOXP3^+$ regulatory T cells *in vitro*

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ARTICLE INFO

Article history:
Received 17 November 2010
Accepted 15 March 2011
Available online 17 March 2011

Keywords:
Dendritic cells
Neutrophils
Elastase
TGF-β
Regulatory T cells

ABSTRACT

We have previously shown that neutrophilic elastase converts human immature dendritic cells (DCs) into TGF-β secreting cells and reduces its allostimulatory ability. Since TGF-β has been involved in regulatory T cells (Tregs) induction we analyzed whether elastase or neutrophil-derived culture supernatant treated DCs induce $CD4^+FOXP3^+$ Tregs in a mixed lymphocyte reaction (MLR). We found that elastase or neutrophil-derived culture supernatant treated DCs increased TGF-β and decreased IL-6 production. Together with this pattern of cytokines, we observed a higher number of $CD4^+FOXP3^+$ cells in the MLR cultures induced by elastase or neutrophil-derived culture supernatant treated DCs but not with untreated DCs. The higher number of $CD4^+FOXP3^+$ T cell population was not observed when the enzymatic activity of elastase was inhibited with an elastase specific inhibitor and also when a TGF-β1 blocking antibody was added during the MLR culture. The increased number of $CD4^+$ that express FOXP3 was also seen when $CD4^+CD25^-$ purified T cells were cocultured with the TGF-β producing DCs. Furthermore, these $FOXP3^+$ T cells showed suppressive activity *in vitro*.

These results identify a novel mechanism by which the tolerogenic DCs generated by elastase exposure contribute to the immune regulation and may be relevant in the pathogenesis of several lung diseases where the inflammatory infiltrate contains high numbers of neutrophils and high elastase concentrations.

1. Introduction

The immunological system has mounted mechanisms to prevent self reactions, at central and peripheral levels, in order to maintain tolerance against self antigens.

Centrally, self reactive T cell clones are eliminated during thymus differentiation and peripherally various mechanisms exist to inactivate those clones that escaped central tolerance.

One of these mechanisms is based on regulatory T cells (Tregs). These cells play a critical role in suppressing the immune response and its deficiency and/or deregulation has been linked to several autoimmune and inflammatory diseases [1,2].

Their principal characteristic phenotype is $CD4^+CD25^+$ and they also express the transcription factor FOXP3, which has been identified not only as a molecular marker, but also as a key regulatory gene for the development and function of Tregs [3,4].

Based on the cytokine expression profile and surface markers, these cells can be divided in two different groups: naturally occurring Tregs and acquired Tregs [5–7]. Human naturally occurring Tregs, similarly to the murine counterpart, are generated in the thymus and are characterized by the ability to suppress proliferation of responder T cells through a cell–cell contact-dependent mechanism, the inability to produce IL-2, and their anergic phenotype *in vitro* [8]. Whereas acquired or induced Tregs (iTregs) are $CD4^+CD25^+FOXP3^+$ T cells differentiated from $CD4^+CD25^-FOXP3^-$ T cells in the periphery. Conventional T cells are able to gain immune suppressive activities and become acquired Tregs [9,10].

TGF-β is a multifaceted cytokine and regulates multiple cellular functions including proliferation, differentiation, migration, and survival [11]. This cytokine suppresses immune responses through two means: inhibiting the function of inflammatory cells and promoting the function of Tregs. TGF-β inhibits immune responses through promotion of the generation of Tregs by inducing FOXP3 expression [12]. Several studies demonstrated that TGF-β is able to convert $CD4^+CD25^-$ non-Tregs murine cells into $CD4^+CD25^+$ Tregs cells, and this conversion was accompanied by increased FOXP3 expression [13,14], however, a substantial portion of

Abbreviations: PMN, neutrophils or polymorphonuclear leukocytes; DC, dendritic cells; CS, culture supernatants; moDC, monocyte derived DC; PBMC, peripheral blood mononuclear cells; HSA, human serum albumin; SLPI, human secretory leukocyte protease inhibitor; MLR, mixed lymphocyte reaction; Tregs, regulatory T cells.

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0008-8749/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellimm.2011.03.013

FOXP3$^+$ Tregs cells are negative for CD25 [15,16]. Wan and Flavell also demonstrated that TGF-β induced *de novo* FOXP3 expression in FOXP3$^-$CD4$^+$ T cells; they also showed that only FOXP3$^+$CD4$^+$ cells but not FOXP3$^-$CD4$^+$ counterparts possessed regulatory activities. Furthermore, some tumors promote the differentiation of DCs producing IL-10 that induce the expansion of CD4$^+$CD25$^+$ regulatory T cells which contribute to tumor escape [17,18].

