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ORIGINAL ARTICLE

Adipose-derived mesenchymal stromal cells induce immunomodulatory macrophages which protect from experimental colitis and sepsis

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ABSTRACT

Objective To investigate the effect of adipose-derived mesenchymal stromal cells (ASCs) on the activation state of macrophages (MΦ) in vitro, and the potential therapeutic effect of these cells in experimental colitis and sepsis.

Design Murine bone marrow-derived macrophages were cultured with ASCs or with ASC conditioned media (ASC-MΦ) and characterised for the expression of several regulatory macrophage markers, including enzymes and cytokines, and for their immunomodulatory capacity in vitro. The therapeutic effect was investigated of ASC-MΦ in two models of experimental inflammatory colitis induced by trinitrobenzene sulphonic acid and dextran sodium sulphate, and in polymicrobial sepsis induced by caecal ligation and puncture.

Results ASC-MΦ showed a phenotype that clearly differed from the classically activated macrophages or the alternatively activated macrophages induced by interleukin (IL)-4, characterised by high arginase activity, increased production of IL-10 upon restimulation and potent immunosuppressive activity on T cells and macrophages. Activation of cyclo-oxygenase-2 on ASCs seems to be critically involved in inducing this phenotype. Systemic infusion of ASC-MΦ inhibited colitis in mice, reducing mortality and weight loss while lowering the colonic and systemic levels of inflammatory cytokines. Importantly, therapeutic injection of ASC-MΦ in established chronic colitis alleviated its progression and avoided disease recurrence. Moreover, ASC-MΦ protected from severe sepsis by reducing the infiltration of inflammatory cells into various organs and by downregulating the production of several inflammatory mediators, where ASC-MΦ-derived IL-10 played a critical role.

Conclusion ASCs induce a distinct regulatory activation state of macrophages which possess potent immunomodulatory ability and therapeutic potential in inflammatory bowel diseases and sepsis.

Significance of this study

What is already known on this subject?

- Among the different macrophage types, the regulatory macrophages (MΦreg) have shown therapeutic potential in inflammatory disorders, including inflammatory bowel disease (IBD). The way in which MΦreg are induced is largely unknown.
- Mesenchymal stem cells (MSCs) from different sources show potent immunosuppressive and anti-inflammatory action and have emerged as attractive candidates to treat autoimmune diseases. These effects are partially exerted by educating other immune cells to be tolerant.

What are the new findings?

- Adipose-derived MSCs (ASCs) promote the conversion of macrophages to a unique regulatory phenotype through the production of soluble factors. The ASC-induced MΦreg show potent suppressive effects on T cells and macrophages.
- Treatment with ASC-induced MΦreg ameliorates colitis and decreases mortality in experimental IBD and sepsis by downregulating the production of a wide panel of inflammatory mediators by resident and infiltrating cells in target organs.
- Resident MSCs of the colonic mucosa could induce physiologically MΦreg to maintain immune tolerance in the gut.

How might it impact on clinical practice in the foreseeable future?

- We are proposing a new therapeutic tool for the treatment of IBD and sepsis based on the use of MΦreg generated from autologous blood monocytes isolated from the patient in the presence of conditioned media of ASCs from unrelated donors.
- Importantly, the entire process is rapid, safe and can be performed in good manufacture procedures (GMP) conditions, and the conditioned media from ASCs can be stored frozen and ready to use in any clinical facility.

INTRODUCTION

Macrophages are phagocytic cells resident in tissue which play important roles in steady-state tissue homoeostasis, removing cellular debris and apoptotic cells, and in the defence against intracellular pathogens. Macrophages respond rapidly to environmental signals using a multitude of receptors, which results in a specific and optimised activation state. Mosser and Edwards have proposed three main macrophage activation states based upon function. These include classically activated macrophages involved in host defence; wound healing macrophages, also known as alternatively activated macrophages (AAM) induced by

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interleukin (IL)-4 that express the markers arginase I, Fizz1, IGF1, CCL22 and Ym1; and regulatory macrophages (MΦreg) involved in immune regulation and anti-inflammatory activities that express IL-10, LIGHT, CCL1 and arginase II.[1]

In addition to their critical role in innate immunity, considerable evidence indicates that macrophages are involved in the pathogenesis of several inflammatory and autoimmune diseases, including multiple sclerosis and inflammatory bowel disease (IBD). IBD is the result of an aberrant immune reaction to the intestinal microflora in genetically susceptible individuals. Two main inflammatory conditions with overlapping and idiosyncratic symptoms can be discerned—namely, Crohn's disease (CD) and ulcerative colitis (UC). CD is driven by T helper (Th) 17/Th1 effector cells and UC is thought to depend upon a Th2 response, while inflammatory macrophages play a role in the onset and disease severity of both IBD conditions.[2 3] Both in human IBD and experimental colitis, monocytes/macrophages migrate into the inflamed colonic mucosa, where they produce IL-1, IL-6, tumour necrosis factor α (TNFα), IL-12 and IL-23.[4 5] Blockade of this migration or depletion of macrophages ameliorates disease severity.[5 6] However, other studies have shown that macrophages can also protect against colitis. Hunter *et al* reported that depletion of macrophages from the lamina propria is followed by reduction of colitis,[7] and the presence of colonic AAM is probably involved in the induction of gut immunotolerance. Indeed, infection with intestinal parasites generates resistance to colitis through the induction of colonic AAM, and administration of AAM generated in vitro reduced colonic inflammation in mice.[7 8]

Mesenchymal stromal cells (MSCs) have emerged as a promising tool for the treatment of colitis and clinical trials using MSCs for the treatment of CD is well underway.[9 10] Administration of syngeneic, allogeneic and xenogeneic MSCs obtained from different sources ameliorates experimental colitis by downregulating both the autoreactive and inflammatory responses.[11 12] We have recently shown that the adipose tissue-derived MSC (ASC)-mediated inhibition of T cell proliferation depended upon the number of monocytes in the co-culture.[13] Other evidence suggests that MSCs may induce AAM or MΦreg in vivo in animals with inflammatory disorders. Thus, intravenous injection of bone marrow-derived MSCs into mice induced IL-10 production in macrophages which protected against sepsis,[14] and gingival tissue-derived MSCs could home to the site of injury and promote skin wound healing through the induction of AAM.[15] Finally, Parekkadan and collaborators recently showed that the protective effect of bone marrow-derived MSCs in experimental colitis depends on their interaction with splenic CD11b macrophages and suggested that immune cells re-educated by MSCs might be used instead of the stromal cells for the treatment of inflammatory disorders.[16] However, no study has investigated the effect of ASCs on the function of macrophages and the potential therapeutic effect of ASC-modified macrophages on inflammation in vivo. The aim of our study was to investigate the effect of ASCs on the phenotype of macrophages and to characterise their function on the immune system, using various experimental models of colitis and sepsis. We found that ASCs induce macrophages with a regulatory function, distinct from AAM, which can inhibit immune responses both in vitro and in vivo.

MATERIALS AND METHODS

Cell isolation and cultures

ASCs were isolated from mouse epididymal and inguinal fat and from human lipoaspirates, and macrophages were generated from mouse bone marrow and cultured as described in the online supplementary methods.

Induction and treatment of colitis

Acute and chronic colitis were induced in Balb/c mice by intracolonic administration of a single dose of trinitrobenzene sulphonic acid (TNBS, 3 mg in 50% ethanol) or of four increasing doses of TNBS (once a week), respectively, as described in the supplementary methods. Control mice received 50% ethanol. Alternatively, acute and chronic colitis were induced in C57BL/6 mice by oral administration of dextran sulphate sodium (DSS) in a single cycle or two cycles, respectively, as described in the supplementary methods. At different times after TNBS and DSS infusion, mice were injected intraperitoneally (IP) with 10^6 syngeneic macrophages cultured for 48 h in complete Mesencult (control-MΦ) or in ASC-conditioned medium (ASC-MΦ) or with phosphate-buffered saline (vehicle control). Mice injected IP with syngeneic mASCs (10^6 cells/mouse) were used as reference control. Animals were monitored daily for body weight loss, colitis and survival. At the peak of the disease, serum, colon and mesenteric lymph nodes (MLNs) were collected and processed as described in the supplementary methods.

To trace the injected cells in vivo, ASC-MΦ were labelled with 5,6-carboxyfluorescein succimidyl ester before injection and analysed as described in the supplementary methods.

Induction and treatment of sepsis

Sepsis was induced in Balb/c mice by caecal ligation and puncture (CLP) as described in the supplementary methods. Mice were treated IP with phosphate-buffered saline or with 10^6 syngeneic control-MΦ or ASC-MΦ at different times after caecum perforation, and monitored for survival. Serum, peritoneal exudates, liver, lungs and small intestines were collected at 18 h for histopathology examination and cytokine determination as described in the supplementary methods.

Statistical analysis

Data are presented as mean (SD). The Mann–Whitney U test to compare non-parametric data for statistical significance was applied on all clinical results and cell-culture experiments. Body weight changes were compared by the Wilcoxon matched-pair signed rank test. Survival was analysed by the Kaplan–Meier log-rank test. $p<0.05$ was considered significant.

RESULTS

ASCs generate macrophages with a regulatory phenotype

As previously demonstrated for human monocytes,[13] the addition of increasing numbers of murine bone marrow-derived macrophages to mouse ASCs–CD4 T cell co-cultures progressively inhibited the proliferative response to a T-cell mitogen (online supplementary figure 1). This suggests that macrophages could increase the immunosuppressive activity of ASCs, or alternatively, that ASCs could convert macrophages into a suppressive phenotype. We found that murine ASCs increased the activity of arginase, a signature molecule of AAM and some types of MΦreg, in syngeneic macrophages (figure 1A). This effect seemed to be mediated through soluble factors produced by ASCs, because ASC-conditioned media (ASCcm) induced arginase activity (supplementary figure 2A). With the exception of arginase I, ASCcm-induced macrophages (ASC-MΦ) did not express other conventional AAM markers (ie, Ym1, Fizz1 and CCL22 induced by IL-4), but showed markers associated with MΦreg, such as haeme oxygenase-1 (HO-1), LIGHT and arginase II (figure 1B). Resting ASC-MΦ did not possess inducible

