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Mesenchymal stem cells promote the sustained expression of CD69 on activated T lymphocytes: roles of canonical and non-canonical NF-κB signalling

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Abstract

Mesenchymal stem cells (MSCs) are known to induce the conversion of activated T cells into regulatory T cells *in vitro*. The marker CD69 is a target of canonical nuclear factor kappa-B (NF-κB) signalling and is transiently expressed upon activation; however, stable CD69 expression defines cells with immunoregulatory properties. Given its enormous therapeutic potential, we explored the molecular mechanisms underlying the induction of regulatory cells by MSCs. Peripheral blood $CD3^+$ T cells were activated and cultured in the presence or absence of MSCs. $CD4^+$ cell mRNA expression was then characterized by microarray analysis. The drug BAY11-7082 (BAY) and a siRNA against v-rel reticuloendotheliosis viral oncogene homolog B (RELB) were used to explore the differential roles of canonical and non-canonical NF-κB signalling, respectively. Flow cytometry and real-time PCR were used for analyses. Genes with immunoregulatory functions, CD69 and non-canonical NF-κB subunits (RELB and NFKB2) were all expressed at higher levels in lymphocytes co-cultured with MSCs. The frequency of $CD69^+$ cells among lymphocytes cultured alone progressively decreased after activation. In contrast, the frequency of $CD69^+$ cells increased significantly following activation in lymphocytes co-cultured with MSCs. Inhibition of canonical NF-κB signalling by BAY immediately following activation blocked the induction of CD69; however, inhibition of canonical NF-κB signalling on the third day further induced the expression of CD69. Furthermore, late expression of CD69 was inhibited by RELB siRNA. These results indicate that the canonical NF-κB pathway controls the early expression of CD69 after activation; however, in an immunoregulatory context, late and sustained CD69 expression is promoted by the non-canonical pathway and is inhibited by canonical NF-κB signalling.

Keywords: CD69 • immunoregulation • mesenchymal stem cells • NF-κB • RELB • T lymphocytes

Introduction

Bone marrow stromal cells capable of transferring the microenvironment of hemopoietic tissues were first described by Friedenstein et al. [1]. These cells were later renamed MSCs [2] and were broadly defined as spindle-like plastic-adherent cells endowed with the potential to differentiate *in vitro* toward osteogenic, chondrogenic and adipogenic lineages [3]. Despite the controversies generated by this broad definition and the limitations of the *in vitro* assays routinely used to assess the differentiation potential of MSCs thoroughly discussed by Bianco [4], these cells have attracted the attention of many researchers over the years, in part because of their ability to support haematopoiesis.

Our research group was the first to comprehensively characterize the gene expression profile of human bone marrow stromal stem cells and to compare it to the profile of haematopoietic stem cells, thereby contributing to the scientific community by providing a large amount of valuable information [5]. We later isolated cells with similar functional and phenotypic characteristics from the sub-endothelial layer of umbilical cord veins [6]. Further characterization revealed that the cells obtained from both sources were very similar at the transcriptional level, although small

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differences indicated specific features related to their anatomic location [7]. We later demonstrated that these expandable spindle-like plastic-adherent cells could be obtained from virtually all adult and foetal human tissues [8]; this finding has also been demonstrated in mice [9]. After extensive characterization, we demonstrated that MSCs were related to diverse known cell types, closely resembling pericytes and hepatic stellate cells and, to a lesser extent, their differentiated 'more-restricted' counterparts (smooth muscle cells and stellate myofibroblasts, respectively), as well as fibroblasts [8].

Reports of the distribution of these cells in the vascular wall throughout the entire organism began to make the *in vivo* physiological role of these cells clearer. The wide distribution of MSCs suggests that these cells may function as a cell repository for tissue repair and could potentially contribute to tissue and immune system homeostasis [8, 10, 11].

In support of this hypothesis, MSCs were shown to possess many immunomodulatory properties, including the ability to suppress the proliferation of T lymphocytes activated by diverse stimuli such as allogeneic cells, mitogens (such as phytohemagglutinin or concanavalin A) and antibodies (anti-CD2/CD3/CD28) mimicking T cell receptor (TCR) activation [12–16].

Upon T cell activation, the immune response is orchestrated by different signalling pathways, including the canonical NF-κB pathway, which plays a central role in regulating the production of inflammatory cytokines and other important molecules [17]. Among the proteins induced by NF-κB, CD69 [18] and CD25 are expressed at the cell surface and are considered classical markers of activated effector T lymphocytes [19–21].

Interestingly, regulatory T cells (Tregs), which act by suppressing the immune response carried out by effector T cells, are also characterized by the expression of CD25 or CD69 [22–27].

Besides suppressing T cell proliferation, MSCs are also known for their ability to induce classical $CD4^+CD25^{hi}FOXP3^+$ Tregs [12, 27–30]. Interestingly, the induction of immunoregulatory cells by MSCs parallels that of tumour stromal cells in a way that is not surprising because many immunomodulatory factors (such as IDO, PGE2 and TGF-β) are similarly secreted by the tumour stromal microenvironment [31, 32] and by MSCs [12, 33, 34]. Although MSCs present in the tumour niche may provide an immune escape mechanism influencing cancer growth and spread [35], MSCs located in the wall of the vasculature throughout the body [8] could contribute to the peripheral homeostasis of the immune system [36]. In fact, mechanisms controlling the induction of tolerance in the periphery are directly implicated in diverse autoimmune diseases and in the immune responses against pathogens, tumours and allografts [24, 37].

In light of their immunological properties, their potential therapeutic uses and the implications of these uses in diverse pathological situations, further dissection of the mechanisms by which MSCs modulate signalling pathways in activated T lymphocytes is of great interest [38].

In this work, we explored MSC-induced changes in the transcriptional profile of activated T lymphocytes using whole-genome microarrays. Our results show that several pathways related to T cell activation and proliferation and the induction of a regulatory phenotype are modulated in lymphocytes co-cultured with MSCs. Moreover, we show evidence that in activated T cells co-cultured with MSCs, canonical NF-κB signalling is inhibited and is replaced with non-canonical signalling. Furthermore, we demonstrate that this change in NF-κB signalling correlates with the acquisition of a regulatory phenotype that includes the sustained expression of the surface marker CD69 and increased transcript levels of Treg-related genes.