The methods to reprogram human CD4$^+$CD25$^-$ T cells to Tregs *in vitro* have been recently investigated. It has been reported that *in vitro* TCR-mediated stimulation of CD4$^+$CD25$^-$ T cells can result in the development of suppressive CD4$^+$FOXP3$^+$ Tregs cells [19–21].

However, several reports indicate that even in the presence of exogenous TGF-β, TCR stimulation of human CD4$^+$CD25$^-$ T cells does not consistently generate Tregs cells despite high FOXP3 expression within these cells [22–24]. Moreover, the stability of the suppressive capacity of these *in vitro* generated Tregs cells has not been fully established.

In infection or tissue injury, DC activation and maturation occur rapidly, typically noted within 1–4 h [25], often preceding the peak of PMN accumulation at the site. DCs have indeed the capacity to recruit and activate cells of the innate immune system, even PMNs and immature DCs. Once in the inflammatory site, PMNs may interact with DCs to modulate their function and the induced T-cell responses.

The main intercellular physiological function of neutrophil elastase is the degradation of foreign organic molecules phagocytosed by neutrophils, whereas the main target for extracellular elastase is elastin. In neutrophils, the concentration of neutrophil elastase exceeds 5 mM and its total cellular amount has been estimated to be up to 3 pg [26]. Such a high concentration of elastase is tightly regulated by compartmentalization in the azurophil granules.

Neutrophil elastase has been linked to the pathophysiology of acute lung injury and impaired wound healing [27]. At inflammatory sites this enzyme appears to remain active due to an imbalance between its own elevated level and that of the endogenous protease inhibitors [28].

In a previous work, we have shown that neutrophilic elastase or the PMNs culture supernatant converted human immature dendritic cells (iDCs) into TGF-β producing iDCs, with the concomitant reduced ability to induce lymphoproliferation [29].

In this work, we show for the first time that these iDCs treated with neutrophilic elastase or polymorphonuclear culture supernatant (PMN-CS), also promote the generation of CD4$^+$FOXP3$^+$ Tregs *in vitro*.

2. Materials and methods

2.1. Blood samples

Peripheral blood was obtained from healthy donors in accordance with local ethical committee approval.

2.2. Blood monocyte isolation and monocyte derived DCs (moDCs) culture

Human DCs were generated from monocytes as previously described with minor modifications [30]. Briefly, blood was obtained from healthy donors and the peripheral blood mononuclear cells (PBMC) were isolated by centrifugation through a Ficoll-Hypaque (Pharmacia LKB Biotech., Piscataway, NJ) gradient. To further isolate monocytes, the PBMC were isolated by centrifugation through Percoll (Pharmacia Fine Chemicals, Dorval, PQ) gradient as described elsewhere [29]. Once the purest monocyte fraction was recovered, it was cultured (5×10^6 cells/ml) for 5 days in RPMI-1640 supplemented with 2 mM L-glutamine, 50 mg/ml gentamicin, 50 mM 2-Mercaptoethanol, 800 U/ml GM-CSF, 500 U/ml IL-4 (Sigma, St. Louis, MO) and 10% heat inactivated pooled AB human serum (HS) from 3 to 4 normal donors. DCs were identified by immunofluorescence staining of the cells. A number of monoclonal antibodies (mAbs) that recognize antigens present on DCs were used *in vitro*. These included mAbs anti-CD1b (Wm25, IgG1 from Chemicon, CA), anti-HLA-DR (HB-55, IgG2a from ATCC, Rockville, MD), anti-CD86 (FUN-1, IgG1, Pharmingen, CA) and anti-CD83 (HB15A, IgG2b, Immunotech, France). Other mAbs used were anti-CD14 (3C10, IgG2b from ATCC), anti-CD11b (2LPM19c IgG1 from DAKO, CA), anti-CD54 (84H10, IgG1, Immunotech, France), and anti-CD40 (Pharmingen, CA).

To generate dendritic cells culture supernatants (DC-CS), the cells were treated or not with 100 µl of PMN-CS or with 30 nM elastase (SIGMA, St. Louis) for 3 h. Thereafter, the cells (2×10^6 moDCs/ml) were washed with PBS and incubated for 80 h in RPMI 1640 supplemented with 2 mM L-glutamine, 50 mg/ml gentamicin, 50 mM β-ME, 800 U/ml GM-CSF, and 500 U/ml IL-4. Supernatants were centrifuged at 1200 rpm for 10 min, and immediately dispensed in aliquots and frozen at −70 °C.

2.3. Human PMN leukocyte purification

Human PMNs were purified as described previously [29] from ACD-heparin-anticoagulated venous blood from healthy donors. Briefly, red cells were sedimented with 6% dextran-saline (Rivero, Buenos Aires), leukocyte-rich plasma was collected, and PMN leukocytes were purified by discontinuous Percoll gradient centrifugation, washed, and resuspended to 2.5×10^6 PMN/ml in RPMI 1640, 0.5% human serum albumin (HSA), 10 mM HEPES, pH 7.418. This method yielded PMN of >95% purity with essentially no red cell contamination and >98% cell viability.

