Rapamycin, unlike cyclosporine A, enhances suppressive functions of in vitro-induced CD4⁺CD25⁺ Tregs

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Abstract

Background. A growing body of data shows that CD4⁺CD25⁺ regulatory T cells (Tregs) can induce transplantation tolerance by suppressing immune responses to allograft antigens. However, both the generation and the suppressive capacity of CD4⁺CD25⁺ Tregs can be substantially affected by different immunosuppressive drugs used in clinical transplantation. The goal of this study was to compare the effects of cyclosporine A and rapamycin on the induction and suppressive functions of human CD4⁺CD25⁺ Tregs in vitro.

Methods. CD4⁺CD25⁺ Tregs were induced in two-way mixed lymphocyte reaction (MLR) in the presence of rapamycin (Treg-Rapa) or cyclosporine A (Treg-CsA). Tregs were identified in MLR cultures by flow cytometry using anti-CD4, anti-CD25, anti-CTLA-4, anti-CD122, anti-

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GITR mAbs and ant-PE-FOXP3 staining sets. Suppressive capacity of induced Tregs was evaluated by their capability to inhibit anti-CD3 Ab-triggered proliferation of peripheral blood mononuclear cells (PBMCs), as measured by flow cytometry. The concentration of TGF-β1 in culture supernatants was measured by enzyme-linked immunosorbent assay.

**Results.** Although both rapamycin and cyclosporine A suppressed the induction of CD4⁺CD25⁺ Tregs during MLRs, this effect was significantly more pronounced in cells cultured with cyclosporine. On the other hand, only rapamycin significantly decreased the percentage of CD4⁺CD25⁺ Tregs which expressed GITR, a negative regulator of Treg's suppressive capacity. Importantly, Treg-Rapa, unlike Treg-CsA, displayed significant suppressive activity and were capable of inhibiting the proliferation of anti-CD3 Ab-activated PBMCs. This activity was likely mediated by TGF-β1.

**Conclusions.** Rapamycin, unlike cyclosporine A, does not inhibit the function of CD4⁺CD25⁺ Tregs. This implies that rapamycin could contribute to the development of transplantation tolerance by promoting the induction of functional CD4⁺CD25⁺ Tregs. Moreover, our results suggest that rapamycin could be combined with functional Tregs.

**Keywords:** cyclosporine A; rapamycin; suppressive functions; TGF-β1; Treg cells

**Introduction**

CD4⁺CD25⁺ regulatory T cells (Tregs) are a T-cell subset endowed with the capacity to suppress immune responses to both self and foreign antigens. Although a specific Treg marker has not been identified as yet, Tregs do display a typical 'pattern' of a constitutively increased expression of several molecules/receptors including CD25, CTLA-4, CD122, GITR and FOXP3 [1].

A growing number of experimental and clinical studies show that Tregs can induce transplantation tolerance by suppressing immune responses to allograft antigens [2]. In a murine transplantation model, indefinite vascularized allograft survival was associated with the expansion of recipient CD4⁺CD25⁺ FOXP3⁺ Tregs [3,4], and the adoptive transfer of de novo generated donor alloantigen-specific Tregs resulted in specific suppression of donor skin allograft rejection [5]. In line with these findings are results of first clinical studies performed largely on renal allograft recipients, which confirm that Tregs can suppress immune responses to allograft antigens [6]. Moreover, there is emerging evidence that allograft function may correlate with the level of Tregs [7]. There is a real need to identify a specific marker of Tregs, which would allow the monitoring of this cell population in the human organism.

In view of their natural tolerogenic properties, Tregs are currently considered a potential means of inducing donor-specific tolerance in clinical transplantation [8]. However, in the context of a potential therapeutic use of Tregs, it needs to be considered that both their induction and suppressive functions can be considerably affected by immunosuppressive drugs, including cyclosporin A (CsA), which is a calcineurin inhibitor, and rapamycin (RAPA), which is an mTOR inhibitor [9]. While the majority of previous studies focused largely on the effects of CsA and RAPA on the suppressive capacity of isolated nTregs, we asked how these drugs affect Tregs of extrathymic origin. In particular, we evaluated how immunosuppressive drugs influence the alloantigen-triggered generation of human Tregs in vitro. Moreover, we determined the effects of both drugs on the suppressive activity of Tregs induced in the presence of either drug.