Materials and methods

All samples were obtained after informed consent had been obtained from the patients. The study was approved by the institutional ethics committee.

Isolation and characterization of MSCs

MSCs were isolated from bone marrow aspirates by plastic adherence as previously described [5]. Cells were cultured in α-MEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 15% foetal calf serum (Hy Clone, Logan, UT, USA), 2 mmol/l **l**-glutamine and 100 U penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

Plastic-adherent multipotent mesenchymal stromal cells were characterized after the fourth passage, as previously described [5], based on their potential to differentiate into adipocytes (Sudan II and Scarlet stains for lipid accumulation), osteocytes (von Kossa stain for calcium deposition) and chondrocytes (immunostaining with anti-type II collagen) and on their immunophenotype as determined using the following monoclonal antibodies: CD33-FITC, CD45-FITC, CD31-FITC, HLA-DR-FITC, Cadherin-5-FITC, Glycophorin-FITC, CD73-PE, CD146-PE, CD90-PE, CD29-PE, CD44-PE, CD13-PE, CD49e-PE, HLA-ABC-PE, CD34-PE, CD14-PE, CD54-PE, CD166 and AC133-PE (Pharmigen, BD Biosciences, Franklin Lakes, NJ, USA). An Axioskop 2.0 microscope equipped with an Axiocam camera (Zeiss, Germany) and a FACSort flow cytometer (BD Biosciences) were used in the analyses.

Isolation of peripheral blood mononuclear cells (PBMCs) and immunomagnetic selection of T lymphocytes

PBMCs were isolated from the blood of healthy volunteers by centrifugation using Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) and washed three times with PBS. Isolated PBMCs were used directly in co-culture experiments or were used for the immunomagnetic selection of $CD3^+$ lymphocytes using the Pan T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. In brief, all blood cells (except $CD3^+$ T cells) were depleted from PBMCs using a mixture of primary antibodies and magnetic microbead-conjugated secondary antibodies using magnetic columns and a VarioMACS magnet (Miltenyi Biotec).

For microarray or real-time PCR gene expression analyses, $CD4^+$ and $CD8^+$ T cells were positively selected using anti-CD4- or anti-CD8-linked magnetic beads (Miltenyi Biotec) on the fifth day of culture. The purities of

the selected cell populations used in this work were all above 95% as determined by flow cytometry.

T cell activation

Anti-biotin magnetic beads pre-loaded with biotinylated antibodies against human CD2, CD3 and CD28 (T Cell Activation/Expansion Kit; Miltenyi Biotec) were used (1–2 beads/cell) to mimic antigen-presenting cells and to activate resting T cells isolated from PBMCs or purified $CD3^+$ T cells. Following activation, cells were cultured in RPMI (Invitrogen Life Technologies, Carlsbad, CA, USA), and on the third day, 20 U/ml of human recombinant IL-2 (Peprotech Inc, Rocky Hill, NJ, USA) was added to the culture. Cultures were maintained for two additional days before final evaluation.

Proliferation assay

Following activation, 5×10^5 PBMCs or $CD3^+$ T cells (per ml) were cultured in 12-well plates in the presence or absence of 1×10^5 previously adhered MSCs. Following a period of 5 days, incorporation of bromodeoxyuridine into newly synthesized DNA over the period of an additional hour was measured by flow cytometry using an APC-anti-BrdU antibody according to the manufacturer's instructions (BrdU Flow Kit, BD Biosciences).

Microarray gene expression profiling

Microarray profiling was performed using cells from three distinct individuals. Briefly, purified $CD3^+$ T cells were activated and cultured (RPMI, 10% FCS) in three 100-mm plates (7.5×10^6 cells/plate): two containing 1.5×10^6 MSCs (adhered 24 hrs earlier) and one without MSCs. Following a 5-day incubation, $CD4^+$ T cells were immunomagnetically purified, and total RNA was extracted using TRIZOL-LS (Invitrogen). After DNase treatment and purification using an RNeasy kit (Qiagen, Valencia, CA, USA), RNA quality was assessed by agarose gel electrophoresis. RNA quantitation was performed with a NanoDrop 1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

Expression profiling was performed as previously described [39]. Briefly, a one-colour Quick Amp Labeling Kit (Agilent, 5190-0442) was used to generate Cy3-labelled cRNA, which was then fragmented and hybridized to Whole Human Genome Oligo microarrays (G4112F; Agilent, Palo Alto, CA, USA) containing 41,000 distinct probes.

Microarray slides were scanned at 535 nm with 5 μm/pixel resolution using a GenePix 4000B scanner and GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA, USA). Images were analysed with the Agilent Feature Extraction software (version 9.5.3.1), and data were normalized to the 75th percentile expression value of the whole array.

Complete microarray data sets have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE31153 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31153).

Microarray data analysis

The Gene Expression Profile Analysis Suite-GEPAS (an online platform accessible at http://gepas.bioinfo.cipf.es/) was used to identify genes that were differentially expressed between activated $CD4^+$ lymphocytes cultured with MSCs and those cultured without MSCs. A low stringency *P* value was used ($P < 0.05$) to allow the selection of a larger set of transcripts that were further analysed using the Ingenuity Pathway Analysis-IPA (http://www.ingenuity.com/) tool to search for signalling pathways potentially modulated by MSCs.

Real-time quantitative PCR

Total RNA from $CD4^+$ lymphocytes was reverse transcribed using the High Capacity cDNA Archive Kit, and real-time PCR was performed using TaqMan probes and MasterMix (Applied BioSystems, Foster City, CA, USA) following the manufacturer's instructions. Probes used were: FOXP3 (Hs00203958_m1), IL-10 (Hs00174086_m1), CTLA4 (Hs00175480_m1), GITR/TNFRSF18 (Hs00188346_m1) IRAK3 (Hs00200502_m1), A20/TNFAIP3 (Hs00234713_m1), BTRC/βTrC (Hs00182707_m1), NFKB1 (Hs00765730_m1), NFKB2 (Hs00174517_m1), RELA (Hs00153294_m1) and RELB (Hs00232399_m1). Relative expression was calculated by the formula $2^{-\Delta\Delta Ct}$ [40], using GAPDH and β-actin to normalize sample loading; the median Ct values of the samples from lymphocytes cultured alone were used as a reference. Prism 4 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform all statistical analyses and to generate graphs. When appropriate, statistical significance was determined using a one-tailed non-parametric Mann–Whitney *U*-test, a paired *t*-test or one-tailed unpaired *t*-test.