In order to obtain PMN-CS, PMNs were primed with IL-8 (50 ng/ml) for 10 min, or used unprimed. Next, the cells (10^7 PMNs/ml) were washed with PBS and incubated in serum-free RPMI 1640 + 5 mg/ml HSA at 37 °C for 3 h. Thereafter, supernatants were centrifuged at 1200 rpm for 10 min, and immediately dispensed in aliquots and frozen at −70 °C. For some experiments, the PMN-CS were incubated with different concentrations of the elastase specific inhibitor, secretory leukocyte protease inhibitor (SLPI), in order to measure the residual proteolytic activity in the CS.

2.4. Mixed lymphocyte reaction (MLR)

DCs were treated or not with PMN-CS or human neutrophil elastase (SIGMA, St. Louis) to a final concentration of 75 µM for 3 h at 37 °C. DCs (35×10^3) were then thoroughly washed and cocultured in 96-well microplates (round bottom) with 10^5 allogeneic PBMC. On day 5 cells were harvested, immune stained, and analyzed in a flow cytometer. For some experiments, on day 5 cells were pulsed with [^{3}H]-thymidine (1 µCi/well, specific activity 5 mCi/mMol; Amersham Life Sciences). Thymidine incorporation was measured after 18 h of the initial pulse. For some experiments and in order to inhibit elastase activity, recombinant human SLPI (rhSLPI) was added to a final concentration of 4 µg/ml. Then, cells were thoroughly washed and cocultured with allogeneic PBMC.

In some experiments, a neutralizing mouse mAb anti-human TGF-β1 was added at the beginning of MLR assay. The antibody used in these experiments is a monoclonal antibody anti-human TGF-β1 (clone 9016, IgG1, R&D System Inc., MN, USA). According to the manufacturers data sheet this antibody has been selected for its ability to neutralize biological activity of TGF-β1 and TGF-β1.2 and for use as a capture antibody in sandwich ELISAs.

2.5. Isolation of $CD4^+CD25^-$ T cells from blood

The $CD4^+CD25^-$ T cells were obtained by labeling the cells with anti-CD4 PE and anti-CD25 FITC antibodies and then sorted via a FACS Aria cell sorter (BD Biosciences) with >96% purity. The FACS-sorted $CD4^+CD25^-$ T cells (5×10^5) were cocultured in 96-well microplates with 3×10^5 DCs pre treated or not with human neutrophil elastase (SIGMA, St. Louis) to a final concentration of 75 μM for 3 h at 37 °C. On day 5, cells were analyzed for CD4, CD25 and FOXP3 expression by flow cytometry and used in a T cell suppression assay.

2.6. Analysis by flow cytometry

After 5 day of coculture, DCs and PBMC were harvested and washed. Cells (1×10^6) were stained for surface CD4 and CD25 expression with monoclonal antibodies conjugated with PE-Cy5 and FITC, respectively (Caltag Lab, CA). After washing the cells in cold PBS, the pellets were then resuspended with a pulse vortex and 1 ml of freshly prepared fixation/permeabilization working solution was added to each sample and incubated at 4 °C for 30–60 min in the dark. Then cells were washed once by adding 2 ml 1X Permeabilization Buffer followed by centrifugation and decanting of the supernatant. Then, blocking solution (2% normal rat serum in 1X Permeabilization Buffer) was added, in approximately 100 μl volume, and incubated at 4 °C for 15 min.

Afterwards, 20 μl of fluorochrome conjugated anti-human FOXP3 antibody or isotype control (Caltag Lab, CA) was added in 1X Permeabilization Buffer and incubated at 4 °C for at least 30 min in the dark. The cells were then washed and analyzed by flow cytometry in a FACS-Calibur cytometer using WinMDI software (BD Biosciences). While performing the flow cytometric analysis, $CD4^+$ population was gated for further analyzing the FOXP3 expression.

2.7. Suppressive functional assay

To further analyzed the suppressive functional properties of the cells, DCs treated or not with elastase and $CD4^+CD25^-$ FACS-sorted T cells were cocultured for five days, afterwards they were harvested and plated in triplicate wells with 3×10^4 cells per well and cocultured with 3×10^4 allogeneic PBMC as responder cells and 5×10^4 syngeneic (regarding the $CD4^+CD25^-$) mitomycin treated PBMC as antigen presenting cells per well. Cells were cultured in 96-well flat bottom tissue cultures plates with 10 μg/ml of plate bound anti-CD3 (OKT3) in culture medium RPMI at 37 °C. Thymidine incorporation was measured on day 5 by 18 h pulse with [^{3}H]-thymidine (1 μCi/well, specific activity 5 mCi/mMol; Amersham Life Sciences).