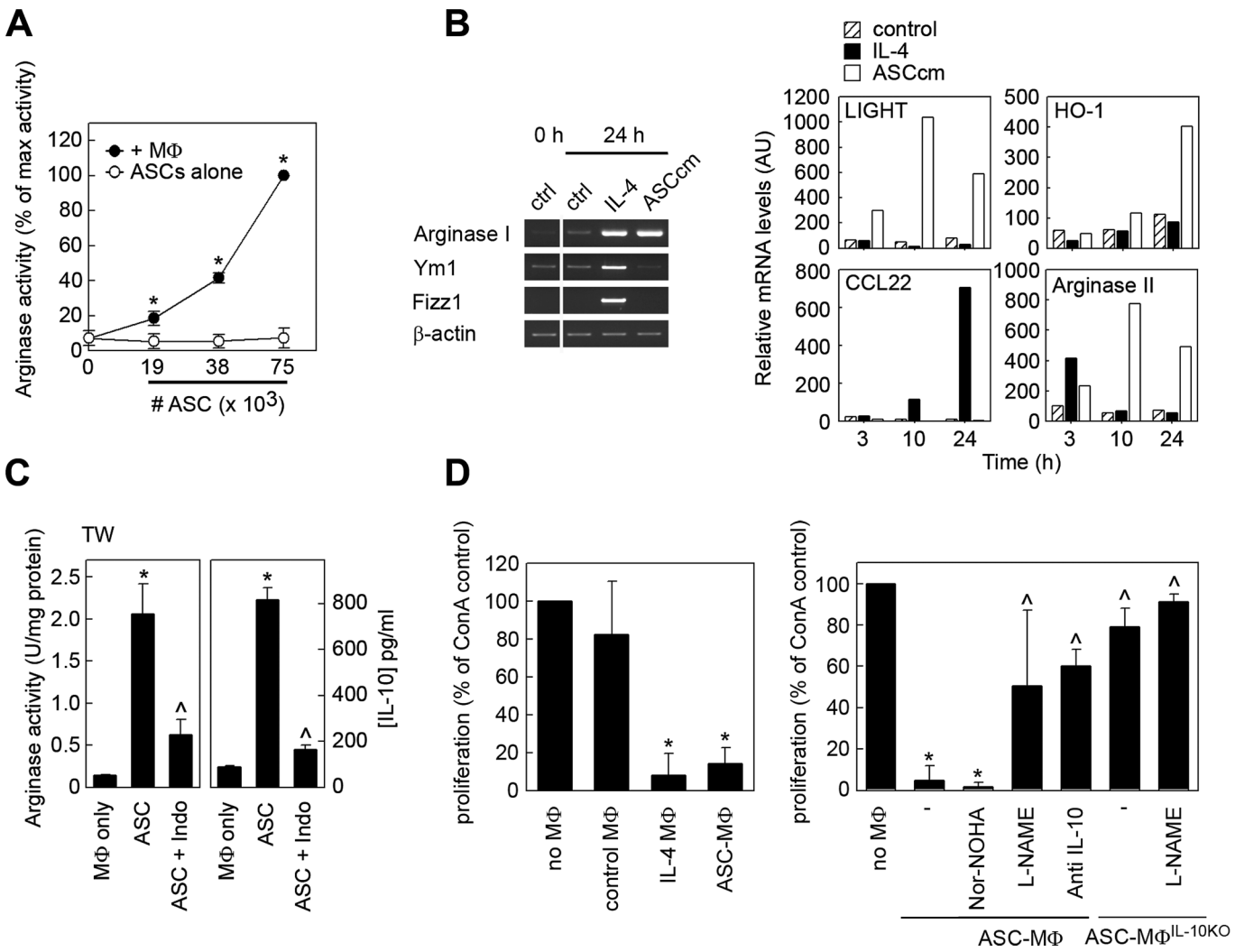

Figure 1 Adipose-derived mesenchymal stromal cells (ASCs) induce a regulatory activation state in macrophages, distinct from classically activated macrophages and alternatively activated macrophages. (A) Macrophages (MΦ, 1.5×10^5) were cultured with different numbers of ASCs for 48 h and arginase activity was measured in cell lysates. The mean arginase activity of co-cultures of macrophages (1.5×10^5) with ASCs (75×10^3) was 201 mU/10^6 macrophages (considered as maximal activity of reference). n=4 experiments performed in duplicate. *p<0.05 versus control without ASC (0). (B) Macrophages (8×10^5) were cultured with medium (ctrl) or stimulated with interleukin (IL)-4 (10 ng/ml) or ASC conditioned media (ASCcm) for different times and gene expression of different macrophage markers was analysed by qPCR. Data shown are representative of three independent experiments. (C) Macrophages (3×10^5 cells/insert) were cultured in transwell cultures with medium (MΦ only) or with ASCs (2×10^5 cells/well) in the presence or absence of indometacin (Indo, 20 μM). After 48 h, arginase activity was measured in cell lysates or cells were harvested and restimulated with lipopolysaccharide for 24 h and IL-10 content measured in supernatants by ELISA. *p<0.05 versus MΦ only; ^p<0.05 versus ASC-treated samples. n=3–4 experiments performed in triplicate. (D) Balb/c macrophages (8×10^5 cells/well) were cultured with medium (control-MΦ) or stimulated with IL-4 (10 ng/ml, IL4-MΦ) or ASCcm (ASC-MΦ) for 48 h. Cells (5×10^4) were then harvested and added to Balb/c splenocytes (2×10^5 cells) stimulated with concanavalin A (ConA, 2.5 μg/ml). When indicated, Balb/c splenocytes were co-cultured with ASC-MΦ generated from Balb/c wild-type or IL-10KO mice (ASC-$MΦ^{IL10KO}$, at a ratio of 20:1) and stimulated with ConA (2.5 μg/ml) in the absence (–) or presence of L-NAME (1 mM), nor-NOHA (50 μM) or anti-IL-10 antibodies (20 μg/ml). The proliferative response was determined after 72 h and expressed as a percentage of proliferation versus untreated ConA-stimulated splenocytes (no MΦ). *p<0.05 versus no MΦ. n=3–5 experiments performed in duplicate or triplicate. ^p<0.05 versus ASC-MΦ-treated co-cultures (–). Data are shown as mean (SD).

nitric oxide synthase (iNOS) activity or express IL-12, but produced high levels of transforming growth factor (TGF) β1, distinguishing them from lipopolysaccharide (LPS)/interferon γ-induced classically activated macrophages (supplementary figure 2B). ASC-MΦ restimulated with LPS significantly increased the expression of IL-10, another MΦreg marker, as well as iNOS, while producing low levels of the inflammatory cytokines TNFα and IL-12 (supplementary figure 2C). LPS stimulation of macrophages co-cultured with ASCs using transwells also resulted in high IL-10 levels and arginase activity, confirming that cell contact is not required; and indometacin (a cyclo-oxygenase 2 (COX-2) inhibitor), but not IL-4-blockade, significantly reduced the macrophage arginase activity and IL-10 production (figure 1C, supplementary figure 3), suggesting that among the ASC-derived soluble factors, prostaglandin E2 (PGE2) plays a major role in the generation of this MΦ phenotype. Indeed, mouse ASCs constitutively secreted high PGE2 levels (supplementary figure 3A) and constitutively expressed COX-2 (not shown).

Whether human macrophages can express arginase I is still controversial. Neither human ASCs nor human ASCcm induced arginase activity in human THP-1 macrophages; however, both

human ASCs and human ASCcm significantly increased IL-10 and reduced IL-12 secretion upon LPS-restimulation (supplementary figure 4), which is consistent with a regulatory phenotype.

Because the suppression of CD4 T-cell proliferation in ASC co-cultures depends upon the number of macrophages (supplementary figure 1), we investigated the regulatory potential of ASC-MΦ on splenocyte proliferation. ASC-MΦ and IL-4-induced AAM, but not control-MΦ, showed potent suppressive activities on activated splenocytes at ratios as low as 20:1 (figure 1D). Despite the high expression/activity of arginase in ASC-MΦ, we could not demonstrate a role for this enzyme in the inhibition of splenocyte proliferation. Neither the arginase inhibitor nor-NOHA (figure 1D) nor excess of L-arginine (not shown) could reverse the inhibitory effect of ASC-MΦ on proliferation. However, the iNOS inhibitor L-NAME and IL-10-blocking antibodies significantly reversed their suppressive activity (figure 1D), suggesting that IL-10 and nitric oxide are critically involved in the suppressive activity of ASC-MΦ on T cells. Indeed, ASC-MΦ from IL-10KO mice failed to suppress T cell proliferation, especially in presence of iNOS inhibition (figure 1D).

ASC-MΦ inhibit murine experimental colitis

Once the immunosuppressive activity of ASC-MΦ in vitro was confirmed, we next investigated their potential therapeutic action in two experimental models of acute and chronic colitis induced by intrarectal infusion of TNBS and by oral DSS administration, which displays human IBD-like clinical, histopathological and immunological features.

Mice treated with a high dose of TNBS developed a severe acute illness characterised by bloody diarrhoea, rectal prolapse, pancolitis accompanied by extensive wasting syndrome, and continuous weight loss, resulting in 90% mortality (figure 2A). Macroscopic examination of colons showed striking hyperaemia, inflammation, necrosis and shortening (figure 2B). ASC-MΦ-treated, but not control-MΦ-treated mice, had an

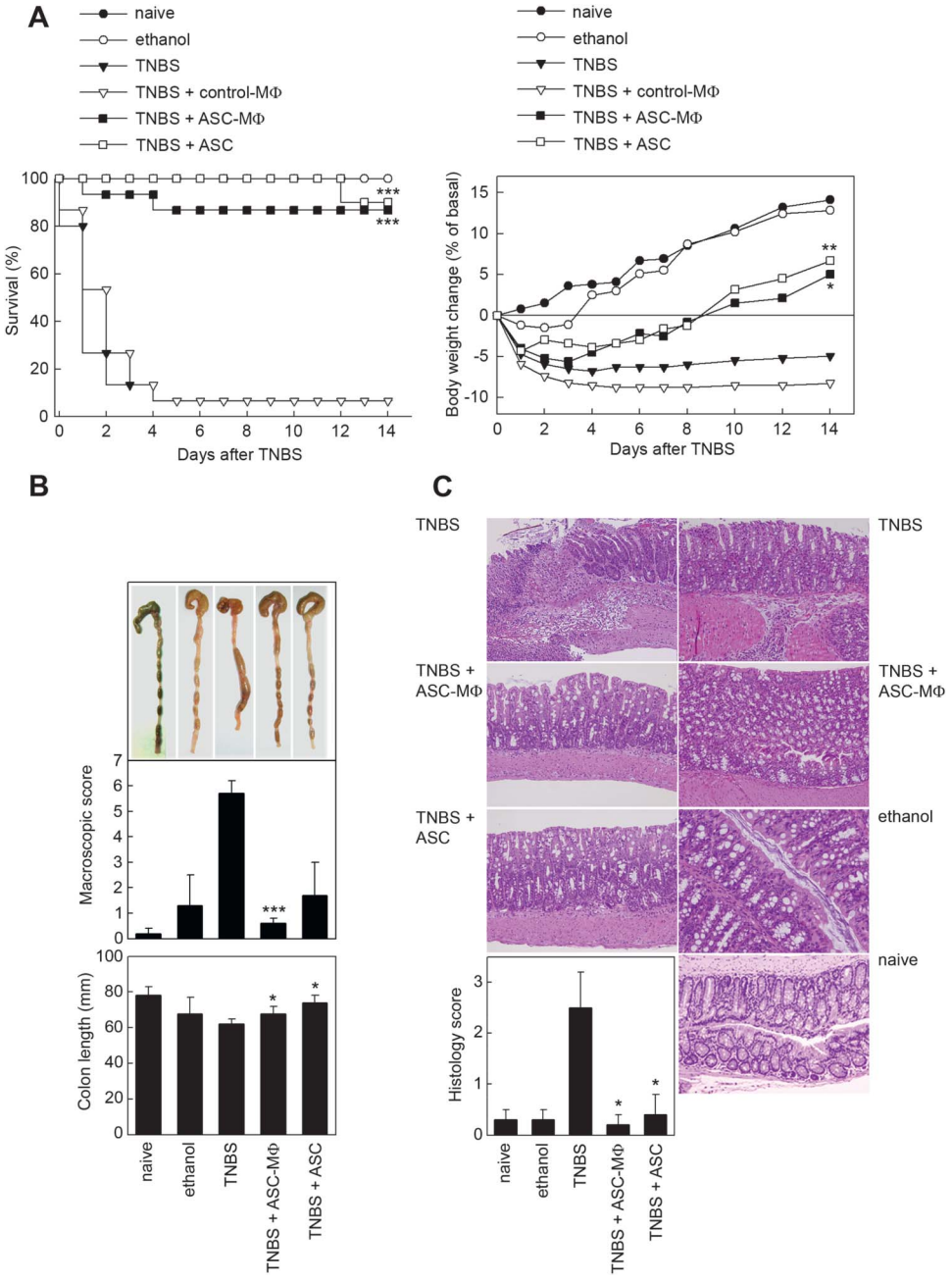

Figure 2 ASC-MΦ protect from TNBS-induced acute colitis. Acute colitis was induced in male Balb/c mice by intracolonic administration of TNBS (3 mg/mouse) in 50% ethanol. Mice were immediately injected intraperitoneally with phosphate-buffered saline (vehicle) or with 10^6 macrophages previously cultured for 48 h with complete medium (control-MΦ) or with ASCcm (ASC-MΦ). Mice treated intraperitoneally with 10^6 ASCs were used as reference control. Naïve mice and mice injected intrarectally with 50% ethanol were used as basal controls. Disease evolution and severity was monitored by survival and weight loss (A). At the peak of the disease (day 3), colons were subjected to macroscopic (B) and histopathological (C) evaluation of damage. (n=10–15 mice/group for panel A and n=5 mice/group for panels B and C, performed in two independent experiments). *p<0.05; **p<0.01; ***p<0.001 versus untreated TNBS-colitic mice. ASCs, adipose-derived mesenchymal stromal cells; ASCcm, ASC conditioned medium; MΦ, macrophages; TNBS, trinitrobenzene sulphonic acid.

increased survival rate, rapidly recovered body weight, had improved wasting disease, regained a healthy appearance and showed only slight signs of colonic inflammation, similar to control mice treated with vehicle (ethanol) or ASC-treated colitic mice (figure 2A,B). Histological examination of the colons showed that ASC-MΦ treatment reduced the TNBS-induced transmural inflammation, mucin-producing goblet cell depletion, epithelial ulceration, disseminated fibrosis, focal loss of crypts and infiltration of inflammatory cells in the lamina propria (figure 2C). Based on these results, there is the possibility of using ASCcm to ameliorate colitis through the induction of MΦreg in resident macrophages. However, in contrast to treatments with ASCs or ASC-MΦ, we observed no therapeutic effect in TNBS-induced acute colitis by the intraperitoneal administration of ASCcm (at days 1 and 2, data not shown).