Temporal evaluation of CD69 expression on $CD3^+$ lymphocytes

PBMCs from six individuals were activated with anti-CD2/CD3/CD28 beads, and 2.5×10^5 cells were cultured in 24-well plates in the presence or absence of 5×10^4 MSCs. Cells were examined on the first, third and fifth days after activation. The percentage of $CD69^+$ cells within the $CD3^+$ lymphocyte population was evaluated by flow cytometry. Cells were also characterized by size and complexity.

Expression of CD69 in lymphocyte sub-populations

PBMCs from three individuals were activated and cultured as described earlier. On the fifth day of culture, the expression of CD69 was evaluated by flow cytometry in the following subtypes of gated $CD3^+$ lymphocytes: total $CD4^+$ and $CD8^+$, $CD4^+CD25^{hi}$, $CD8^+CD25^{hi}$ and $CD8^+CD28^-$. The antibodies used included APC-conjugated anti-CD4, anti-CD8 and anti-CD28; FITC-conjugated anti-CD8 and anti-CD25; PerCP-conjugated anti-CD3; PE-conjugated anti-CD69 and corresponding isotype controls (Pharmingen).

Evaluation of RelB protein expression by intracellular flow cytometry

Immunomagnetically selected $CD3^+$ lymphocytes were activated and cultured in the presence or absence of MSCs as described. Intracellular expression of RELB was evaluated in gated $CD4^+$ T lymphocytes on the fifth day after activation by flow cytometry using a rabbit polyclonal anti-RELB primary antibody and an FITC-conjugated goat anti-rabbit secondary antibody (sc-226 and sc-3839, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Inhibition of canonical NF-κB signalling and CD69 expression

Immunomagnetically selected $CD3^+$ lymphocytes were activated and cultured in 24-well plates as described earlier. The NF-κB inhibitor BAY11-7082 (Calbiochem) was added at a final concentration of 10 μM immediately following activation or on the third day (together with IL-2). The percentage of $CD69^+$ cells within the $CD3^+$ lymphocyte population was evaluated by flow cytometry on the fifth day. Cells were also characterized by size and complexity.

RELB RNA interference and CD69 expression

Following immunomagnetic selection, 2.5×10^5 $CD3^+$ lymphocytes were resuspended in 75 μl siPORT™ siRNA electroporation buffer containing 50 pmol of RELB siRNA (ID: s11918; Ambion, Austin, TX, USA) or of negative control no. 1 siRNA (Cat. No. 4390843; Ambion) and electroporated in a 1-mm cuvette using a Gene Pulser Xcell Electroporation System (single 0.4 msec. pulse, 150 V; Bio-Rad). After electroporation, cells were left to rest for 4 hrs before being activated and cultured in the presence or absence of MSCs as described earlier. Transcript levels of RELB were evaluated using RNA extracted on the second day after activation, and the percentage of $CD69^+$ cells among total T lymphocytes ($CD4^+$ and $CD8^+$) was evaluated by flow cytometry on the fifth day.

Results

MSC characterization

Cultured MSC cells derived from bone marrow showed a homogeneous capacity for differentiation into adipocytes, chondrocytes and osteocytes. Furthermore, a typical MSC immunophenotype was observed in the cultured samples used in this work. MSCs were positive for CD73, CD90, CD29, CD13, CD44, CD49e and HLA-class I markers and were negative for markers of haematopoietic cells (CD34, CD14, CD45, glycophorin A), endothelial cells (CD31, KDR) and HLA-class II (data not shown). Thus, these cells displayed all distinguishing characteristics of MSCs [3].

Suppression of T lymphocyte proliferation by MSCs

MSCs inhibited the proliferation of activated T lymphocytes, as co-cultured T lymphocytes proliferated less than T lymphocytes cultured alone. In general, only half of the cells co-cultured with MSCs incorporated BrdU. Figure 1 is representative of the results observed. Although 42.4% of $CD3^+$ T lymphocytes cultured alone actively incorporated BrdU into their DNA, indicating their proliferation, only 22.1% of the lymphocytes co-cultured with MSCs did so. Similar results were obtained in the other experiments conducted in this study.

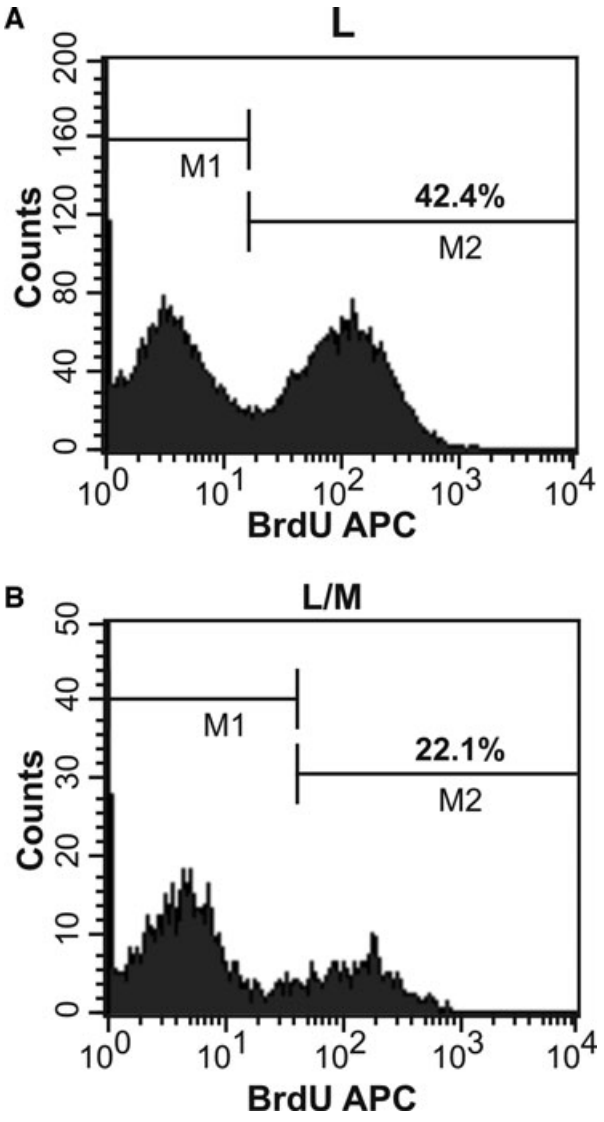

Fig. 1 Inhibition of lymphocyte proliferation by MSCs. Representative flow cytometry plots show BrdU incorporation by $CD3^+$ lymphocytes. Total PBMCs were activated by anti-CD2/CD3/CD28 beads and cultured alone (upper) or in the presence of MSCs (lower). Evaluation was performed on the fifth day following activation.