**Methods**

**Isolation of cells**

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of healthy donors by density-gradient centrifugation over Gradiol L (Aqua Medica, Łódź, Poland).

**Primary two-way mixed lymphocyte reaction**

PBMCs were resuspended at a concentration of 1×10⁶ cells/ml in complete medium: RPMI-1640 (Biomed Lublin, Poland) containing 10% heat-inactivated fetal calf serum (FCS) (Gibco), 20mM l-glutamine (Gibco), 10μg/ml gentamycine (Biochemie) and 1μM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) (Gibco). The cells of two individuals were mixed at a ratio of 1:1 for the two-way mixed lymphocyte reaction (MLR). The mixed cells were cultured in 24-well plates at 37°C for 6 days in 5% CO₂ with drugs: 20ng/ml rapamycine (Wyeth–Lederle, Novartis Pharma GmbH) or 200ng/ml cyclosporine A (Novartis) and without them.

**Identification of Tregs by flow cytometry**

To identify Tregs, allostimulated PBMCs from primary MLRs were stained with the following mAbs: anti-CD4 (PE or PerCP), anti-CD25 fluorescein isothiocyanate (FITC) or allophycocyanin (APC), anti-CTLA4 (PE), anti-CD122 (PE) and anti-GITR (APC) (all from Becton Dickinson). Isotype controls were cells stained with IgG1 conjugated with the respective fluorochromes. Intracellular staining of FOXP3 was performed using the anti PE-FOX3 staining set (eBioscience) according to the manufacturer's instructions. Flow cytometry was carried out on a FACS Calibur using Cell Quest software.

**Isolation of Tregs from primary MLR cultures**

CD4⁺CD25⁺ Tregs induced in primary MLRs were isolated from cells harvested from cultures on day 6 using a Dynabeads Regulatory CD4⁺CD25⁺ T Cell Kit (Dynal) according to the manufacturer's instructions. This kit allowed for isolation of two separate subsets of T cells: CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ T cells. The purity of the isolated Tregs was evaluated by flow cytometry analysis using anti-CD4-PerCP and anti-CD25-APC Abs.

**Secondary MLR coculture**

CD4⁺CD25⁻ T cells induced during primary MLRs were sorted using a FACs Calibur sorter on day 6 of culture. Subsequently, secondary MLR cocultures were performed by culturing 10⁶ mitomycin-blocked stimulator PBMCs with 0.5×10⁵ responder PBMCs and 0.5×10⁵ sorted CD4⁺CD25blo T cells. The cells were cultured for 6 days in 96-well plates. On day 6, proliferation of cells was measured by ³H-thymidine incorporation assay.

**Cocultures of anti-CD3 Ab-stimulated PBMCs and CD4⁺CD25⁺ Tregs**

PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE) using a Cell Trace CFSE Cell Proliferation Kit (Invitrogen) according to the manufacturer's instructions. Subsequently, PBMCs were stimulated with anti-CD3 Ab (Becton Dickinson) at a concentration of 1μg/ml. To
Fig. 1. Identification of CD4+CD25+ Tregs induced during primary MLR. (A, C) Representative flow cytometric results of CD25 expression within the CD4+ T-cells population on day 6 of MLR. About 20% of CD4+ T cells have the CD25 low and 4% have the CD25 high phenotype. CD4+CD25high T cells showed a typical 'pattern' of the expressions of the markers FOXP3, CTLA-4 and CD122 associated with Treg and other than the activated CD4+ T cells (CD4+CD25low). The dashed line represents the isotype control (IgG1-PE). (B) Each day of culture, cells were stained with anti-CD4 PerCP and anti-CD25 APC Abs. The results show the percentage of CD4+CD25high in the CD4+ T-cell population. The experiment was repeated seven times.
evaluate the suppressive capacity of the CD4⁺CD25⁺ Tregs, cocultures were then set up in which 5 x 10⁴ anti-CD3 Ab-activated PBMCs were cultured with (i) 5 x 10⁴ of CD4⁺CD25⁺ Tregs induced in primary MLR in the presence of CsA, (ii) 5 x 10⁴ of CD4⁺CD25⁺ Tregs induced in primary MLR in the presence of RAPA and (iii) 5 x 10⁴ of CD4⁺CD25⁺ Tregs induced in primary MLR in the absence of both drugs. Cells were cultured for 72h in 96-well plates in culture medium containing RPMI-1640 (Biomed Lublin) supplemented with 10% heat-inactivated FCS (Gibco), 20mM L-glutamine (Gibco), 10μg/ml gentamycine (Biochemie) and 1M HEPES (Gibco) at 37 °C in 5% CO₂. Subsequently, cells were stained with anti-CD4 Ab, which enabled the narrowing of the proliferation analysis to CD4⁺ T cells. Controls included cells which were not stimulated with anti-CD3 Ab. As additional control, Tregs were isolated stained with CFSE and stimulated with anti-CD3 Ab.