The therapeutic effect of ASC-MΦ was not exclusive to the TNBS-induced colitis model. ASC-MΦ treatment was proved to be effective in ameliorating acute colitis induced with 5% DSS. Injection of ASC-MΦ, but not of control-MΦ, significantly reduced the clinical activity, as shown by improvement of stool consistency, reduced rectal bleeding, decrease in colon shortening, reduced colon neutrophil infiltration (measured as myeloperoxidase activity), amelioration of colon damage and improvement in survival (figure 3).

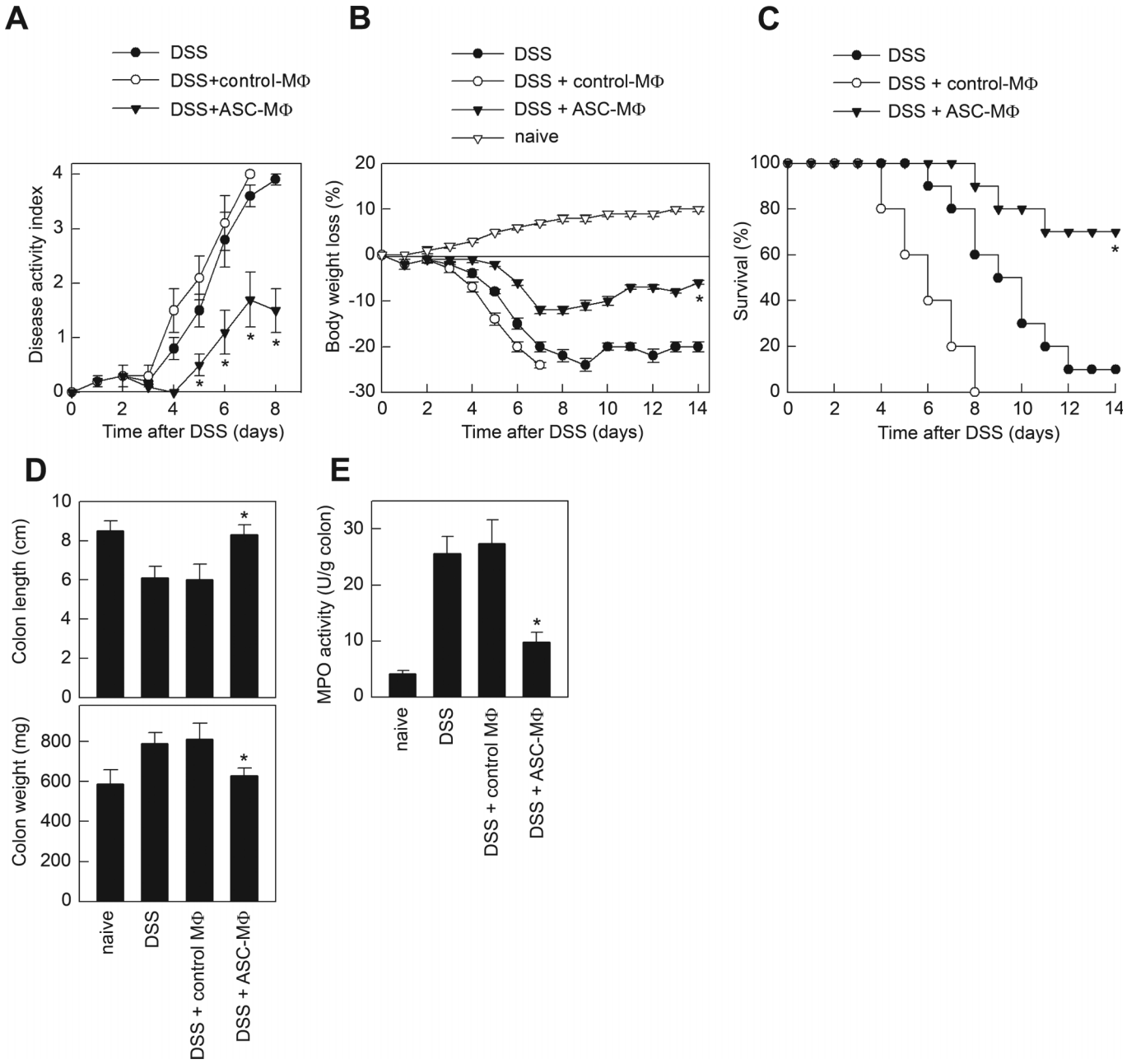

Figure 3 ASC-MΦ protect from DSS-induced acute colitis. Mice received 5% DSS with their drinking water from day 0 to day 7. Control-MΦ or ASC-MΦ (10^6 cells) were infused intraperitoneally on day 2. Disease activity scores (A), weight loss (B) and mortality (C) were determined daily. Colon length and weight (D) were determined at day 6. Neutrophil infiltration was determined by measuring myeloperoxidase (MPO) activity in colonic protein extracts obtained on day 6 (E). Mice receiving tap water instead of DSS were used as controls (naive). n=5–10 mice/group, performed in two independent experiments. *p<0.01 versus untreated DSS-colitic mice. ASCs, adipose-derived mesenchymal stromal cells; DSS, dextran sulphate sodium; MΦ, macrophages.

We next examined the efficiency of ASC-MΦ in chronic and recurrent colitis and during the later phases of the disease when colitis was more established. Repetitive weekly infusion of low doses of TNBS resulted in progressive weight loss and chronic colitis maintained for more than 4 weeks (figure 4A). Initiation of ASC-MΦ treatment in mice with established disease (day 8, during the second TNBS infusion) significantly improved weight loss and survival, and a second late ASC-MΦ injection (during the third TNBS-infusion) fully protected from colitis (figure 4A). In TNBS-induced acute colitis, ASC-MΦ injection 24 h after disease onset almost completely reversed disease evolution, whereas control-MΦ worsened it; and initiation of the treatment in mice showing severe clinical signs of colitis and a mortality of 20% (48 h after TNBS infusion), partially, albeit not significantly, improved survival rate (supplementary figure 5). On the other hand, administration of 3% DSS in two cycles resulted in two peaks of colitis characterised by continuous weight loss, diarrhoea and bloody stools, causing a mortality of 80% (figure 4B). ASC-MΦ injection during the first acute peak of colitis (day 4, with a clinical score >2) significantly reduced the clinical activity by day 6, as shown by the improvement of stool consistency, reduction of rectal bleeding and improved survival (figure 4B). Importantly, a single ASC-MΦ infusion at the first acute colitis peak conferred significant resistance to disease activity during a second cycle of DSS administration (figure 4B). A second ASC-MΦ injection during the second cycle of DSS infusion completely abrogated the clinical signs (figure 4B).

To better understand the half-life and trafficking of the infused ASC-MΦ, we injected 5,6-carboxyfluorescein succimidyl ester-labelled ASC-MΦ into DSS-colitic mice. We detected the inoculated cells in the MLNs and inflamed colon 2 days after the injection, but not in non-inflamed intestine or kidney (figure 5).

ASC-MΦ reduce systemic and mucosal inflammatory responses in colitic mice

We next investigated the in vivo effect of ASC-MΦ on the production of inflammatory mediators that are mechanistically linked to colitis in patients with IBD and animals. Colons of ASC-MΦ-treated mice contained reduced levels of inflammatory cytokines (TNFα, IL-6, IL-1β) and the chemokine RANTES in comparison with untreated TNBS- and DSS-colitic mice (figure 6A,B). However, the immunosuppressive cytokines IL-10 and

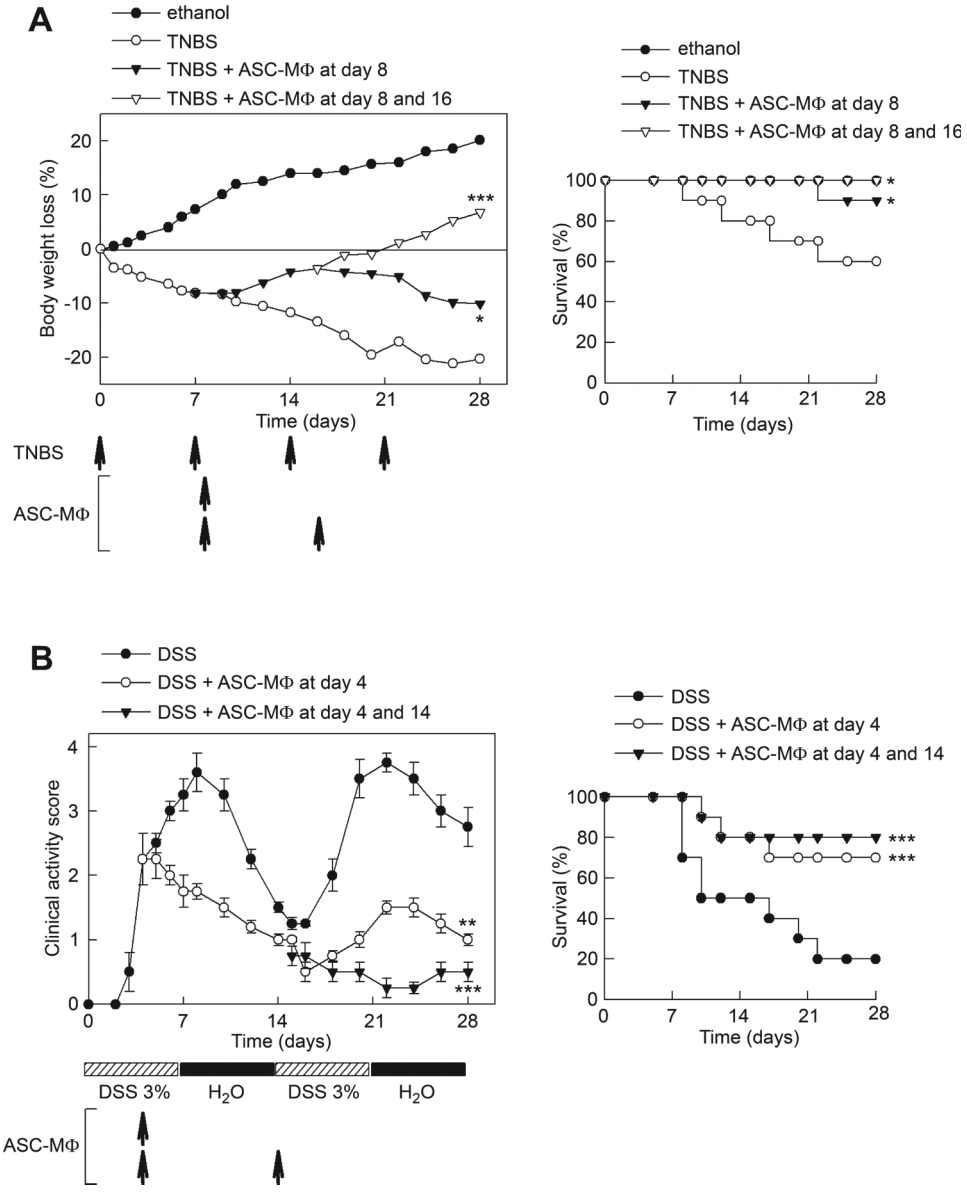

Figure 4 ASC-MΦ ameliorate established TNBS-induced and DSS-induced chronic colitis. (A) Mice were injected intracolonically once a week with increasing doses of TNBS (0.8 mg/mouse at day 1, 1 mg/mouse at day 7, 1.2 mg/mouse at day 14 and 1.5 mg/mouse at day 21). ASC-MΦ (10^6 cells) were infused intraperitoneally on day 8 or on days 8 and 16. Mice injected with 50% ethanol on days 1, 7, 14 and 21 were used as basal controls. Disease evolution and severity was monitored by survival and weight loss (n=9–12 mice/group, performed in two independent experiments). *p<0.05; ***p<0.001 versus untreated TNBS-colitic mice. (B) Mice received 3% DSS with their drinking water in a cyclic manner. Each cycle consisted of 7 days of DSS followed by a 7-day period without DSS supplementation. ASC-MΦ (10^6 cells) were infused intraperitoneally on day 4 or on days 4 and 14. Disease activity scores and mortality were determined daily. Mice receiving tap water instead of DSS were used as controls and showed no clinical signs. n=8–10 mice/group, performed in two independent experiments. **p<0.01; ***p<0.001 versus untreated DSS-colitic mice. ASCs, adipose-derived mesenchymal stromal cells; DSS, dextran sulphate sodium; MΦ, macrophages; TNBS, trinitrobenzene sulphonic acid.