2.8. Reagents

rhSLPI was cloned and expressed as described previously [29]. It was used in a final concentration of 4 μg/ml. This concentration was seen to inhibit elastase activity in PMN-CS and commercial human neutrophilic elastase.

Human neutrophilic elastase was purchased from SIGMA (Sigma, St. Louis, MO), and diluted to a final concentration of 75 μM.

2.9. Cytokine measurements

Determination of IFN-γ, TNF-α, IL-2, IL-10, IL-6 and IL-4 were measured by the BD cytometric bead array kit (BD Biosciences, San Diego, CA), following the manufacturer's instructions.

TGF-β1 was detected by sandwich ELISA as described in [29].

2.10. Elastase activity assay

PMN-CS was diluted 1:3 in Tris buffered saline (pH 7.5) containing the substrate *N*-methoxysuccinyl-Al-Ala-Pro-Val *p*-nitro-anilide (Sigma Aldrich) to a final concentration of 0.6 mM. At the same time, reaction wells with a known concentration (20 nM) of human leukocyte elastase (Sigma Aldrich) instead of PMN-CS were assayed, and the kinetics of the enzyme reaction was measured in an ELISA plate reader at 2 min intervals over a 20 min period. Then the velocity of the enzyme reaction was calculated from an optical density/time plot, and the constant for this reaction (k) was deduced from the standard reaction with a known concentration of elastase. The formula used for the determination of neutrophil elastase concentration was: $V = [\mathrm{Et})\ k2[S]/[S] + \mathrm{km}$. If [$S$] km, then $V_{max} = k2$ [Et], where km elastase = 0.21 mmol/L ([Et], elastase concentration; and [S], substrate concentration).

2.11. Statistical analysis

Statistical significance was determined using Student's t test for unpaired samples with unequal variance, with p-values <0.05 regarded as statistically significant.

3. Results

3.1. PMN-CS or elastase action on human DCs

We have previously shown that elastase converts human immature DCs into TGF-β secreting cells and reduces their allostimulatory ability. In order to determine whether elastase or PMN-CS might change the expression of other cytokines on immature DCs, DCs derived-CS were analyzed with a BD cytometric bead array (BD Biosciences, San Diego, CA) for IFN-γ, TNF-α, IL-2, IL-10, IL-6, IL-4 and with a sandwich ELISA for TGF-β1. Fig. 1 shows that elastase-treated DCs secreted higher amounts of TGF-β and lower amounts of IL-6 compared to untreated DCs. The same was observed with IL-8 primed PMN and unprimed PMN derived CS-treated DCs (data not shown). IL-8 primed PMNs or unprimed PMNs had both similar amounts of elastase released to the medium. However, the reduction on IL-6 secretion was more pronounced for elastase than

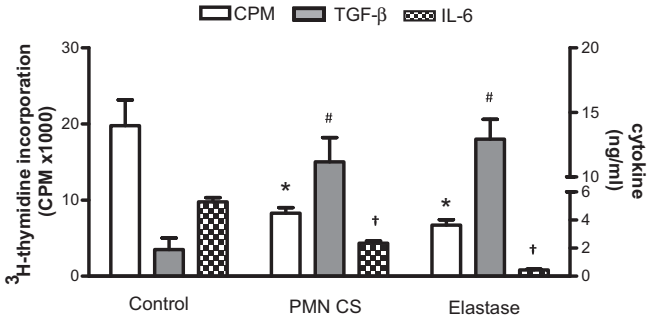

Fig. 1. Cytokine expression and proliferative capacity of DCs treated with PMN-CS or elastase. To analyze the DCs cytokine expression, DCs were washed and incubated for 48 h in RPMI-HAS, after 3 h of treatment with PMN-CS or elastase. Then the supernatants were recovered, diluted and assessed for TGF-β1 by ELISA and IL-6 by the BD cytometric bead array kit, as described in Section 2. To quantify the proliferative capacity, another group of DCs previously treated with PMN-CS or elastase were cocultured with allogeneic PBMC, on day 5 cells were pulsed with [^{3}H]-thymidine and its incorporation was measured after 18 h of the initial pulse. Data represent the mean ± SEM of three different experiments. $^*p < 0.05$ for CPM between PMN-CS or elastase treated DCs compared to control untreated DCs, $^\#p < 0.05$ for TGF-β expression between PMN-CS or elastase treated DCs compared to control untreated DCs, $^\dagger p < 0.05$ for IL-6 expression between PMN-CS or elastase treated DCs compared to control untreated DCs.