Cytokine concentration measurement

Concentrations of TGF-β and IL-10 were measured in supernatants from the cocultures of anti-CD3 Ab-stimulated PBMCs with CD4⁺CD25⁺ Tregs using enzyme-linked immunosorbent assay kits (BD Pharmingen) according to the manufacturer’s instructions.

Statistical analysis

Data are presented as median and median absolute deviation (MAD). Statistical analyses were performed by the Kruskal–Wallis test and the Wilcoxon test. Differences were considered significant at P<0.05.

Results

Induction of functional CD4⁺CD25⁺ Tregs during two-way primary MLR

As a model of alloantigen-triggered CD4⁺CD25⁺ Treg induction, we employed two-way MLR. Aside from activated CD4⁺CD25³ T cells, a separate population of CD4⁺CD25⁺ T cells could be identified in cultures. To this end, 6-day cultures were set up in which Tregs were identified based on their higher expressions of the surface markers CD25 (CD4⁺CD25⁺), CD122 and CTLA-4 and of the intracellular marker FOXP3 (Figure 1A, C). The percentage of CD4⁺CD25⁺ T cells rose gradually on consecutive days of cultures, reaching its maximum level on days 5 and 6 (Figure 1B). To confirm the suppressive capacity of these cells, secondary MLR cocultures were performed in which CD4⁺CD25⁺ T cells isolated from the primary MLR on day 6 inhibited alloantigen-triggered PBMC proliferation (Figure 2). Thus, two-way MLR enabled the generation of functional CD4⁺CD25⁺ Tregs.

The effects of CsA and RAPA on the induction of CD4⁺CD25⁺ Tregs during PBMC allostimulation

To evaluate whether CsA or RAPA suppresses the induction of CD4⁺CD25⁺ Tregs following PBMC allostimulation, we performed MLR assays in which these drugs were added to culture medium in separate cultures (final concentrations of 200 and 20ng/ml for CsA and RAPA, respectively). On day 6 of MLR, we analysed the percentages of CD4⁺CD25⁺ T cells expressing FOXP3, CTLA-4, CD122 and GITR. We found that both CsA and RAPA significantly decreased the percentage of CD4⁺CD25⁺ T cells compared with the control cultures, to which neither drug was added (Figure 3A). The reduction of the percentage of CD4⁺CD25⁺ T cells was significantly larger when they were generated in the presence of CsA than in the presence of RAPA. Also, only CsA significantly reduced the percentage of the CD4⁺CD25⁺FOXP3⁺, CD4⁺CD25⁺CTLA-4⁺ and CD4⁺CD25⁺CD122⁺ compared with the control cultures and RAPA (Figure 3B-D). In the presence of RAPA, the decrease of the percentage of Treg was negligible or insignificant. Thus, the presence of a calcineurin inhibitor resulted in a larger decrease in the percentages of all the analysed subsets of CD4⁺CD25⁺ Tregs than the presence of an mTOR inhibitor.