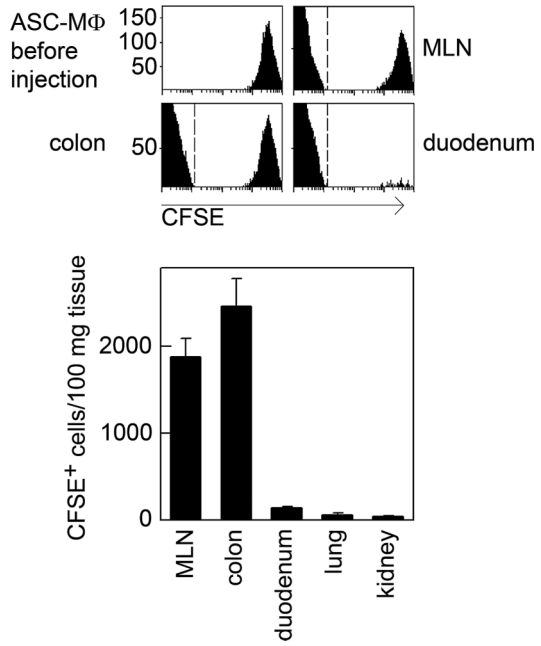

Figure 5 ASC-MΦ are preferentially recruited by lymphoid organs and inflamed colon. 5,6- Carboxyfluorescein succimidyl ester (CFSE)-labelled ASC-MΦ (10^6) were injected intraperitoneally on day 2 to DSS-colitic mice and their presence in various organs determined by flow cytometry 2 days after ASC-MΦ injection. The profile of CFSE-labelled ASC-MΦ before injection is included. Bottom panel represents the number of CFSE cells per 100 mg tissue. n=3 mice/group, each mouse analysed in triplicate. ASCs, adipose-derived mesenchymal stromal cells; DSS, dextran sulphate sodium; MLN, mesenteric lymph node; MΦ, macrophages.

TGFβ1 did not change upon ASC-MΦ treatment (figure 6A,B). The broad anti-inflammatory activity of ASC-MΦ in the colon was accompanied by downregulation of the systemic inflammatory response (figure 6C). This effect was not only a consequence of a diminished inflammatory infiltration in the mucosa, because mononuclear cells isolated from the lamina propria of ASC-MΦ-treated DSS-colitic mice produced less TNFα, IL-12 and IL-1β on ex vivo culture (supplementary figure 6A), suggesting that ASC-MΦ deactivated the colonic inflammatory response. This hypothesis is corroborated by data obtained in gut-draining MLNs, one of the major sites where naive T cells respond to intestinal pathogens and generate a dysregulated T cell response causing severe colitis. MLN cells from ASC-MΦ-treated mice reactivated ex vivo produced lower levels of inflammatory cytokines and slightly increased IL-10 and TGFβ1 levels than untreated TNBS-colitic mice (supplementary figure 6B). However, ASC-MΦ treatment did not limit the proliferative responses of MLNs to a polyclonal restimulation (supplementary figure 6C), suggesting that ASC-MΦ could act by impairing the differentiation/activation of inflammatory cells in the colon and MLNs.

ASC-MΦ protect against sepsis

We next investigated whether the therapeutic effect of ASC-MΦ could be extended to other inflammatory disorders caused by systemic infection with gut bacteria. We evaluated the action of ASC-MΦ in systemic inflammation and sepsis by using a model of diffuse peritonitis induced by CLP, which mimics the clinical situation of patients with colonic leakage following surgical procedures. Treatment with ASC-MΦ, but not with control-MΦ, protected against mortality caused by CLP (figure 7A). Experiments of delayed injection of ASC-MΦ showed a therapeutic window of 6–12 h after caecum perforation (figure 7B). The pathogenesis of sepsis is characterised by overwhelming inflammatory and immune responses that can lead to tissue damage, multiple organ failure and death. ASC-MΦ injection of septic animals decreased the levels of inflammatory mediators (TNFα, IL-6, IL-1β, interferon γ) in serum and reduced the inflammatory infiltration into the most affected organs such as peritoneal cavity, lung, liver and intestine (figure 7C–E). This was corroborated at the histopathological level in lung and intestine, where ASC-MΦ treatment significantly ameliorated the inflammatory infiltration and disseminated coagulation seen in the untreated septic mice (figure 7E). These results indicate that ASC-MΦ rescue mice from septic death by downregulating the exacerbated inflammatory response characteristic of this disorder.

To investigate whether ASC-MΦ regulated the inflammatory response by acting directly on inflammatory cells, we co-cultured ASC-MΦ with activated macrophages isolated from septic mice. ASC-MΦ inhibited TNFα, IL-6 and IL-12 production by macrophages, especially under inflammatory stimulation (figure 8A). This inhibitory effect was partially dependent on IL-10 secretion (figure 8A). ASC-MΦ generated from IL-10KO mice did not protect from sepsis and colitis (figure 8B,C), suggesting a critical role for IL-10 in the suppressive/protective activity of ASC-MΦ in these inflammatory disorders.

ASC generate MΦreg from both healthy and diseased donors

Because the use of ASC-MΦ is limited to autologous treatment and an inflammatory milieu could affect the conversion of these macrophages, we finally investigated the ability of ASCs to generate a regulatory phenotype in macrophages isolated from diseased donors. We found that ASC-MΦ generated from peritoneal macrophages isolated from septic or colitic mice showed similar anti-inflammatory and protective activities in vitro and in vivo in colitis and sepsis compared with those of ASC-MΦ isolated from healthy donors (supplementary figure 7).

DISCUSSION

IBD is a family of chronic, idiopathic, relapsing and tissue-destructive diseases characterised by dysfunction of mucosal T cells and altered cellular inflammation that ultimately leads to damage of the distal small intestine and the colonic mucosa. Available treatments for IBD based on immunosuppressive agents are directed towards diminishing the inflammatory response and treating the sequel of uncontrolled inflammation, but they are not entirely effective, are non-specific and result in multiple adverse effects; in most cases, surgical resection is the ultimate alternative. This illustrates the need for new therapeutic approaches that specifically modulate both components of the disease (ie, the inflammatory and Th1-driven responses).

In this study, we propose a new cell-based therapeutic strategy for IBD using regulatory macrophages generated with ASCs. ASC-MΦ provided a highly effective treatment for acute and chronic colitis induced by DSS and TNBS. This work also provides important evidence that ASC-MΦ might represent feasible therapeutic agents to treat sepsis. Sepsis, a life-threatening complication of bacterial infections and the most common cause of death in intensive care units, is characterised by a hyperactive and out-of-balance network of endogenous proinflammatory cytokines. Our work showed that a single systemic injection of

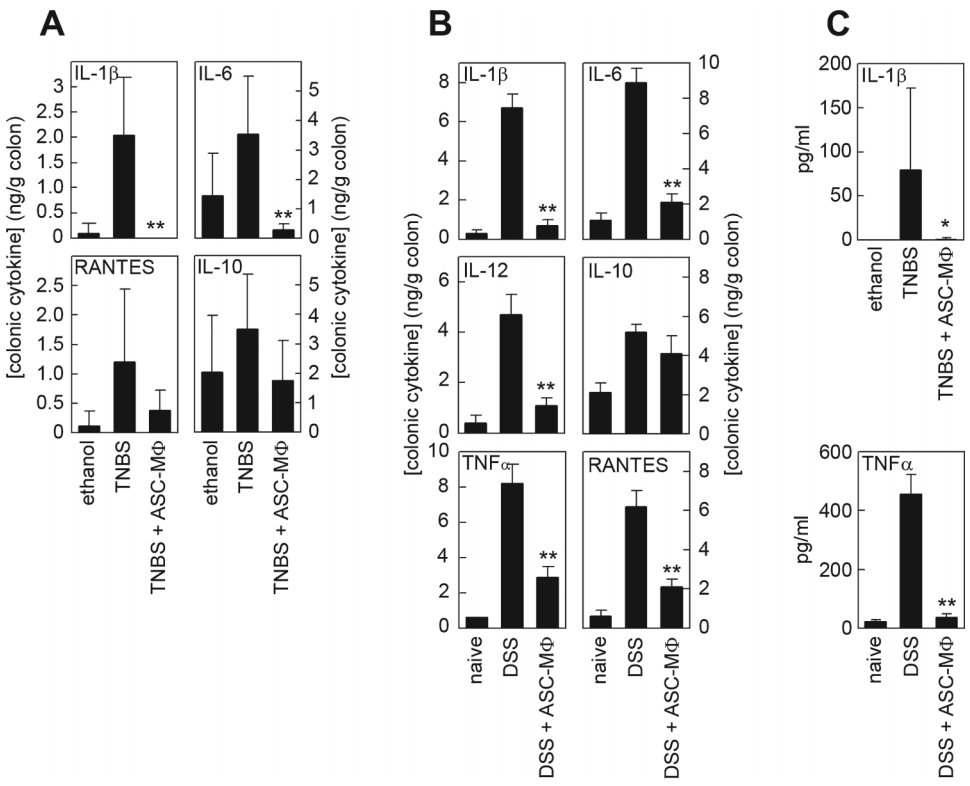

Figure 6 ASC-MΦ reduce systemic and mucosal inflammatory responses in colitic mice. Colitis was induced by intrarectal infusion of 3 mg TNBS or by oral administration of 5% DSS. Mice were treated intraperitoneally with phosphate-buffered saline (vehicle) or ASC-MΦ immediately after TNBS injection or 2 days after initiation of DSS administration. Mice treated with 50% ethanol or mice receiving tap water instead of DSS (naïve) were used as controls. Cytokine and chemokine contents were determined in colonic protein extracts (A and B) or serum samples (C) isolated at the peak of the disease (day 3 for TNBS and day 7 for DSS). Data are shown as mean (SD). n=5-6 mice/group, each mouse analysed in triplicate. *p<0.05, **p<0.01 versus untreated TNBS- or DSS-colitic mice. ASCs, adipose-derived mesenchymal stromal cells; DSS, dextran sulphate sodium; IL, interleukin; MΦ, macrophages; TNBS, trinitrobenzene sulphonic acid; TNF, tumour necrosis factor.

ASC-MΦ at the onset of IBD or sepsis significantly ameliorated the clinical and histopathological signs and reduced the high mortality caused by both diseases. Of obvious therapeutic importance is that the delayed treatment with ASC-MΦ reduced the severity of colitis in animals with established chronic disease and that an initial ASC-MΦ injection prevented the recurrence of the disease after subsequent infusions of TNBS or DSS.