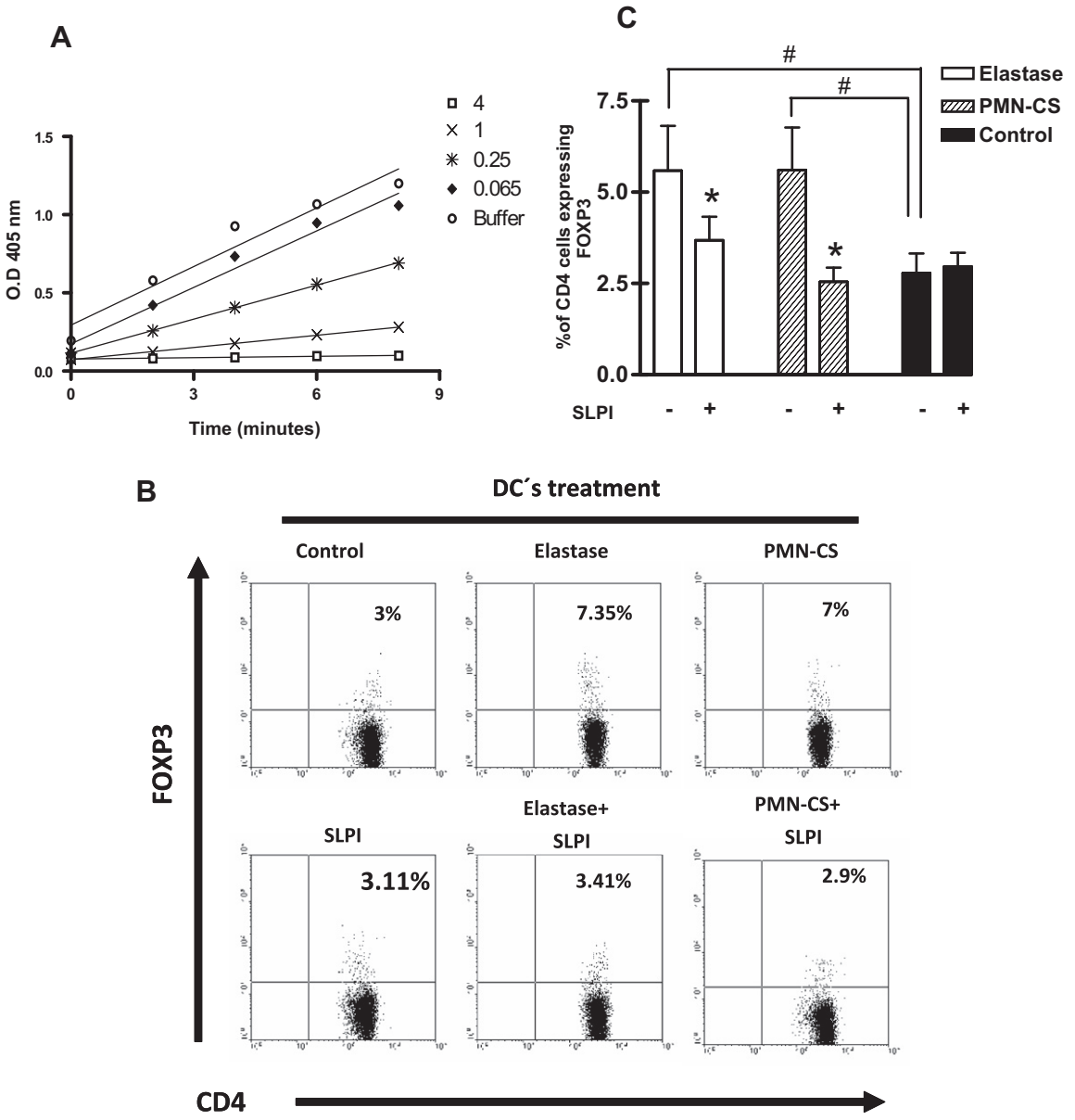

Fig. 2. Effect of elastase and PMN-CS on DCs ability to induce $CD4^+FOXP3^+$ cells. (A) To determine the amount of SLPI needed to inhibit the elastase activity, different concentrations of the inhibitor (μg/ml) or control buffer were co-incubated with PMN-CS, and the remaining elastase activity was measured in a colorimetric specific assay. (B and C) DCs were treated with PMN-CS or elastase in the presence or not of SLPI for 3 h, and then cocultured with 10^5 PBMC. After five days, cells were stained with surface CD4-PE-Cy5 and CD25-FITC antibodies, then fixed, permeabilized and stained with FOXP3-PE mAb. Finally cells were analyzed by flow cytometry as described in Section 2. In panel (B) one representative donor is depicted, numbers in corners indicate the percentage of positive cells in each quadrant. Data in (C) represented as mean ± SEM of five donors; percentages of $CD4^+FOXP3^+$ are indicated. Statistical significance was determinate by comparison to the same treatment without SLPI ($^*p < 0.05$) and to control without SLPI ($^\#p < 0.05$).

PMN-CS treated DCs ($p < 0.05$). The other cytokines measured by the kit were below the detection limit of the assay.