MSCs promote CD69 expression in $CD3^+$ lymphocytes

Upon engagement of the T cell receptor/CD3 complex, induction of CD69 peaks between 16 and 24 hrs and then gradually declines [41,42]; nevertheless, there are conflicting reports in the literature about the effects of MSCs on the expression of CD69 by activated lymphocytes [43–45]. Therefore, we evaluated the kinetics of CD69 expression in activated lymphocytes cultured in the

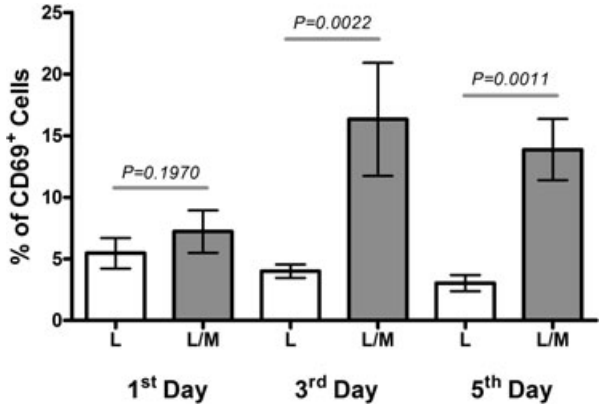

Fig. 2 Sustained increase of CD69. Flow cytometry evaluation of CD69 expression in lymphocytes cultured alone (L, white bars) or in the presence of MSCs (L/M, grey bars). Evaluations were performed on the first, third and fifth days following activation with anti-CD2/CD3/CD28 beads. Statistical significance was calculated using a one-tailed non-parametric Mann–Whitney *U*-test.

presence or absence of MSCs. As expected, expression of CD69 in activated lymphocytes cultured alone was highest on the first day and decreased subsequently. In contrast, although similar proportions of co-cultured and independently cultured T lymphocytes expressed CD69 on the first day, the percentage of lymphocytes co-cultured with MSCs expressing CD69 increased notably on subsequent days; this finding is consistent with CD69 being a marker of cells with regulatory potential [23, 25, 26] and with the induction of regulatory cells by MSCs (Refs. 12 and 27–30; Fig. 2).

Expression of CD69 is not restricted to any of the evaluated sub-populations

Because the expression of CD69 defines a population of cells with regulatory potential [23, 25, 26] and because MSCs are known to induce classical $CD4^+CD25^{hi}$ Tregs [12, 27–30], we wished to determine whether the increased percentage of $CD69^+$ cells among T lymphocytes co-cultured with MSCs was due solely to the increased generation of Tregs in co-cultures or whether this was due to a general increase in the expression of CD69. To address this question, we performed an additional experiment designed to evaluate the percentage of $CD69^+$ cells among total $CD4^+$ and $CD8^+$ cells as well as in different populations with immunophenotypes associated with regulatory potential, including $CD4^+CD25^{hi}$ Tregs, $CD8^+CD25^{hi}$ T cells [46] and $CD8^+CD28^-$ T cells [47]. Our results demonstrate that the proportion of cells expressing CD69 among all analysed populations was higher in co-cultured lymphocytes than in lymphocytes cultured alone (Fig. 3).