Previous studies reported the therapeutic effect on experimental colitis and sepsis of some AAM and MΦreg generated by genetic manipulation or with cytokines, immune complexes or glucocorticoids.[1 7 17–20] We show that ASCs convert macrophages into a distinctive regulatory phenotype that shares markers with AAM and MΦreg, such as high expression of arginase I and II, HO-1 and LIGHT and production of nitric oxide and IL-10 upon stimulation, but lack of classical markers of AAM (ie, Ym1, CCL22 or Fizz1). The cell contact between ASCs and macrophages is not necessary to generate this MΦreg population, and conditioned medium from ASC cultures fully converts them. As previously suggested for other MSCs,[14 21] COX-2 activation seems to play a major role in the generation of these MΦreg by ASCs. However, apart from PGE2, we cannot exclude the possibility of involvement of other soluble factors or apoptotic bodies, since treatment with PGE2 alone did not fully induce the MΦreg phenotype (MD, personal communication).[21]

Our data demonstrated that ASC-MΦ efficiently suppress both T cell proliferation and macrophage inflammatory responses, acting at low cell-to-cell ratios, and that IL-10 plays a critical role in the suppressive activity of ASC-MΦ in vitro and in vivo, although iNOS, HO-1 and TGFβ1 may also play a part. Noteworthy for a future clinical application is the fact that ASCs also promoted a regulatory phenotype in human monocytes characterised by high IL-10 and low IL-12 expression. On the other hand, although this is the first report describing ASC-induced arginase activity in murine macrophages, contrary to its well-established involvement in the immunosuppressive action of AAM,[22] the role of arginase in ASC-MΦ-induced T-cell suppression seems immaterial, as it simply acts as a phenotypic marker of ASC-MΦ. The concept of re-education of immune cells by MSCs was previously proposed by others, and as with ASCs, MSCs from other sources (ie, bone marrow) induce an alternatively activated/regulatory phenotype of macrophages with therapeutic potential in sepsis, colitis and wound healing.[14–16 21 23]

There are several potential mechanisms for the therapeutic effect of ASC-MΦ on the effector phase of colitis. In both IBD and experimental colitis, activated Th1 cells promote an exaggerated macrophage and neutrophil infiltration and activation, giving rise to a transmural inflamed intestinal mucosa, characterised by prolonged and uncontrolled production of inflammatory cytokines and chemokines. Cytokines and free radicals produced by infiltrating cells and resident macrophages play a critical role in colonic tissue destruction. ASC-MΦ regulate the two arms of the immune response involved in the pathogenesis of IBD. They strongly reduce colonic inflammation by downregulating the production of a wide panel of inflammatory/cytotoxic mediators by mucosal immune cells. Moreover, the inhibition of the production of chemokines is critical to explain the absence of inflammatory infiltrates in the mucosa of ASC-MΦ-treated mice. Similarly, ASC-MΦ function at several levels to regulate many crucial aspects of sepsis, including reduction of the systemic levels of a wide panel of inflammatory cytokines and chemokines and inhibition of leucocyte infiltration of various targets organs. Most therapeutic strategies in sepsis have targeted single inflammatory mediators, but they did not improve survival of septic patients when studied in large multicentre clinical trials. The regulation of a plethora of inflammatory mediators by ASC-MΦ obviously supposes a therapeutic advantage. Importantly, the therapeutic window of ASC-MΦ (6–12 h) in sepsis corresponds to the peak of the cytokine storm

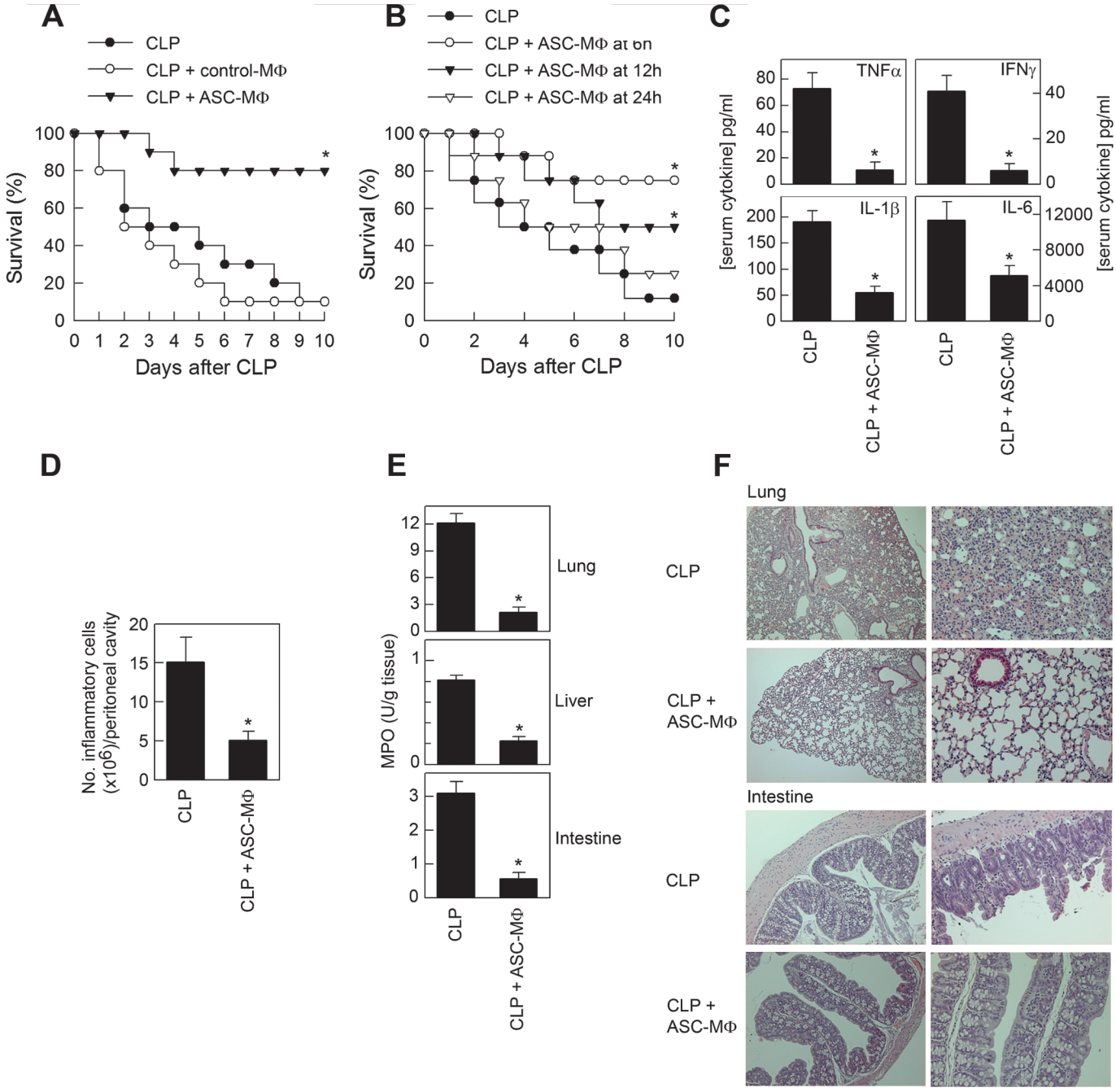

Figure 7 Treatment with ASC-MΦ protects from sepsis. Mice were subjected to caecal ligation and puncture (CLP) and treated intraperitoneally with phosphate-buffered saline, control-MΦ or ASC-MΦ at different times later (4 h for A, C–F). (A, B) Survival was monitored every 24 h. (C) Cytokine levels were determined by ELISA in serum samples isolated 18 h after the CLP procedure. (D) The number of inflammatory cells infiltrating the peritoneal cavity was determined 18 h after CLP. (E) Neutrophil infiltration in target organs was determined by measuring myeloperoxidase (MPO) activity in protein extracts obtained 18 h after CLP. (F) Histopathological signs of inflammatory infiltration and disseminated coagulation were evaluated in lung and intestine isolated 18 h after CLP. Data are shown as mean (SD). n=10–12 mice/group, performed in two independent experiments, samples analysed in triplicate. *p<0.01 versus untreated CLP controls. ASCs, adipose-derived mesenchymal stromal cells; IFN, interferon; IL, interleukin; MΦ, macrophages; TNF, tumour necrosis factor.

characteristic of this disorder, after which ASC-MΦ treatment is ineffective. Deactivation of resident and infiltrating mucosal macrophages might be a major mechanism involved in the anti-inflammatory action of ASC-MΦ in colitis and sepsis, and IL-10 plays a critical role in this suppressive effect. Similar to other AAMs,[7 8 24] ASC-MΦ preferentially home to the inflamed colon where they are induced to produce the immunosuppressive cytokine IL-10 in an inflammatory milieu and deactivate resident and infiltrating inflammatory cells preventing tissue destruction and disease. Beside this local effect, ASC-MΦ could initiate their protective response in the periphery, because they also home to gut-draining MLNs, where they deactivate Th1-driven inflammatory responses in colitic mice. Moreover, the fact that ASC-MΦ treatment confers some resistance to disease recurrence suggests that these cells could be maintained for a long period of time in both colonic mucosa and MLNs

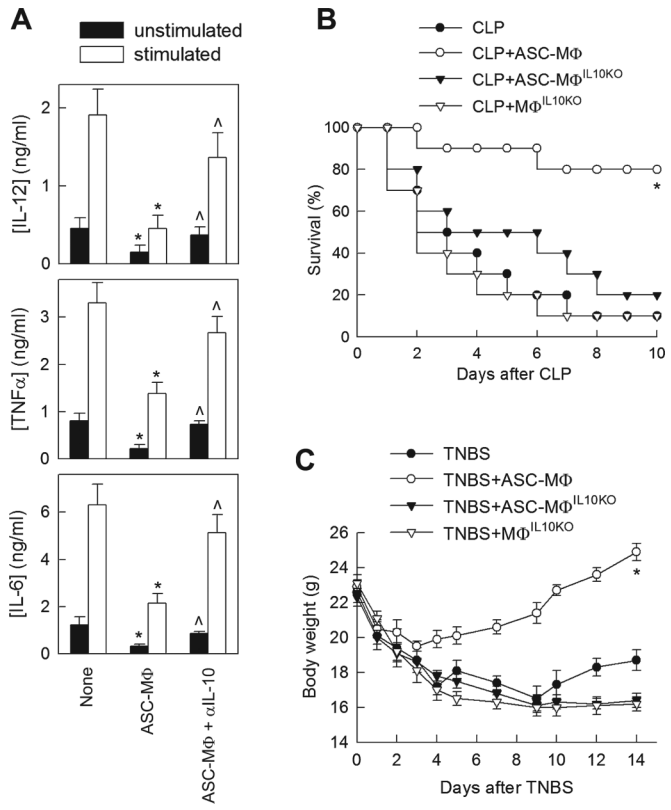

Figure 8 ASC-MΦ downregulate the inflammatory response of activated macrophages through an IL-10-dependent mechanism. (A) Peritoneal macrophages (5×10^4) isolated from mice with caecal ligation and puncture (CLP) were cultured with medium (unstimulated) or with lipopolysaccharide (stimulated) in the absence (none) or presence of ASC-MΦ (5×10^4). When indicated, neutralising anti-interleukin 10 (αIL-10) antibodies were added to co-cultures. After 24 h, cytokine contents in the supernatants were determined by ELISA. Data are shown as mean (SD). n=4 experiments performed in duplicate. *p<0.01 versus untreated controls (none); ^p<0.01 versus ASC-MΦ–macrophage co-cultures without αIL-10 antibodies. (B) Mice were subjected to CLP and treated intraperitoneally 4 h later with PBS or with 10^6 bone marrow macrophages generated from wild-type or IL-10KO mice and cultured for 24 h with medium ($M\Phi^{IL10KO}$) or with ASCcm (ASC-MΦ or ASC-$M\Phi^{IL10KO}$). Survival was monitored every 24 h. n=8–10 mice, performed in two independent experiments. *p<0.001 versus PBS-treated CLP mice. (C) Mice were infused intrarectally with TNBS (3 mg in 50% ethanol) and immediately treated with $M\Phi^{IL10KO}$, ASC-MΦ or ASC-$M\Phi^{IL10KO}$. Progression of colitis was assessed by measuring body weight loss. n=7–11 mice/group, performed in two independent experiments. *p<0.001 versus untreated TNBS-colitic mice. ASCcm, ASC conditioned medium; ASCs, adipose-derived mesenchymal stromal cells; IL, interleukin; MΦ, macrophages; PBS, phosphate-buffered saline; TNBS, trinitrobenzene sulphonic acid; TNF, tumour necrosis factor.

and exert longlasting protective effects, or alternatively, that ASC-MΦ could re-educate other immune cells that perpetuate the tolerance response.