The cytokine pattern secreted by elastase or PMN-CS treated-DCs of high TGF-β and low IL-6, and its decreased allostimulatory ability, led us to study the subset of $CD4^+FOXP3^+$ cells induced by these DCs in a mixed lymphocyte reaction (MLR).

3.2. Elastase or PMN-CS treated DCs promote the generation of $FOXP3^+$ cells

To asses the concentration of active elastase present in the PMN-CS, the velocity of the enzyme reaction was calculated as described in Section 2, from standard reaction with a known concentration of elastase (not shown). The active elastase concentration present in the PMN-CS was found to be among 5–30 nM. In order to calculate the concentration of an elastase specific inhibitor (SLPI) needed to inhibit the enzyme present in the PMN-CS, a dose dependent experiment was performed. The optimal concentration of SLPI needed to inhibit completely the elastase activity was found to be 4 μg/ml (Fig. 2A).

Fig. 2 B and C shows that the number of $CD4^+FOXP3^+$ cells in the MLR induced by elastase or PMN-CS treated DCs was generally two fold higher compared with the MLR induced by untreated DCs.

When we analyze the elastase specific activity on the promotion of the tolerogenic characteristics of elastase and PMN-CS treated DCs, we could see that the inhibitor SLPI prevented the

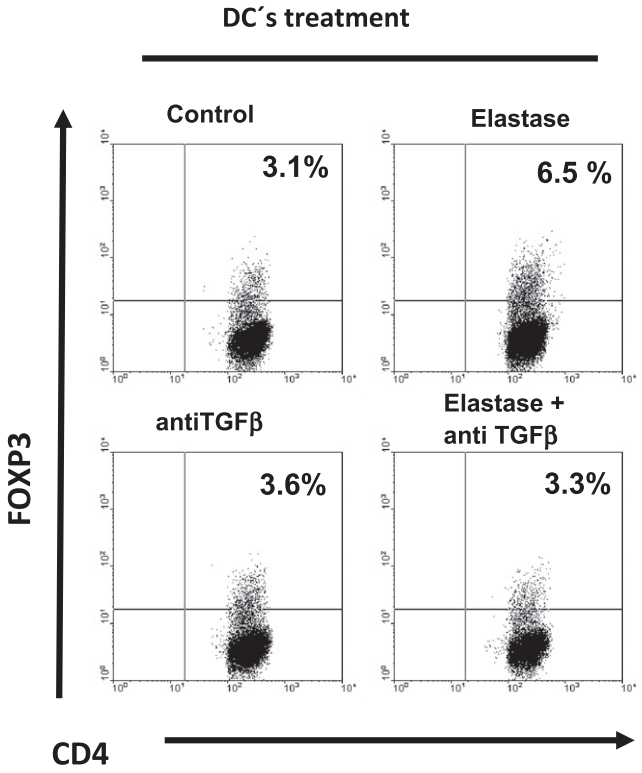

Fig. 3. Effect of a mAb against TGF-β1 on DCs-induced $CD4^+FOXP3^+$ cells. DCs were treated or untreated for 3 h with elastase. Afterwards, the cells were washed and incubated with allogeneic lymphocyte in the presence of 20 μg of TGF-β1 mAb or isotype control mAb. After 5 days, cells were harvested and stained with CD4 and FOXP3 mAb as described in Section 2. Data are representative of an experiment of three independent experiments. The numbers in each quadrant represent the percentage of cells expressing the antigen.