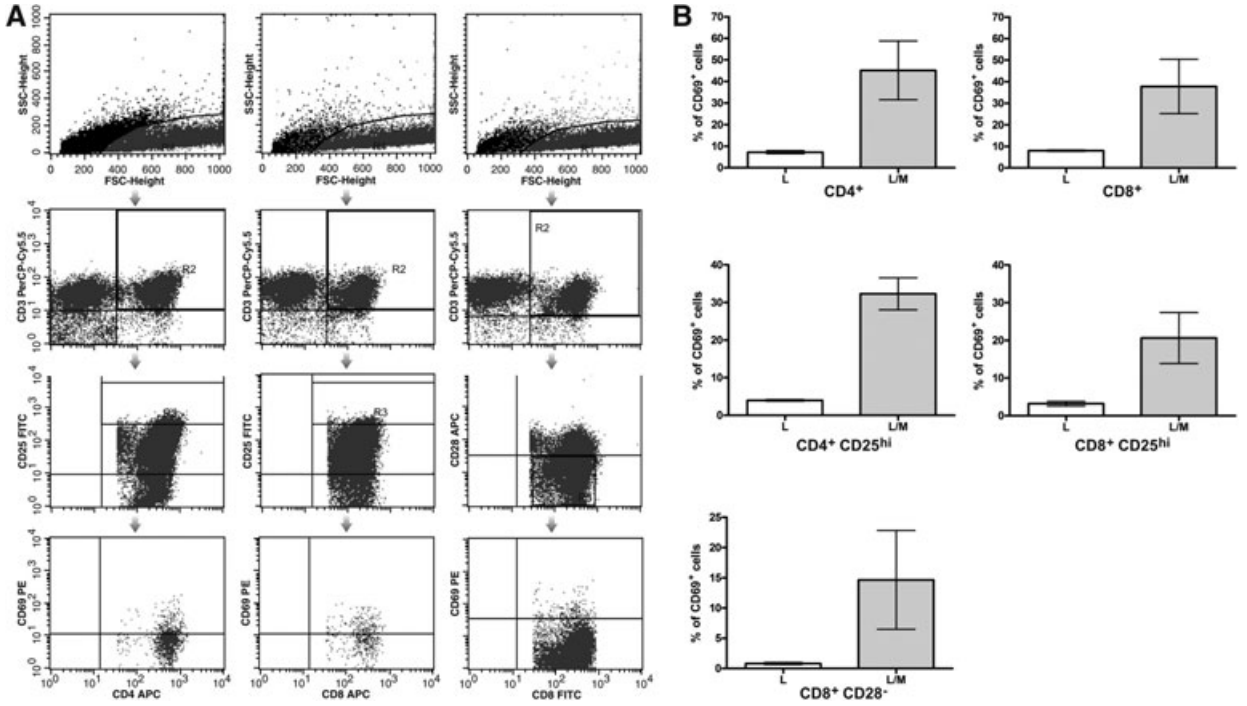

Fig. 3 Flow cytometry evaluation of CD69 in selected lymphocyte sub-populations. PBMCs were activated with anti-CD2/CD3/CD28 beads and cultured alone (L, white bars) or in the presence of MSCs (L/M, grey bars) and were then evaluated by flow cytometry on the fifth day after activation. (**A**) Gating strategy. Lymphocytes were characterized by size and complexity. $CD3^+$ lymphocytes were evaluated for the expression of CD69 among total $CD4^+$ and $CD8^+$ cells and among $CD4^+CD25^{hi}$, $CD8^+CD25^{hi}$ and $CD8^+CD28^-$ regulatory T cell subsets. (**B**) Results from the analysis. Data from three distinct samples were graphed and analysed using a one-tailed non-parametric Mann–Whitney *U*-test.

$CD4^+$ lymphocytes co-cultured with MSCs display transcriptional changes characteristic of regulatory cells

Based on our preliminary results, we established an experimental design to evaluate the transcriptional changes induced by MSCs in both $CD4^+$- and $CD8^+$-activated T cells. After activation and culture of $CD3^+$ T cells in the presence or absence of MSCs, $CD4^+$ and $CD8^+$ T cells were immunomagnetically separated for transcriptional analysis. To preliminarily determine whether MSCs were able, in our experimental setting, to induce changes known to be associated with a regulatory phenotype, transcriptional levels of FOXP3, CTLA-4 [48, 49], GITR [50] and IL-10 [27] were evaluated by real-time PCR in $CD4^+$ lymphocytes. All were found to be expressed at higher levels in lymphocytes cultured with MSCs than in those cultured alone.

Global transcriptional changes induced in $CD4^+$ lymphocytes by MSCs

From a total of 43,376 genes present on the microarray chip, 11,353 were considered to be differentially expressed; 4855 were expressed at higher levels, and 6498 were expressed at lower levels in $CD4^+$ T cells cultured with MSCs than in those cultured alone. Analysis of this set of genes using the IPA tool revealed the transcriptional modulation of components from many pathways.

Given that inhibition of T cell proliferation is one of the mechanisms by which MSCs modulate the inflammatory response [12–16], we examined the expression levels of transcripts of components involved in T cell activation and proliferation. The proliferative suppression observed in our experimental setting was clearly paralleled by a general transcriptional repression of components related to TCR signalling and to cell cycle progression. For instance, transcript levels of major components that mediate TCR signalling, such as CD3, LCK, Vav, ZAP70, LAT and GRB2 [51], and that control cell cycle progression through the G1 phase, such as Cyclins D1 and E and their corresponding kinases CDK4 and CDK2 [52], were all repressed in lymphocytes co-cultured with MSCs (Figs S1 and S2).

MSCs also induced the generation of classical $CD4^+CD25^{hi}$ $FOXP3^+$ Tregs [12, 27–30]. Because the inhibition of the PI3K/AKT and mTOR signalling pathways in activated T cells has been reported to play a role in the generation of lymphocytes with a Treg phenotype [53], we evaluated the transcript levels of central components of these pathways. Strikingly, our results indicate that these pathways are transcriptionally repressed in activated T cells co-cultured with MSCs; among others, the catalytic subunit of PI3K (PIK3CB, D and G), PDPK1, AKT1 and 2, and FKBP1A were all down-modulated (Fig. S3).

Although the canonical NF-κB pathway is centrally involved in the activation of T cells [17] and in the early transcriptional induction of the activation marker CD69 [18], its role in the late and sustained expression of CD69 in regulatory cells is unexplored. Interestingly, transcript levels of several NF-κB signalling components were higher in $CD4^+$ lymphocytes co-cultured with MSCs than in those cultured alone (Fig. S4).

MSCs induce the expression of non-canonical NF-κB components and canonical inhibitors in $CD4^+$ lymphocytes

There are two NF-κB signalling pathways that are associated with different roles in the immune system: the classic or canonical pathway (mediated mainly by heterodimers encoded by RELA and NFKB1) and the non-canonical or developmental pathway (mediated by heterodimers encoded by RELB and NFKB2); reviewed in [17, 54–57]. Real-time PCR revealed that transcript levels of NFKB2, RELB and RELA (but not NFKB1) were all significantly elevated in $CD4^+$ lymphocytes that were co-cultured with MSCs over those in $CD4^+$ lymphocytes cultured alone (Fig. 4).

Canonical RelA-p50 heterodimers are rendered inactive in the cytoplasm by inhibitory IκB proteins (such as IκBs and unprocessed p105/NFKB1 and p100/NFKB2) [58]. In sharp contrast, non-canonical RelB heterodimers are held inactive in the cytoplasm only by the unprocessed p100 subunit [59]. Other inhibitors of the canonical NF-κB pathway, such as TNFAIP3/A20 [60] and IRAK3/IRAKM [61], interfere with the downstream signalling receptors involved in the activation of this pathway [17, 56, 62–64]. As indicated by microarray analysis and validated by RT-PCR, the expression levels of IRAK3, A20 and the E3 ubiquitin ligase βTrCP were higher in lymphocytes co-cultured with MSCs (Fig. 4). Specific E3 ubiquitin ligases are involved in the selective ubiquitination and degradation (or processing) of different IκBs or of NF-κB subunits [65]. βTrCP is essential for the NIK-induced processing of p100 into p52 that leads to the activation of the non-canonical NF-κB pathway [66]; however, it also mediates the ubiquitination and consequent degradation of Bcl10, a component centrally involved in the activation of canonical NF-κB signalling, in response to TCR activation. Overall, these results suggest a negative-feedback loop that selectively inhibits the canonical pathway [67].

Increased RELB expression in $CD4^+$ lymphocytes co-cultured with MSCs

Increased expression of RELB was also demonstrated at the protein level. On the fifth day after activation, only 39.8% of the $CD4^+$ lymphocytes cultured alone expressed intracellular RELB, but over 77.7% of the $CD4^+$ lymphocytes co-cultured with MSCs did so (Fig. 5).