Suppressive capacity of CD4⁺CD25⁺ Tregs induced during PBMC allostimulation in the presence of CsA or RAPA

The suppressive capacity of CD4⁺CD25⁺ Tregs generated during two-way MLR in the presence of CsA or RAPA was measured by their capability to inhibit T cell receptor (TCR)-mediated proliferation of PBMCs. To that end, CD4⁺CD25⁺ Tregs isolated from MLR on day 6 were mixed at a ratio of 1:1 with PBMCs. PBMC proliferation was triggered by anti-CD3 Ab. Using CFSE allowed the analysis of the intensity of the PBMC proliferative response and their percentage in cell culture after consecutive cell divisions. Staining with anti-CD4 Ab enabled us to restrict measurements of cell proliferation to the CD4⁺ T-cell subset. The proliferation of the anti-CD3 Ab-activated CD4⁺ T cells was intensive, while CD4⁺CD25⁺ Tregs did not proliferate. Only Tregs generated in the presence of RAPA (Treg-Rapa) inhibited CD4⁺ T-cell proliferation, whereas those induced in the presence of CsA (Treg-CsA) or without drugs (Treg-control) failed to inhibit TCR-mediated CD4⁺ T-cell proliferation (Figure 5). In

Fig. 2. Suppressing PBMC proliferation by Tregs induced during primary MLR. Secondary MLR cocultures were set up in which mitomycin C-blocked stimulator PBMCs isolated from the blood of one donor were mixed with: (A) CD4⁺CD25⁺ T cells sorted on day 6 of primary MLR, (B) responder PBMCs from the second donor and (C) responder PBMCs from the second donor mixed with CD4⁺CD25⁺ T cells (1:1). Either CD4⁺CD25⁺ or mitomycin C-blocked cells cultured separately did not proliferate. P<0.03 compared with B. The experiment was repeated four times.
fact, the intensity of the proliferation of CD4⁺ T cells cultured with Treg-CsA was comparable to that of CD4⁺ T cells cultured in the absence of Tregs. Moreover, we measured the concentrations of TGF-β in cocultures of CD4⁺CD25⁺ Tregs and anti-CD3 Ab-activated PBMCs. The concentrations of TGF-β observed in cultures to which Tregs were added compared with the control cultures containing no Tregs (Figure 6). In particular, the concentration of TGF-β in cocultures of activated PBMCs and Treg-Rapa was significantly higher than that in cocultures of PBMCs and Tregs induced in the presence of CsA (Treg-CsA). The concentration of TGF-β observed when PBMCs were cultured with Treg-CsA was comparable to that measured after culturing PBMCs with Treg-control, whereas the concentration of this cytokine was lowest after culturing PBMCs with Treg-CsA (data not shown).
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After 72h of coculture, the concentration of TGF-β1 was measured in the supernatants. The experiment was repeated four times. *P<0.04 compared with Treg-CsA.

**Discussion**

Severe side effects of immunosuppressive drugs resulting in graft failure have created a need to develop non-toxic, antigen-specific therapies promoting the induction of transplantation tolerance. A growing body of data shows that therapeutic protocols involving CD4^+CD25^+ Tregs may allow the reduction of doses of immunosuppressors and lead to the development of tolerance.

In this study, we evaluated the potential effects of two widely used immunosuppressive drugs, CsA and RAPA, on the induction and the suppressive functions of CD4^+CD25^+ Tregs in vitro. We found that cells displaying the phenotype and the suppressive activity of Tregs are indeed generated following the in vitro allostimulation of PBMCs. In our experiments, CD4^+CD25^+ T cells induced during MLR had higher expressions of FOXP3, CTLA-4 and CD122 compared with activated T cells (CD4^+CD25^-), consistent with results obtained in other studies [10,11]. CD4^+CD25^+ T cells sorted after 6-day MLR cultures inhibited the proliferation of T cells in secondary MLR cocultures, which confirmed their suppressive capacity.

We showed that RAPA and CsA, which inhibit the activation of especially allospecific CD4^+ T cells, can also influence both the induction and the suppressive capacity of CD4^+CD25^+ Tregs generated during PBMC allostimulation. Although inhibiting the activities of both calcineurin (by CsA) and mTOR kinase (by RAPA) suppressed the generation of CD4^+CD25^+ Tregs, this effect was more potent in cells cultured in the presence of CsA. Moreover, the expressions of FOXP3 and CTLA-4 (both of which are essential for the induction and the suppressive function of Tregs [11]) in CD4^+CD25^+ Tregs induced in medium containing CsA were reduced to a greater degree.