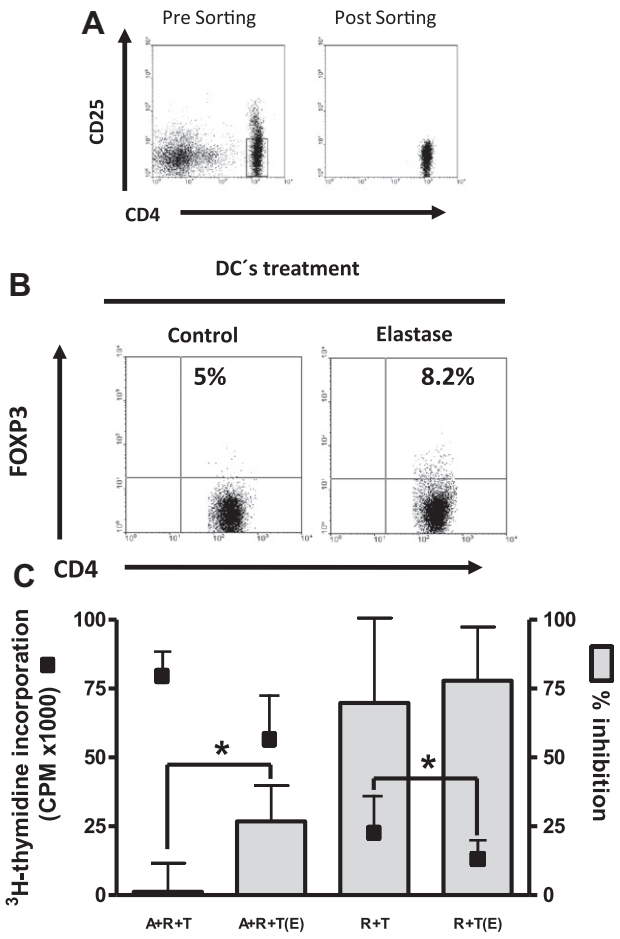

Fig. 4. Generation of $FOXP3^+$ T cells from $CD4^+CD25^-$ after 5 days cocultured with elastase treated DCs and analysis of their regulatory activity. (A) Typical flow cytometric analysis before (pre sorting) and after cell sorting (post sorting) of PBMC stained with CD4 PE and CD25 FITC. (B) Expression of FOXP3 in FACS-sorted $CD4^+CD25^-$ T cells after 5 days cocultured with elastase treated or control DCs. A representative experiment of three is depicted. Numbers in each quadrant as in Fig. 3. (C) In order to determine if $FOXP3^+$ generated cells were indeed Tregs, a suppression assay was performed. Cell proliferation assay was carried out with: FACS-sorted T cells after 5 days culture with DCs treated with elastase (T-E) or DCs alone (T), allogeneic PBMC as responder cells (R) and syngeneic mitomycin treated PBMC as antigen presenting cells (A). [^{3}H]-thymidine incorporation and percentage of inhibition are depicted. The 100% of proliferation and 0% inhibition correspond to the A + R culture CPM (not plotted). Data represent mean ± SEM of three different experiments (*$p < 0.05$).

increment of $CD4^+FOXP3^+$ cell subset from the PMN-CS or elastase treated DCs groups (Fig. 2C). We can conclude that elastase activity is needed for the higher levels of TGF-β expression in DCs (as we described previously, see [29]) and the increased number of $CD4^+FOXP3^+$ cells.

3.3. TGF-β is essential for the generation of the $CD4^+FOXP3^+$ subset

TGF-β is considered a key role cytokine in the generation of Tregs. To demonstrate that this cytokine was involved in the generation of the $CD4^+FOXP3^+$ subset, a blocking antibody for TGF-β1 was added to the MLR during the five days culture (Fig. 3). This antibody reversed the increment in the number of $CD4^+FOXP3^+$ cells that the elastase treated DCs induced. This result confirms that TGF-β1 generated by DCs is essential for the generation of this T cell subset.

3.4. Elastase treated DCs induce de novo generation of $FOXP3^+$ from sorted $CD4^+CD25^-$ cells

In order to determine whether the increased number of FOXP3 was actually induced in the culture, we used a FACS-sorted population of $CD4^+CD25^-$ T cells (Fig. 4A). These cells were cocultured with DCs or elastase treated DCs, and after 5 days the number of $CD4^+FOXP3^+$ cells was analyzed as previously described. In these experiments, we could see that elastase treated DCs induced *de novo* expression of FOXP3 from a subset of $CD4^+CD25^-$ isolated cells (Fig. 4B).

3.5. $CD4^+FOXP3^+$ T cells induced by DCs treated with elastase function as regulatory T cells and suppress the proliferation of T lymphocytes

To test whether the $CD4^+FOXP3^+$ T cells induced by DCs were regulatory T cells, we assessed their suppressive function. For this purpose we performed a suppressive test as described in Section 2. T cells that were previously cocultured with elastase treated DCs had an increased suppressive activity in comparison to T cells generated by DCs alone (Fig. 4C).

4. Discussion

In this study we demonstrate for the first time that neutrophils are capable to modulate the immune response through the release of the serine protease elastase, and its action on DCs. As we previously described, neutrophilic elastase converts immature myeloid DCs into TGF-β-secreting cells, and in this work we show that these DCs are capable of inducing Tregs cells *in vitro*.

Neutrophil-derived elastase is a serine protease involved in host defense against bacterial pathogens [31]. Elastase is capable of hydrolyzing a broad spectrum of extracellular matrix and cellular proteins, such as elastin, interstitial collagens, proteoglycans,

fibronectin, laminin, and others [32], and it has been reported to exhibit several functions in addition to its proteolytic activity, such as induction of IL-8 [33], SLPI [34], or TGF-β [29].

When human myeloid immature DCs were incubated with neutrophil elastase or neutrophil CS, they shift to a TGF-β producing phenotype. And when we analyzed a more broad range pattern of cytokines we could see that the production of IL-6 was also affected, but in a down regulated manner. This two cytokine pattern led us to consider the possibility that these DCs could be inducing the generation of $CD4^+FOXP3^+$ Tregs.