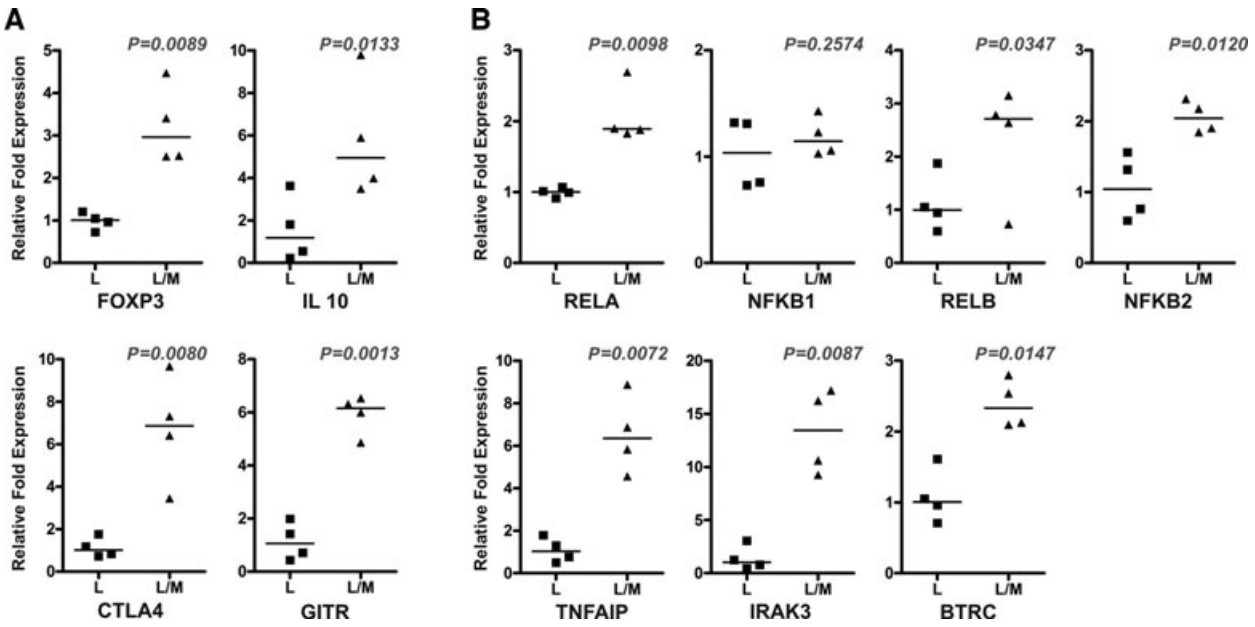

Fig. 4 Gene expression analysis of selected transcripts. PB $CD3^+$ T cells from four individuals were activated with anti-CD2/CD3/CD28 beads and cultured alone (L) or in the presence of MSCs (L/M). On the fifth day following activation, $CD4^+$ T cells were immunomagnetically purified and profiled by real-time PCR to evaluate gene expression in regulatory cells (**A**) and selected components of NF-κB signalling (**B**). The relative fold change values were calculated by the formula $2^{-\Delta\Delta Ct}$ using the median Ct value from the samples of lymphocytes cultured alone as a reference. Statistical significance was evaluated using a one-tailed paired *t*-test.

Inhibition of the canonical NF-κB pathway at distinct time points has opposite effects on CD69 expression

Based on the above results, we hypothesized that a shift from canonical to non-canonical signalling could be involved in the late sustained increase in CD69 expression promoted by MSCs, with non-canonical RelB dimers controlling late CD69 transcription. Moreover, because non-canonical RelB activity has been shown to be repressed by direct binding to RelA in the nucleus [68], the late increase in CD69 could result from the inhibition of canonical signalling, with reduced nuclear localization of RelA and the consequent de-repression of RelB. If true, early inhibition of canonical NF-κB would abrogate CD69 expression. In turn, its later inhibition after the exchange of canonical for non-canonical dimers would be expected to further induce CD69 expression.

To evaluate this hypothesis, we used BAY11-7082, an irreversible inhibitor of IκBα phosphorylation and of the consequent nuclear translocation of the canonical NF-κB complex [69]. In support of our hypothesis, inhibition of the canonical NF-κB pathway immediately following activation completely abrogated the expression of CD69 on $CD3^+$ lymphocytes, but inhibition of the canonical NF-κB pathway on the third day following activation did not; in fact, it had a completely opposite effect, further increasing the percentage of $CD3^+$ lymphocytes expressing the immunomodulatory CD69 molecule (Fig. 6A). This further increase in CD69 expression occurred in both co-cultured and independently cultured T lymphocytes, although a significantly greater proportion of co-cultured cells expressed CD69 (~20%) than did cells cultured alone (~10%).

RNA interference of RELB eliminates the late expression of CD69 on T lymphocytes co-cultured with MSCs

To directly evaluate the role of RelB in the late expression of CD69, we used RNA interference (Fig. 6B). Consistent with our previous results, T lymphocytes cultured alone did not express CD69 on the fifth day following activation, but T lymphocytes co-cultured with MSCs were positive for CD69 at this time point. T lymphocytes transfected with a non-specific control siRNA behaved similarly. In sharp contrast, and in agreement with our hypothesis, in T lymphocytes that were transfected with siRNA against RELB and that were co-cultured with MSCs, the expression of CD69 was almost completely inhibited.

Altogether, these results indicate that the canonical and non-canonical NF-κB signalling pathways may play distinct roles in the expression of CD69. Although the canonical NF-κB pathway could control the early expression of CD69 (as an activation marker), its late and sustained expression (as an immunoregulatory molecule) could