In addition to their therapeutic potential in IBD, our findings also have relevance from a physiological point of view in the maintenance of gut homoeostasis. The gut mucosa contains the largest population of resident macrophages, which are thought to maintain immunological tolerance towards the microflora.[25] ASC-MΦ show many phenotypic/functional similarities to these macrophages. Intestinal macrophages exhibit a quiescent phenotype, but show high phagocytic capacity and IL-10 production, especially after bacterial stimulation.[26 27] Increasing evidence indicates that IL-10 and PGE2 play a major role in the desensitisation of intestinal macrophages and IBD pathogenesis,[26 28] and a population of MSCs identified in the colon seems to be an important local PGE2 source.[29] Furthermore, conditioned medium from intestinal stromal cells induces an inflammatory anergy in human monocytes.[25] Finally, the interaction of ASCs with macrophages/monocytes induces secretion of high levels of IL-10 in both cells.[13] These findings suggest that one role of resident intestinal MSCs might be to induce or maintain the inflammation anergy of intestinal macrophages in the healthy colon.

The use of allogeneic ASC holds great promise as a treatment of many autoimmune/inflammatory diseases, including IBD. Similar to other IBD treatments, including anti-TNFα antibodies and glucocorticoids,[30] ASCs could act through the in vivo induction of IL-10-producing MΦreg. Indeed, as discussed above, various reports demonstrated the involvement of macrophages/monocytes with an alternatively activated/regulatory phenotype in the therapeutic effect of MSCs in experimental inflammatory disorders.[14–16 21 23] Although our findings show that ASC-MΦ could be rapidly (in few hours) generated from cells of diseased patients with frozen-stored ASCcm, treatments based on ASC-MΦreg are limited to their use only in an autologous manner. This suggest an obvious disadvantage versus treatments with allogeneic ASCs or MSCs, which have been efficiently used without major histocompatibility complex matching in preclinical studies. However, until clinical trials in IBD, which are in progress, demonstrate the efficiency of allogeneic MSCs, autologous MΦreg generated ex vivo with ASCcm emerge as an attractive and alternative treatment for patients with CD or UC. Moreover, although most of the performed studies do not report any adverse effects of MSC treatment, concerns about safety related to tumour progression/promotion still need to be resolved.[31–34] Monocytes/macrophages are non-oncogenic and human MΦreg can readily be generated from peripheral blood monocytes and be administered safely through central venous infusion,[35] and have been used for promoting transplant tolerance[36] and for the treatment of chronic wounds.[37]

Contributors PA, MD: study design; EG-R, PA, LS-M, MC, MM, FOV: acquisition of data; EG-R, MD, PA: analysis and interpretation of data and statistical analysis; PA, MD: manuscript preparation.

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Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

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ONLINE SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Animal procedure guidelines

Balb/c and C57Bl/6 mice were purchased from Charles River and IL-10KO (in a Balb/cJ background) mice were obtained from Jackson Laboratories. All experiments were performed according to the institutional guidelines for the care and use of laboratory animals in research and with the approval of the local committee in the Consejo Superior de Investigaciones Cientificas.

Preparation of mesenchymal stem cells from adipose tissue (mASC) and mASC-conditioned media

Abdominal (epididymal) and subcutaneous (inguinal) fat from female Balb/c mice (10-12 weeks) was aseptically removed and washed twice in Hank´s balanced salt solution (HBSS; Invitrogen). The fat tissue was weighed, cut into small pieces (2 mm^3) and resuspended in 2.5 ml of HBSS containing 2 mg/ml collagenase type I (Sigma) per gram fat tissue and incubated for 30 minutes at 37°C, swirling the tube every 5 minutes. The digest was washed twice with 10 ml HBSS and filtered each time through a 100-µm nylon mesh. Finally, cells were resuspended in complete MesenCult (Stem Cell) medium containing 100 units/ml penicillin/streptomycin (Gibco, Invitrogen) and 20% mouse mesenchymal supplements (Stem Cell) and plated at a density of 15,000-30,000 cells/cm^2 and cultured at a 20% O_2/5% CO_2 atmosphere. Non-adherent cells were removed after 24 hours in culture. Subsequent passages were plated at 10,000 cells/cm^2 in complete MesenCult medium. ASCs were used between passage 3-7. These cells showed a fibroblast-like morphology and differentiation capacity to the adipocytic and osteocytic lineages. To obtain ASC conditioned media (ASCcm), ASC were plated at 10,000

cells/cm^2 in T75 flasks in 13 ml complete MesenCult medium. After 3 days, the ASCcm was collected, filtered using 0.22 μm pore filters and used the same day.

Human adipose-derived ASCs (hASCs) were obtained from lipoaspirates of adipose tissue. Lipoaspirates were washed twice with PBS to remove contaminating debris and red blood cells, and digested at 37ºC for 30 minutes with 0.075% collagenase (Type I, Invitrogen) in PBS. The digested sample was washed with 10% of fetal bovine serum (FBS), treated with 160 mM $ClNH_4$ to eliminate remaining red blood cells, suspended in complete DMEM medium (DMEM containing 10% fetal bovine serum, 2 mM glutamine and 1% penicillin/streptomycin) and filtered through a 100-μm nylon mesh. Cells were seeded (2-3x10^4 cells/cm^2) onto tissue culture flasks and expanded at 37ºC and 5% CO_2, changing the culture medium every 3-4 days. Cells were passed to a new culture flask (1,000 cells/cm^2) when cultures reached 90% of confluence. A total of six different samples with population doublings 6-9 were used in the study.

Macrophage isolation and culture

Murine macrophages were generated from bone marrow from Balb/c, C57Bl/6 or IL-10KO mice. Briefly, bone marrow cells (0.4 x 10^6/ml) were cultured in DMEM (2 mM L-Glutamine, 100 units/ml penicillin/streptomycin and 20% heat-inactivated FCS, all from Gibco/Invitrogen) containing 20 ng/ml M-CSF (Peprotech) for 7-8 days. Differentiated macrophages were detached by incubating the plates with 2mM EDTA/PBS at 37ºC for 10 minutes. Cell preparations generally consisted of >95% $CD11b^+CD11c^-$ macrophages. Bone marrow derived macrophages were plated at 1.5 x 10^5 cells/well in 24-well plates or at 3 x 10^5 cells in transwell inserts (Milipore) or 8 x 10^5 cells/well in 6-well plates. After 4 hours of adherence, macrophages were washed with PBS and stimulated with IFNγ (BD Bioscience; 10 ng/ml) and LPS (Sigma; isotype B4:11, 1 μg/ml) or IL-4 (BD Bioscience; 10 ng/ml) or ASC-conditioned media

(ASCcm) or cocultured with ASCs for 24 and 48 hours. Macrophages cultured in complete MesenCult served as control cells for all ASC- or ASCcm-treated macrophages.

To determine the suppressive activity of macrophages on T cells, Balb/c splenocytes (2 x 10^5 cells/well) were stimulated with concanavalin A (ConA, 2.5 μg/ml) in flat-bottomed 96-well plates in complete RPMI-1640 medium (2 mM L-Glutamine, 100 units/ml penicillin/streptomycin, 50 μM 2-mercaptoethanol and 10% heat-inactivated FCS, all from Gibco/Invitrogen) in the presence of different numbers of syngeneic macrophages previously stimulated as described above. Cells were cultured for 72 hours, pulsed with 0.5 μCi/well [^{3}H]-thymidine (Perkin Elmer) for the last 6 hours of culture and harvested onto glass fiber filters using an LKB 96 well-harvester (Wallac Oy, Turku, Finland) and the uptake of [^{3}H]-thymidine was measured on a 1450 microbeta Trilux scintillation counter (Wallac). In some experiments, a neutralising anti-IL-10 antibody (20 μg/ml, BD Bioscience) was added to co-cultures.

To determine the suppressive activity of macrophages on inflammatory macrophages, peritoneal macrophages ($5x10^4$) were isolated from Balb/c mice with sepsis and cultured with medium or stimulated with LPS (1 μg/ml) in the absence or presence of ASC-MΦ ($5x10^4$). When indicated, a neutralising anti-IL-10 antibody (20 μg/ml) was added to co-cultures. After 24 h, cytokine contents in the supernatants were determined by ELISA.

The human monocytic cell line THP-1 was maintained in complete RPMI 1640. For co-culture experiments, THP-1 cells (2.5 x 10^5/ml) were first differentiated into adherent macrophages with PMA (50 ng/ml) in 24-well plates. After 24 hours, human ASCs (1 x 10^5 cells) or human ASC-conditioned medium were added to the adherent THP-1 macrophages. After 48 hours, cells were analyzed for arginase activity or stimulated with LPS (1 μg/ml) for another 24 hours. Supernatants were collected for cytokine determination by ELISA.

Measurement of arginase activity

To determine the arginase activity, cells were washed with PBS and lysed by adding 2 ml lysis buffer/10^6 cells (25 mM Tris-HCl, pH 7.4; containing 0,1% Triton X-100 and protease inhibitors) on a shaker for 30 minutes at room temperature. One volume of Tris-HCl (25 mM, pH 7.4) was added to the lysates and the enzyme was heat activated by incubating 100 µl lysate with 10 µl $MnCl_2$ (10 mM) at 56°C for 10 minutes. L-arginine (500 mM, pH 9.7; 100 µl/sample) was then added to the activated lysate and L-arginine hydrolysis was carried out at 37ºC for 40 minutes. The reaction was stopped by the addition of 0.9 ml of an acid mixture containing H_2SO_4 (96%), H_3PO_4 (85%) and H_2O (1:3:7, v/v/v). The urea formed was colorimetrically quantified at 540 nm after the addition of 40 µl 9% α-isonitrosopropiophenone (ISPF; Sigma Aldrich; dissolved in 100% ethanol) and heating at 100°C for 20 min. Urea (Sigma) was used for the standard curve. The protein concentrations of cell lysates were measured using the bicinchoninic acid (BCA) assay. One unit of arginase activity is defined as the amount of enzyme that catalyzes the formation of 1 mmol of urea per minute.

Measurement of nitrite production

To assess iNOS activity, supernatants from control and stimulated macrophages were checked for nitrite contents using the Griess assay. In brief, 100 µl of Griess reagent (a 1/1 mixture of 1% p-aminobenzene-sulfonamide in 5% H_3PO_4 and 0.1% naphtylethylene-diamine dihydrochloride in distilled H_2O) were added to 100 µl culture supernatants and standard ($NaNO_2$) in 96-well plates. Plates were incubated at room temperature for 10 min, and absorbance was measured at 550 nm.

Cytokine and PGE2 determination

For cytokine determination in colonic mucosa, protein extracts were isolated by homogenization of colon segments (50 mg tissue/ml) in 50 mM Tris-HCl, pH 7.4, with 0.5 mM DTT, and 10 µg/ml of a cocktail of proteinase inhibitors containing phenylmethylsulfonyl

fluoride, pepstatin and leupeptin (Sigma). Samples were centrifuged at 20,000g for 15 min at 4ºC and the supernatants were stored at –80˚C until cytokine/chemokine determination. Cytokine and chemokine levels in the serum, colonic protein extracts and culture supernatants were determined by specific sandwich ELISAs using capture/biotinylated detection antibodies from BD Pharmingen according to the manufacture's recommendations. The PGE2 secretion by ASCs was measured in cell culture supernatants using the PGE2 ELISA Kit (Cayman Chemicals) according to the manufacturer´s instruction.