Many authors referred about the influence of these cytokines in the generation of Tregs. Luo et al. [35] showed that β-cell peptide-pulsed DCs from non-obese diabetic (NOD) mice can effectively induce $CD4^+CD25^+FOXP3^+$ T cells from naïve isolet-specific $CD4^+CD25^-$ T cells in the presence of TGF-β1.

Although TGF-β promotes Tregs generation *in vitro* in murine models, it has been controversial whether TGF-β is involved in the generation or maintenance of FOXP3 expressing Tregs under physiological conditions.

It is coming to a consensus that TGF-β is required for the maintenance of Tregs in the periphery.

Wang et al. [22] found that FOXP3 was induced when $CD4^+CD25^-FOXP3^-$ human T cells were activated through TCR crosslinking. They also found that these cells were hyporesponsive when stimulated, showing attenuated proliferation and cytokine production. The findings that FOXP3 is induced by TCR engagement, and that those T cells that have up regulated FOXP3 are hyporesponsive, lead to the conclusion that the induction of FOXP3 serves to shut off T cell activation.

Under physiological conditions, it is believed that induced $FOXP3^+$ Tregs are generated mainly in the gut and possibly in other immunological niches that contain high local concentrations of TGF-β and are colonized by specialized types of antigen-presenting cells [36,37].

In this work, we present an important finding, that PMNs can induce the generation of $CD4^+FOXP3^+$ Tregs via DCs. Neutrophilic elastase alone or PMN-CS, which contain high levels of elastase among other proteases, can induce DCs to promote the generation of $CD4^+FOXP3^+$ Tregs cells through the release of TGF-β.

A diminished amount of IL-6 was also detected in the elastase treated-DC supernatant.

Increasing evidence supports a role for IL-6 trans-signaling into T cells as a key pathway that blockades the development of adaptive Tregs cells and might control the balance between effectors and Tregs cells [38–42]. Thomas Korn et al. [43] have discovered a reciprocal developmental relationship between $FOXP3^+$ Tregs and T helper 17 cells because TGF-β triggers the expression of FOXP3 in naïve T cells, whereas IL-6 inhibits the TGF-β-driven expression of FOXP3. They also found that IL-6 critically prevented the conversion of naïve $CD4^+$ T cells into $FOXP3^+$ Tregs *in vivo*. In our experiments we could see that DCs treated with elastase or PMN-CS exerted an even lower release of IL-6 to the medium than control untreated DCs. Whether this decrease in IL-6 secretion is relevant for $FOXP3^+$ generation, is still an issue to be resolved.

An important issue also faced by many authors is whether these $CD4^+FOXP3^+$ cells generated *in vitro* had any regulatory functions. Some authors [44–46] found that in murine studies, induced Tregs can be generated from $CD4^+FOXP3^-$ T cells by TCR stimulation in the presence of IL-2 and TGF-β, and these induced Tregs have all of the phenotypic and functional properties of Tregs and exert potent suppressor function both *in vitro* and *in vivo*.

Several murine studies have also determined the critical relationship between DCs and Tregs for activation and regulation of immune response [47–50].

But Tran et al. [24], could not generate *bona fide* Tregs from human naïve $CD4^+$ T cells isolated from adult peripheral blood or cord blood. Although they could induce more than 70% of naïve $CD4^+FOXP3^-$ T cells to express FOXP3, these cells still lack regulatory functions.

On the other hand, Dumitriu et al. [51] demonstrated that lung carcinoma cells convert fully differentiated immature and mature DCs into cells producing TGF-β1. These TGF-β1-producing DCs are impaired in their ability to activate $CD4^+$ T cells and are prone to generating $CD4^+CD25^+FOXP3^+$ regulatory T cells. However, the authors did not assess the factor or factors responsible to enable human DCs to produce TGF-β1.

We found that neutrophilic elastase converts DCs into TGF-β producing DCs, and they promote the generation of Tregs in a MLR and from a $CD4^+CD25^-$ starting population of cells. If extrapolated to *in vivo* conditions, we could hypothesize that these DCs that migrate from the neutrophil rich inflammatory site would be playing an important role in the generation of Tregs cells.

We believe that the mechanism described herein, could be considered as a way to control an inappropriate immune response, and may be relevant in all those pathologies that present an excessive or sustained PMN infiltration. For example, in the pathogenesis of several lung diseases, where the inflammatory infiltrate contains high numbers of PMNs and high elastase concentrations, together with an imbalance of elastase and its physiological inhibitor, SLPI.

Acknowledgments

We thank Dr. Mercedes Sanchez for critical reading of the manuscript. We are also grateful to Dr. Eduardo Chuluyan for his invaluable help.

This work was supported by grants from the CONICET PIP 845-10, ANPCyT PICT START UP 2008N°2090.

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