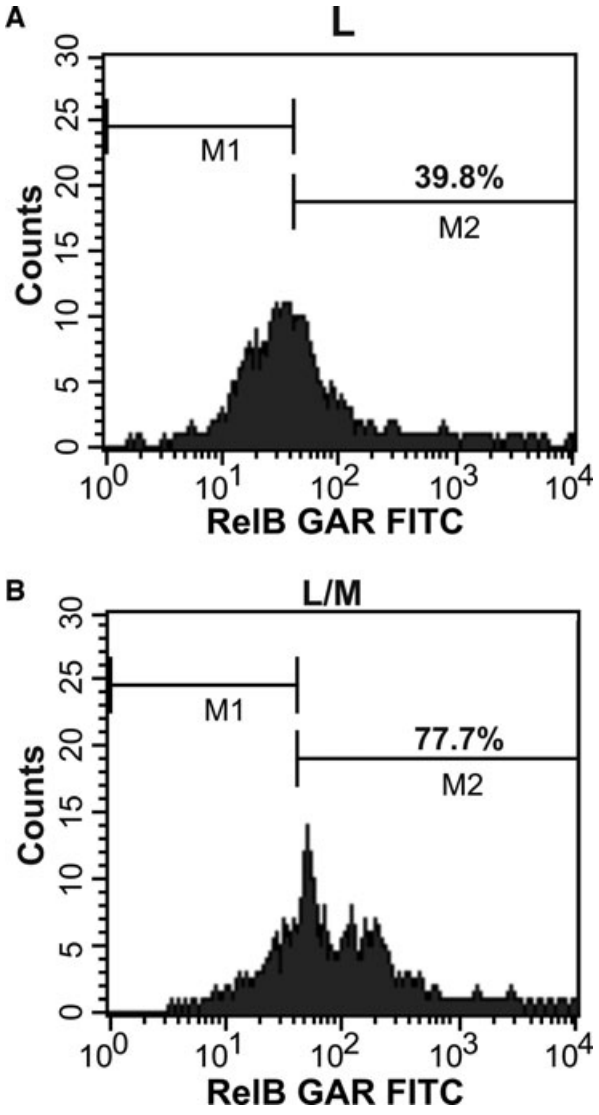

Fig. 5 Intracellular flow cytometry of RelB. Immunomagnetically selected $CD3^+$ lymphocytes were activated with anti-CD2/CD3/CD28 beads and cultured in the presence or absence of MSCs. Cells were collected on the fifth day following activation, and the percentage of cells staining for intracellular RelB was evaluated among total lymphocytes by flow cytometry.

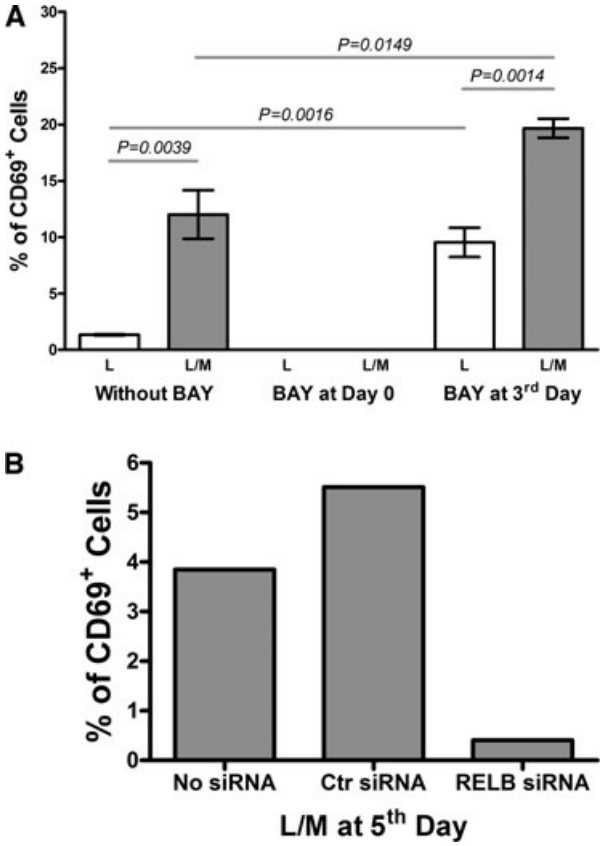

Fig. 6 Differential roles of canonical and non-canonical NF-κB signalling in the early and late expression of CD69. Immunomagnetically selected $CD3^+$ lymphocytes were activated with anti-CD2/CD3/CD28 beads and cultured in the presence (L/M) or absence (L) of MSCs. On the fifth day following activation, cells were collected, and the percentage of $CD69^+$ cells was evaluated by flow cytometry. (**A**) The inhibitor of canonical NF-κB signalling (BAY11-7082) was added to cultures immediately following activation or on the third day. Data derived from three experimental replicates (*P* values from one-tailed unpaired *t*-test). (**B**) Immunomagnetically selected $CD3^+$ lymphocytes were transfected with siRNA directed against the non-canonical NF-κB subunit RELB (RELB siRNA) or with non-specific control siRNA (Ctr siRNA) prior to activation and culture. Because the expression of CD69 was absent in lymphocytes cultured alone, only the results for lymphocytes co-cultured with MSCs (L/M) are shown (data derived from a single experiment).

be controlled by the non-canonical pathway, whereas at the same time being negatively regulated by canonical signalling [23, 25, 26].

Discussion

In this work, we explored the effects of MSCs on activated T lymphocytes. Although MSCs were already known for their ability to induce classical $CD4^+CD25^{hi}FOXP3^+$ Tregs [12, 27–30], this is the first report showing that MSCs promote the expression of CD69 in different T cell populations, including distinct regulatory subsets. These results are attractive in light of the known immunomodulatory role of CD69 [23, 25, 26] because its regulatory function is not necessarily associated with the expression of CD25 and because $CD4^+CD25^-CD69^+$ Tregs have recently been described in tumour-bearing mice [70].

Although others have previously reported the repression of TCR signalling and of cell cycle progression by MSCs [71, 72], our work provides a broader picture of the molecular mechanism by

which this occurs and suggests that a general transcriptional repression may account for the suppression observed. The down-modulation of TCR signalling is involved not only in the suppression of T cell proliferation but also in the induction of Tregs. Although continued TCR stimulation leads to a loss of Foxp3 inducibility, antagonizing TCR stimulation through the inhibition of PI3K/AKT and of mTOR signalling pathways induces transcriptional changes driving the generation of Tregs [53]. Strikingly, our results show that these pathways are transcriptionally repressed in activated T cells co-cultured with MSCs.

The canonical NF-κB pathway is centrally involved in the activation of T cells [17] and in the early transcriptional induction of the activation marker CD69 [18]; nevertheless, the mechanisms responsible for the sustained expression of CD69 in chronic inflammation, as a marker defining a population with immunoregulatory properties, are not known [23, 25, 26].