RAPA, by inhibiting mTOR kinase, arrests the cell cycle in the G1 phase without inhibiting the production of IL-2 (a cytokine playing an important role in the generation and the activity of Tregs [12]) and the expression of IL2R. Although most studies performed both in vitro and in vivo showed that RAPA suppresses T effector cell proliferation while inducing Treg proliferation and preserving their suppressive capacity [3,4,13], our results are consistent with the findings of Valmori et al. who found that RAPA suppresses the proliferation of both CD4^+ non-Treg T cells and CD4^+CD25^+ Tregs. This effect was observed after a single in vitro stimulation and persisted after consecutive stimulations [14]. Moreover, Kopf et al. reported that while RAPA promoted TGF-β1-mediated Treg generation, the total number of generated Tregs might be decreased by RAPA in the concentration range of 1–100ng/ml [15]. There are also some differences of opinion as to whether RAPA stimulates the proliferation of Tregs or rather induces them de novo. Long et al. showed that addition of RAPA to cultures resulted in an increase in the percentage of CD4^+CD25^+FOXP3^+ T cells which did not result from selective FOXP3^+ T-cell proliferation, but rather from their induction in the G0 phase following inadequate activation [12]. Bruno et al. found that PI3K, Akt and mTOR kinases regulate de novo expression of FOXP3 in human CD4^+ T cells when the TCR-mediated signal is inadequate or when the PI3K/Akt/mTOR pathway is inhibited [16].

On the other hand, the inhibition of the induction and the suppressive activity of CD4^+CD25^+ Tregs by CsA may result from several different factors. First, CsA, by inhibiting calcineurin activity, blocks the induction of active transcription factor Nuclear factor of activated T-cells (NFAT). This leads to a decrease in IL-2 and IL-2R gene expression. As shown in earlier studies, the lack of IL-2 or its functional receptor blocked the generation of Tregs both in the thymus and in the periphery. Moreover, the inhibition of calcineurin restricts the access of nuclear factor for FOXP3. Recent findings show that FOXP3 controls the induction and the suppressive capacity of Tregs through the NFAT pathway. Furthermore, the active calcineurin pathway may be involved in transmitting TGF-β1 positive signal for the development and the activity of Tregs [17]. CsA inhibits the in vitro transcription of FOXP3 gene, while RAPA does not substantially affect it. Moreover, CsA decreases the expression of FOXP3 in natural Tregs [9]. Kopf et al. reported that CsA potently suppressed, while
RAPA promoted, TGF-β-mediated generation of murine Tregs [15].

In the present study, we also showed that both drugs affect the suppressive capacity of in vitro-generated Tregs. We found that the percentage of FOXP3+ Tregs expressing GITR was lower in cultures in which Tregs were induced in the presence of RAPA (Treg-Rapa) than in those to which CsA was added. In view of the fact that GITR is a negative regulator of Treg activity [18,19], Treg-Rapa should display a more potent suppressive activity than Treg-CsA. Indeed, Treg-Rapa significantly inhibited anti-CD3 Ab-triggered CD4+ T-cell proliferation, in contrast to Treg-CsA which did not exert this effect. This effect was likely cytokine dependent. TGF-β1 is essential for the induction of Tregs. It is also involved in Treg suppressive activity, especially during the induction of graft tolerance [8]. In our experiments, the concentration of TGF-β1 was higher in all cultures in which Tregs were present than in control cultures, to which no Tregs were added. Treg-Rapa likely produced more TGF-β1 than control Tregs and Treg-CsA. The concentration of TGF-β1 in cultures with Treg-CsA was comparable to that in control cultures. These results suggest that Treg-RAPA retains, and even increases, its suppressive capacity. This was also reported by other investigators who showed a correlation between active suppression and TGF-β1 concentration in the peripheral blood of renal allograft recipients [20].