RT-PCR

Bone marrow derived macrophages (0.8 x 10^5 cells/well in 6-well plates) were stimulated with IL-4 (10 ng/ml) or MSCcm and total RNA was extracted using ultraspec (Biotecx) before and after 3, 10 and 24 hours of culture. The RNA (1 μg/sample) was reverse transcribed using M-MuLV RT (ROCHE) and random hexamer primers. Semiquantitative PCR was performed on an Eppendorf PCR machine and amplified products run on a 2% agarose gel. Quantitative PCR was performed on a Bio-Rad system using the Supermix reagent (Biorad). Primer pairs: IL-10 FW: 5´-GGTTGCCAAGCCTTATCGGA-3´; IL-10 RV: 5´-ACCTGCTCCACTGCCTTGCT-3´; Ym1 FW: 5´-TGTACTCACCTGATCTATGC-3´; Ym1 RV: 5´-CGGAGAGAGTGCCCCTACTA-3´; Fizz1 FW: 5´-ACTTGTTCCCTTCTCATCTG-3´; Fizz1 RV: 5´-TCCACCTCTTCATTCTTAGG-3´; Arginase II FW: 5´-TGTGTATCCTCGTTCAGTGG-3´; Arginase II RV 5´-AGAGGCAAGGTTTGATCCAG-3´; Arginase I FW: 5´- CAGAAGAATGGAAGAGTCAG-3´; Arginase I RV: 5´-CAGATATGCAGGGAGTCACC-3´; LIGHT FW: 5´-CTGCATCAACGTCTTGGAGA-3´; LIGHT RV: 5´-GATACGTCAAGCCCCTCAAG-3´; CCL-22 FW: 5´-TGGAAGACAGTATCTGCTGC-3´; CCL-22 RV: 5´-AGTAGCTTCTTCACCCAGAC-3´; HO-1 FW: 5´-TGCTCGAATGAACACTCTGG-3´; HO-1 RV: 5´-TGTCTGGGATGAGCTAGTGC-3´ ; TNF-alpha FW: 5´-CCCTCACACTCAGATCATCTTCT-3´; TNF-alpha RV: 5´-GCTACGACGTGGGCTACAG-3´; iNOS FW: 5´-GTTCTCAGCCCAACAATACAAGA-3´; iNOS RV: 5´-GTGGACGGGTCGATGTCAC-3´; IL-12p40 FW: 5´-

TGGTTTGCCATCGTTTTGCTG-3´; IL-12p40 RV: 5´-ACAGGTGAGGTTCACTGTTTCT-3´; beta-actin FW: 5´-AATCGTGCGTGACATCAAAG-3´; beta-actin RV: 5´-ATGCCACAGGATTCCATACC-3´.

Induction of colitis with TNBS

Acute colitis was induced in male Balb/c mice (8-weeks, Charles River) by a single injection of high dose of trinitrobenzene sulfonic acid (TNBS, Signma). Mice were lightly anesthetized with Isoflurane and 100 μL of TNBS (3 mg in 50% ethanol) was slowly administered into the colon through a 3.5F catheter inserted intrarectally 4 cm from the anus. Control mice received 50% ethanol alone (100 μL). Immediately after TNBS infusion, mice were injected intraperitoneally (i.p.) with 10^6 syngeneic macrophages (from wild-type Balb/c mice or from IL-10KO mice) cultured for 48h in complete Mesencult (control-MΦ) or in ASCcm (ASC-MΦ) or with PBS (vehicle control). Mice injected i.p. with syngeneic mASCs (10^6 cells/mouse) were used as reference control. For the therapeutic treatment regime, acute colitis was induced with 2.5 mg TNBS and mice were injected with syngeneic control-MΦ or ASC-MΦ (10^6 cells/mouse) 24h or 48h after TNBS administration. Animals were monitored daily for body weight loss and survival. At the peak of the disease (day 3), serum, colon and mesenteric lymph nodes (MLNs) were collected. Cytokine contents in sera and MLN cultures were determined by ELISA as described below. Colons were graded for macroscopic lesions (scale 0–10) based on criteria reflecting inflammation (ie, hyperaemia, bowel thickening, and extent of ulceration), and colonic segments were then frozen on dry ice for protein/RNA extraction or fixed in phosphate-buffered formalin, embedded in paraffin, sectioned, stained with eosin/hematoxylin and histopathologically evaluated as described below.

Chronic colitis was induced by repetitive weekly injections of increasing low doses of TNBS. Mice were lightly anesthetized with Isoflurane and 100 μL of TNBS (0.8 mg at day 1, 1 mg at day 7, 1.2 mg at day 14 and 1.5 mg at day 21; all dissolved in 50% ethanol) were slowly administered into the colon as described above. Control mice received 50% ethanol alone (100

μL) at the same days. Mice were injected intraperitoneally (i.p.) with 10^6 ASC-MΦ or with PBS (vehicle control) on day 8 (single delayed treatment) or on days 8 and 16 (double delayed treatment). Animals were monitored daily for body weight loss and survival.

Induction of colitis with DSS

Acute colitis was induced in male C57Bl/6 mice (7-weeks) by administering 5% dextran sulfate sodium (DSS; molecular weight 10,000 Da; Sigma) from day 0 to day 7 in the drinking water ad libitum. At day 2, mice were i.p. infused with 10^6 syngeneic control-MΦ or ASC-MΦ or with PBS (vehicle control). Colitis severity was assessed daily by scoring (0–4) the clinical disease activity by evaluating stool consistency, presence of faecal blood and weight loss as described below. At day 5, the entire colon was removed from the caecum to the anus, and colon length and weight were measured as indirect inflammation markers. The macroscopic colonic damage score was assessed based on the grade of tissue adhesion, presence of ulceration and wall thickness as described below.

Chronic recurrent-remitting colitis was induced in male C57Bl/6 mice (7-8 weeks) by administering 3% DSS in the drinking water in a cyclic manner, which consisted of two cycles of 7 days with DSS, followed by a 7-day period without DSS supplementation. On day 4 or on days 4 and 14, mice were injected intraperitoneally with syngeneic ASC-MΦ (10^6 cells/mouse) or with PBS (vehicle control). Colitis severity was assessed daily by scoring (0–4) the clinical disease activity by evaluating stool consistency, presence of faecal blood and weight loss as described below.

Induction of sepsis

To induce sepsis, caecum of anesthetised Balb/c mice was ligated 5.0 mm from the caecal tip, punctured once with a 22-gauge needle and the stool then extruded (1 mm). Mice were

treated intraperitoneally with PBS or with 10^6 syngeneic control-MΦ or ASC-MΦ from wild-type Balb/c mice or with 10^6 syngeneic ASC- MΦ from IL-10KO mice, 4 hours after caecum perforation, and monitored for survival. Serum, peritoneal exudates, liver, lungs and small intestines were collected at 18h for histopathology examination and cytokine and MPO activity determinations as described below. To determine the therapeutic window of ASC-MΦ in sepsis, 10^6 syngeneic ASC-MΦ were intraperitoneally injected 6, 12 or 24 hours after caecum perforation and survival was monitored.

Analysis of biodistribution of ASC-MΦ

To trace the injected cells *in vivo*, ASC-MΦ were labelled with 5 μM 5,6-carboxy-succimidyl-fluoresceine-ester (CFSE, Molecular Probes) in PBS/0.1% BSA for 10 min at 37ºC, and after extensive washing in DMEM medium/10% FBS, cells were injected i.p. in 5% DSS-treated animals. At different time points, single-cell suspensions were isolated from spleen, MLNs and various colon segments (inflamed and non-inflamed), following digestion with DNase I (0.1 mg/ml, Sigma) and type IV collagenase (400 U/ml, Sigma) for 20 min at 37ºC in continuous shaking. After filtration, cells were stained with PerCP-anti-mouse CD11b monoclonal antibodies (BD Pharmingen, 5 μg/ml final concentration) at 4^oC for 1 h. After extensive washing, the percentages of $CFSE^+$ $CD11b^+$ ASC-MΦ were determined by flow cytometry on a FACScalibur flow cytometer, and expressed as the number of cells per 100 mg of tissue.

Myeloperoxidase (MPO) assay

Neutrophil infiltration in different tissues was monitored by measuring MPO activity. Briefly, colonic segments, lung and liver from DSS-treated and septic mice were homogenized at 50 mg/ml in phosphate buffer (50 mM, pH 6.0) with 0.5% hexadecyltrimethylammonium bromide. Samples were frozen and thawed 3 times, centrifuged at 30,000g for 20 minutes. The supernatants were diluted 1:30 with assay buffer consisting in 50 mM phosphate buffer pH 6.0

with 0.167 mg/ml o-dianisidine (Sigma) and 0.0005% H_2O_2, and the colorimetric reaction was measured at 450 nm between 1 and 3 min in a spectrophotometer (Beckman Instruments, Irvine, CA). MPO activity per gram of wet tissue was calculated as: MPO activity (U/g wet tissue) = (A_{450}) (13.5)/tissue weight (g), where A_{450} is the change in the absorbance of 450nm light from 1 to 3 min after the initiation of the reaction. The coefficient 13.5 was empirically determined such that 1U MPO activity represents the amount of enzyme that will reduce 1 μmol peroxide/min.

Determination of peritoneal cell suspension

The peritoneal suspension was isolated by lavage with 3 mM EDTA/PBS 18 hours after sepsis induction. Peritoneal suspension was then centrifuged 5 min at 1800*g* and the number infiltrating peritoneal cells were determined in a haemocytometer.

Lamina propria mononuclear cell isolation and culture

To isolate lamina propria mononuclear cells (LPMC), freshly obtained colons were washed in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) medium and then treated with 1 mM EDTA/PBS for 20 minutes to remove the epithelium. The tissue was then digested with type IV collagenase and DNase I (Sigma Chemical Co) for 20 minutes in a shaking incubator at 37°C. Released cells were layered on a 30%–70% Percoll gradient (Amersham Pharmacia, Uppsala, Sweden) and spun at 1800 rpm to obtain the leukocyte-enriched population at the 30%– 70% interface. LPMCs were cultured in complete RPMI 1640 medium for 48 h and the cytokine contents in the culture supernatants determined by ELISA.

Mesenteric lymph node cell isolation and culture

Single-cell suspensions were obtained from mesenteric lymph nodes (MLN) freshly collected at the peak of the disease. MLN cells were enriched in T cells by incubating MLN cells in petri dishes for 2h at 37°C to remove nonadherent cells. LPMC and MLN cells were incubated in complete RPMI 1640 medium at a concentration of 10^6 cells/ml, in the absence (unstimulated) or presence of PMA (10 ng/mL) and Con A (2.5 μg/ml). After 48 h culture, supernatants were assayed for cytokine/chemokine contents by ELISA, and after 72h culture, cells were pulsed with 0.5 μCi/well [^{3}H]-thymidine (Perkin Elmer) for the last 6h of culture and harvested onto glass fiber filters using an LKB 96 well-harvester (Wallac Oy, Turku, Finland) and the uptake of [^{3}H]-thymidine measured on a 1450 microbeta Trilux scintillation counter (Wallac).

Determination of histopathology in colons of TNBS-colitic mice

Histopathology was graded in colon sections by two blinded pathologists from 0 to 4 as follows: 0, no signs of inflammation; 1, low leukocyte infiltration; 2, moderate leukocyte infiltration; 3, high leukocyte infiltration, moderate fibrosis, high vascular density, thickening of the colon wall, moderate goblet cell loss, focal loss of crypts; and 4, transmural infiltrations, massive loss of goblet cells, extensive fibrosis, diffuse loss of crypts.

Determination of macroscopic colonic lesions in TNBS-induced colitis

The macroscopic colonic damage score (scale 0-10) was assessed based on the grade of tissue adhesion, presence of ulceration and wall thickness by two independent researchers in a blinded fashion: ulceration (0 = normal appearance, 1 = focal hyperaemia, no ulcers, 2 = ulceration without hyperaemia or bowel wall thickening, 3 = ulceration with inflammation at 1 site, 4 = two or more sites of ulceration and inflammation, 5 = major sites of damage extending > 1 cm along length of colon), adhesions (0 = no adhesions, 1 = minor adhesions, colon can be

easily separated from the other tissues, 2 = major adhesions), thickness (maximal bowel wall thickness, in mm, measured with a calliper).

Determination of clinical disease activity in DSS-induced colitis

The clinical disease activity (scale 0-4) was determined by two blinded observers by evaluating stool consistency, presence of faecal blood and weight loss, including a summation of the three components: weight loss (0 = 0%, 0.5 = 1–10%, 1 = 11–15%, 1.5 = 16–20%, 2 = >20%), diarrhoea (0 = normal stool, 0.5 = soft stool and minimal wet anal fur/tail, 1 = diarrhoea and moderate-to-severe wet anal fur/tail), and frank rectal bleeding (0 = absent, 0.5 = present but minimal, 1 = moderate/severe).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1.- The presence of macrophages increases the suppressive activity in ASCs-T cell co-cultures. CD4 T cells ($5x10^4$) isolated from lymph nodes of Balb/c mice were co-cultured with ASCs (10^4 cells) in the absence or presence of increasing numbers of Balb/c bone marrow-derived macrophages. T cell activation was followed by the production of IFNγ (at 48h) and proliferation (at 72h). Results are the mean (SD) of three experiments performed in duplicate. *p<0.01 vs samples without macrophages.