During the initial phases of an immune response, expression of inflammatory cytokines, such as TNF-α and IL-1, is directly induced by the canonical NF-κB pathway in activated T cells, delineating amplification loops that provide a rapid and intense inflammatory response [17]. Although beneficial in its initial phase, inflammation may become detrimental if not appropriately terminated, and highly organized mechanisms act in concert to restrain the NF-κB response [63]. Our results (obtained by microarray analysis and validated by RT-PCR) show that MSCs promote the expression of three well-known inhibitors of canonical NF-κB signalling in activated lymphocytes: IRAK3 [61], A20 [60] and the E3 ubiquitin ligase βTrCP [67].

In contrast to its role in the inhibition of the canonical pathway, the E3 ubiquitin ligase βTrCP plays a role in the activation of the non-canonical NF-κB pathway [66]. Moreover, we found increased transcript and protein expression levels of non-canonical subunits of NF-κB. These results indicate that the selective inhibition of the canonical pathway and the concomitant activation of non-canonical NF-κB signalling (see 'Supplementary Discussion') could be one of the mechanisms by which MSCs could modulate T cell responses in an inflammatory response.

This mechanism is consistent with the dogma that under chronic stimulation the initial rapid activation of the canonical NF-κB pathway can be followed by a later increase in non-canonical NF-κB components, promoting a shift from canonical to non-canonical NF-κB signalling [73–76] (see 'Supplementary Discussion'). More specifically, in the context of our experimental design, activation of purified T cells by mAbs against TCR/CD3 or CD2 and CD28 was shown to result in a rapid initial activation of the canonical NF-κB pathway (as early as 30 min., peaking at 5–16 hrs), which was followed by a later increase in non-canonical NF-κB components that peaked around the fourth day [73].

Several lines of evidence from the literature provide support for this mechanism. For instance, the activation of NF-κB in leukocytes at the onset of inflammation is associated with pro-inflammatory gene expression. Later, during the resolution of inflammation, activation of NF-κB is instead associated with the expression of anti-inflammatory genes, suggesting an *in vivo* role for NF-κB in the regulation of resolution of inflammation [77–79]. Moreover, IKKα, which is involved in the activation of the non-canonical pathway, could contribute to the suppression of canonical NF-κB activity, thereby accelerating the turnover of RelA and c-Rel subunits and promoting their removal from pro-inflammatory gene promoters [80]. Both subunits of the non-canonical NF-κB pathway (NFKB2 and RELB) can also act as immunomodulators by negatively regulating the canonical NF-κB pathway. More specifically, while RelB-containing dimers can sustain the expression of the canonical inhibitor IκBα [81], NFKB2/p100 directly sequesters NFKB1-RELA dimers to the cytoplasm, rendering them inactive [82, 83].

Given the known regulation of CD69 by NF-κB, we hypothesized that while the canonical NF-κB pathway could control the early expression of CD69 as an activation marker, the non-canonical NF-κB pathway could be involved in the late sustained increase in CD69 expression promoted by MSCs.

Given that canonical signalling modulates RelB activity in the nucleus through direct binding of RelA [68], we used BAY11-7082 to inhibit the nuclear translocation of the canonical NF-κB complex [69] to evaluate the differential role of the canonical NF-κB pathway in the early induction of CD69 and in its later sustained expression. In support of our hypothesis, although inhibition of the canonical NF-κB pathway immediately following activation completely abrogated the expression of CD69 on $CD3^+$ lymphocytes (as expected), inhibition of the canonical NF-κB pathway on the third day following activation had a completely opposite effect, further increasing the percentage of $CD3^+$ lymphocytes expressing the immunomodulatory molecule CD69. This could be explained by the reduced nuclear localization of RelA and the consequent de-repression of RelB. These results are strikingly consistent with the results obtained by Saccani et al., who found that through the forced expression of the canonical super repressor IκBα SR, the early transcription of NF-κB targets (mediated by canonical dimers) was abrogated, yet the expression of these targets at later times (mediated by non-canonical dimers) was further induced [74]. In line with our hypothesis, the expression of CD69 in T lymphocytes transfected with siRNA against RELB and co-cultured with MSCs was almost completely inhibited.

Altogether, these results indicate that the canonical and non-canonical NF-κB signalling pathways could play distinct roles in the expression of CD69. Although the canonical NF-κB pathway could control the early expression of CD69 (as an activation marker), its late and sustained expression (as an immunoregulatory molecule) could be controlled by the non-canonical pathway and could be negatively regulated by the canonical pathway.

Given the important role played by NF-κB in the transcriptional control of pro-inflammatory genes, we would expect non-canonical signalling to play a much broader role in the transcriptional control of components involved in the development and functional properties of Tregs.

Although a role for NF-κB signalling in the resolution of inflammation has been proposed before [77–79]; only recently, during the course of our research, new lines of evidence brought to light the role of the NF-κB pathway in the generation of Tregs (84–90). Nevertheless, these studies mainly focused on the differential roles of cRel and the known canonical members (RelA and

NFKB1); thus, the role of non-canonical members in this process remains largely unexplored and is worthy of investigation.

Although a role for NF-κB signalling in the resolution of inflammation has been proposed previously [77–79]; our recent research has highlighted the role of the NF-κB pathway in the generation of Tregs [84–90]. Nevertheless, these studies mainly focused on the differential roles of cRel and the known canonical members (RelA and NFKB1); thus, the role of non-canonical members in this process remains largely unexplored and is worthy of investigation.

A role for non-canonical NF-κB signalling in the development and function of Tregs is supported by recent findings showing that Tregs can be defined by the expression of the TNF-α receptor TNFR2 [91, 92], which mediates the activation of the non-canonical NF-κB pathway through ligation of membrane-bound (but not soluble) TNF-α [93]. Interestingly, IL-10 regulates the TNF-α–converting enzyme (TACE/ADAM-17), leading to reduced levels of soluble TNF-α, while simultaneously increasing the levels of membrane-bound TNF-α [94].

These results, together with the inferred mechanism presented in our work, have major implications for the study of tumour immune escape mechanisms [35]; peripheral homeostasis of the immune system [36], autoimmune diseases, immune responses against pathogens and allografts [24, 37].

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 MSC-modulated transcripts in the T cell receptor signalling

Fig. S2 MSC-modulated transcripts in the cell cycle (G_1/S checkpoint regulation)

Fig. S3 MSC-modulated transcripts in the mTOR signalling

Fig. S4 MSC-modulated transcripts in the NF-KB pathway

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