It appears that RAPA and TGF-β1 can promote the induction of Tregs by independent mechanisms. Bruno et al. showed that FOXP3 expression and Treg generation can be induced by TGF-β1 or independently of this cytokine [16]. Gao et al. found that RAPA acts synergistically with TGF-β1 in inducing murine Tregs in vitro. However, the activity of RAPA is independent of TGF-β1 and cannot be replaced by higher concentrations of TGF-β1. These authors also showed that administration of RAPA to mice promoted the de novo generation of antigen-specific FOXP3+ Tregs [5]. It was also found that the TGF-β1 signal and calcineurin activity are required for Treg generation [21,22]. The role of TGF-β1 in Treg’s suppressive activity has not been determined as yet. It is known, however, that neutralization of TGF-β1 does not adversely affect CD4+ CD25+ Treg activity [23].

Previous studies focused on the effects of RAPA and CsA on generation of Tregs and the suppressive capacity of natural Tregs. We showed that Tregs generated in the presence of immunosuppressive drugs display different levels of suppressive activity. These experimental settings mimic those occurring in patients who receive immunosuppressive drugs. Treg-Rapa inhibited anti-CD3 Ab-triggered CD4+ T-cell proliferation, while Treg-CsA did not exert this effect. This activity is likely mediated by TGF-β1.

Our results suggest beneficial effects of combining RAPA with Tregs. Such therapeutic protocol may promote the generation of functional Tregs, thereby facilitating the induction of transplantation tolerance.

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Conflict of interest statement. None declared.

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The CD40–CD154 co-stimulation pathway mediates innate immune injury in adriamycin nephrosis

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Abstract

Background. Blockade of CD40–CD40 ligand (CD154) interactions protects against renal injury in adriamycin nephropathy (AN) in immunocompetent mice. To investigate whether this protection relied on adaptive or cognate immunity, we tested the effect of CD40–CD154 blockade in severe combined immunodeficient (SCID) mice.

Methods. SCID mice were divided into three groups: normal, AN + hamster IgG (ADR+IgG group) and AN + anti-CD154 antibody (MR1) (ADR+MR1 group). AN was induced by tail vein injection of 5.2 mg/kg of adriamycin (ADR). Hamster IgG (control Ab) or MR1 was administered intraperitoneally on days 5, 7, 9 and 11 after ADR injection. Histological and functional data were collected 4 weeks after ADR injection. In vitro experiments tested the effect of soluble and cell-bound CD154 co-cultured with CD40-expressing cells [macrophages, mesangial cells and renal tubular epithelial cells (RTEC)].

Results. All experimental animals developed nephropathy. Compared to the ADR+IgG group, ADR+MR1 animals had significantly less histological injury (glomerulosclerosis and tubular atrophy) and functional injury (creatinine clearance). Kidneys of ADR+MR1 animals had significantly less macrophage infiltration than those of ADR+IgG animals. Interestingly, expression of CD40 and CD41 (a platelet-specific marker) was significantly less in ADR+MR1 animals compared to ADR+IgG animals. In vitro, CD154 blockade significantly attenuated upregulation of CCL2 gene expression by RTEC stimulated by activated macrophage-conditioned medium. In contrast, platelet-induced upregulation of macrophage and mesangial cell proinflammatory cytokine gene expression were not CD154-dependent.

Conclusion. CD40–CD154 blockade has a significant innate renoprotective effect in ADR nephrosis. This is potentially due to inhibition of macrophage-derived soluble CD154.

Keywords: co-stimulation; focal sclerosing glomerulonephritis; macrophages; renal disease; tubulointerstitial injury

Introduction

The CD40–CD154 co-stimulatory pathway is an important regulator of adaptive immunity. Blockade of CD40–CD154 has been shown to significantly ameliorate organ injury in experimental models of islet xenotransplantation [1], allogeneic transplantation (liver [2], islet [3], heart [4] and kidney [5]), thyroiditis [6] and uveoretinitis [7]. In each of these situations, the protective mechanisms were shown or assumed to be lymphocyte-dependent.

CD154 (CD40 ligand) is primarily expressed on activated CD4+ T lymphocytes [8] but is also found in a soluble form [9]. Its expression has subsequently been found on a wide variety of cells, including platelets [10], mast cells and basophils [11], macrophages [12], NK cells, B lymphocytes, as well as non-haematopoietic cells (smooth muscle cells, endothelial cells and epithelial cells) [13]. Basophils, macrophages, smooth muscle cells, endothelial cells and...