Supplementary Figure 2.- ASC-conditioned media induces the conversion of macrophages into a regulatory phenotype. A. ASC-conditioned media stimulates arginase activity in macrophages. Macrophages ($1.5x10^5$) were cultured with medium (control) or stimulated with IL-4 (10 ng/ml) or ASC-conditioned media (ASCcm). After 48h, arginase activity was determined. *p<0.05; **p<0.01 vs control. n=3 experiments performed in duplicate. **B.** Macrophages were cultured in medium (control) or activated with IFNγ (10 ng/ml) plus LPS (1 μg/ml), IL-4 (10 ng/ml) or ASCcm for 24h and 48h and the levels of nitrite and cytokines were

determined in the culture supernatants. The ASCcm did not contain detectable levels of nitrite, IL-12 or TGF-β1. *p<0.05 vs control. n=3-4 experiments performed in duplicate. **C.** Macrophages were cultured with medium (control), IL-4 (10 ng/ml) or ASCcm for 48h. Cells were then harvested and restimulated with LPS (1 μg/ml). After 4h of restimulation, gene expression of iNOS, IL-12, IL-10 and TNFα was determined by qPCR. After 24h of restimulation, culture supernatants were checked for IL-10 and nitric oxide production.*p<0.05 vs control. n=3 independent experiments performed in duplicate.

Supplementary Figure 3.- ASCs constitutively secrete PGE2 and their regulatory effect on macrophages is IL-4-independent. **A.** Production of PGE2 by cultured ASC ($2x10^5$ cells) at different times. **B.** Macrophages ($1.5x10^5$) were cultured in medium (control) or stimulated with IL-4 (10 ng/ml) in the absence or presence of anti-IL-4 neutralizing antibodies (10 μg/ml) for 48h. Arginase activity was measured in cell lysates. **C.** Indomethacin did not affect the induction of arginase in ASCcm-cultured macrophages. Macrophages ($8x10^5$) were cultured with medium (MΦ only) or with ASCcm in the absence or presence of indomethacin (Indo). After 48h, arginase activity was measured in cell lysates. Results are the mean (SD) of three independent experiments performed in duplicate.

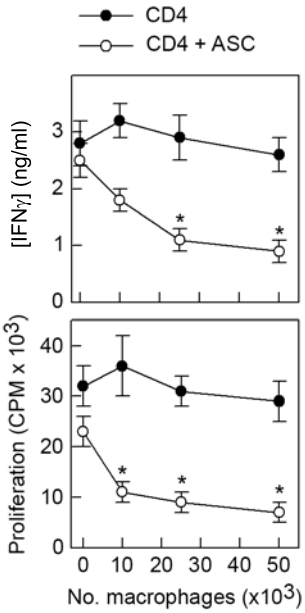
Supplementary Figure 4.- Human ASC-conditioned medium does not induce arginase activity in human monocytes. THP-1 cells ($2.5x10^5$ cells/ml) were stimulated with PMA (50 ng/ml) for 24h to induce macrophage differentiation. The adherent cells were then washed and cultured with complete DMEM medium or human ASC conditioned medium or human ASC ($1x10^5$ cells/ml). After 48h, cells were analyzed for arginase activity (*left panel*) or cultured for another 24h with or without LPS (1 μg/ml) (*medium and right panels*). Supernatants were collected and analyzed for IL-10 and IL-12 levels. Results are the mean (SD) of three independent experiments performed in duplicate. *p<0.05 vs medium-treated controls.

Supplementary Figure 5.- Therapeutic effect of ASC-MΦ on established colitis. Colitis was induced in mice by intracolonic administration of TNBS (2.5 mg/mouse) in 50% ethanol. Mice were treated intraperitoneally with PBS (vehicle), control-MΦ or ASC-MΦ, 24h or 48h after TNBS infusion (n=10 mice/group, performed in two independent experiments). *p<0.05 vs untreated TNBS-colitic mice.

Supplementary Figure 6. ASC-MΦ regulate immune cells of lamina propria and mesenteric lymph nodes in colitic mice. (**A**) Mice received 5% DSS with their drinking water from day 0 to day 7. Control-MΦ or ASC-MΦ were infused intraperitoneally on day 2. Lamina propria cells were isolated at day 7, cultured without stimulation for 24h and the cytokine contents determined in culture supernatants. Mice receiving tap water instead of DSS were used as controls (naive). (**B-C**) Colitis was induced by intrarectal infusion of 3 mg TNBS. Mice were treated intraperitoneally with PBS or ASC-MΦ immediately after TNBS infusion. Mice treated with 50% ethanol were used as basal control. Single cell suspensions from mesenteric lymph nodes (MLN) were isolated at the peak of the disease (day 3) and cultured (10^6 cells/ml) with complete RPMI 1640 medium or stimulated with PMA (10 ng/ml) plus Concanavalin A (ConA, 2.5 μg/ml). Cytokine contents in culture supernatants (**B**) and the proliferative response (**C**) were determined at 48h and 72h, respectively. Results are the mean (SD). n=4-5 mice per group, each mouse analysed in duplicate. *p<0.05 vs untreated colitic mice.

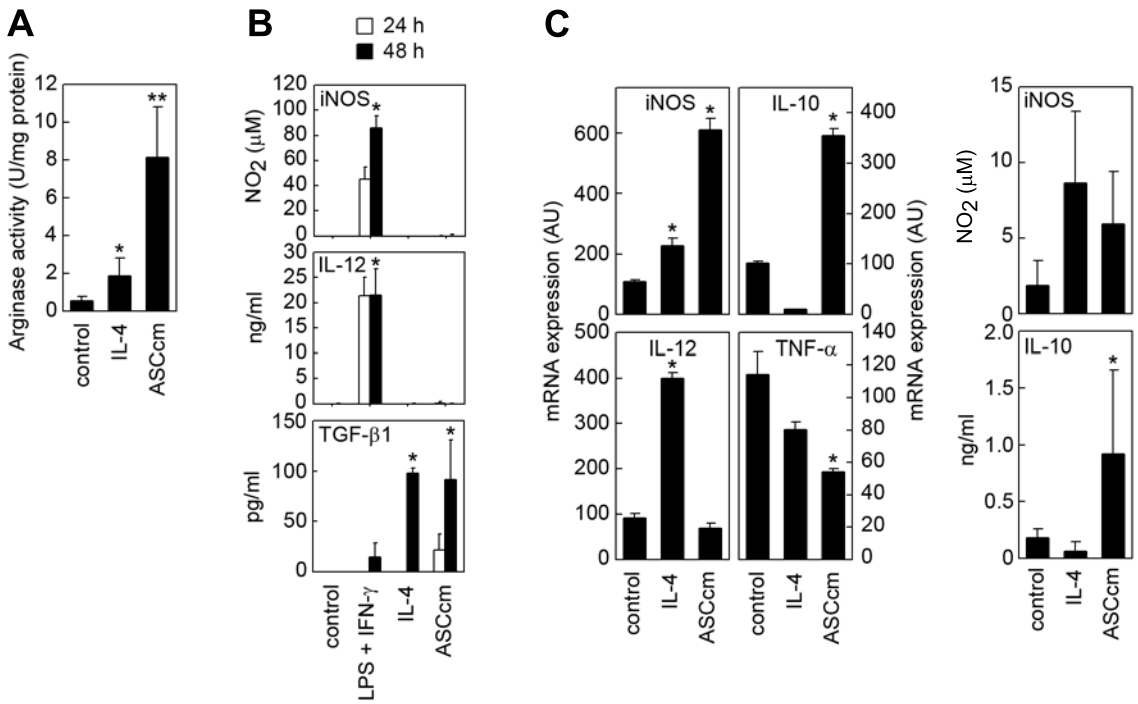
Supplementary Figure 7. ASC-MΦ from diseased donors show similar anti-inflammatory and protective actions than ASC-MΦ from healthy donors. Peritoneal macrophages were isolated from naïve Balb/c mice (ASC-MΦ healthy), from Balb/c mice subjected to CLP-induced sepsis (12 h after CLP; ASC-MΦ septic) or from Balb/c mice subjected to TNBS-induced colitis (2 days after TNBS infusion; ASC-MΦ septic) and cultured for 24h with ASCcm. **A.** Peritoneal

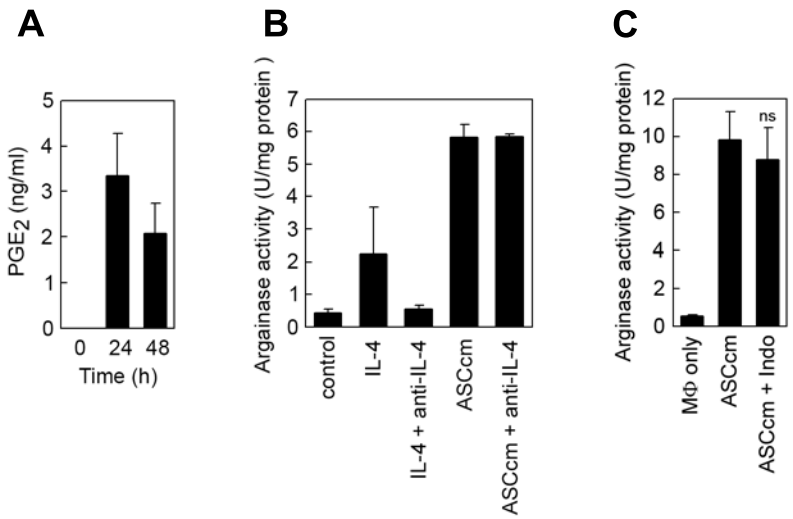
macrophages ($5x10^4$) isolated from naïve Balb/c mice were stimulated with LPS (0.5 μg/ml) in the absence or presence of ASC-MΦ healthy or ASC-MΦ septic ($5x10^4$). After 24 h, cytokine contents in the supernatants were determined by ELISA. Data is shown as mean (SD). n=3-4 experiments performed in duplicate. *p<0.01 vs untreated controls. **B.** Mice with CLP-induced sepsis were injected intraperitoneally with ASC-MΦ healthy or ASC-MΦ septic (10^6 cells/mouse) 4 hours after CLP and monitored for survival. n=8-10 mice/group, performed in two separate experiments. *p<0.001 vs. untreated septic mice. **C.** Mice with TNBS-induced colitis were injected intraperitoneally with ASC-MΦ healthy or ASC-MΦ colitic (10^6 cells/mouse) 6 hours after TNBS-infusion and disease progression was evaluated by body weight loss. n=9-12 mice/group, performed in two independent experiments. *p<0.001 vs. untreated TNBS-infused mice.



Supplementary Figure 1

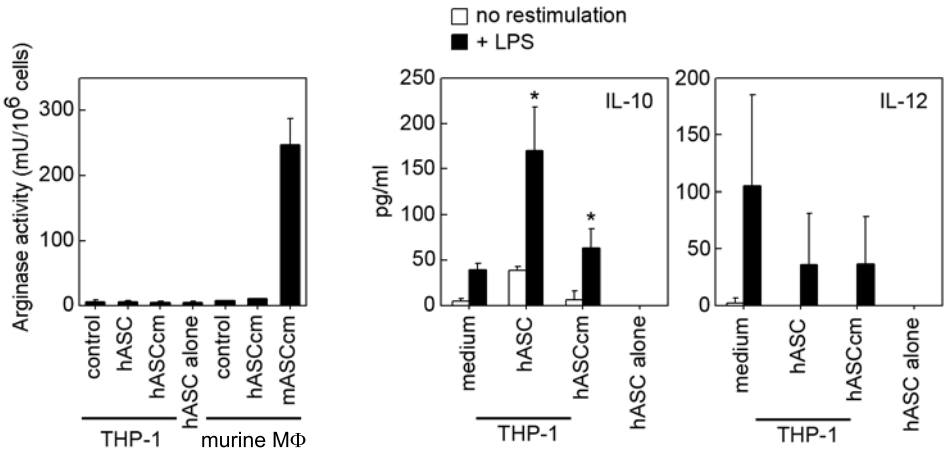
Supplementary fig. 2





Supplementary Figure 3

Supl. fig. 4


Arginase activity (mU/10^6 cells)
300
200
100
0
control
hASC
hASCcm
hASC alone
control
hASCcm
mASCcm
THP-1
murine MΦ
no restimulation
+ LPS
pg/ml
250
200
150
100
50
0
IL-10
*
*
medium
hASC
hASCcm
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200
150
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50
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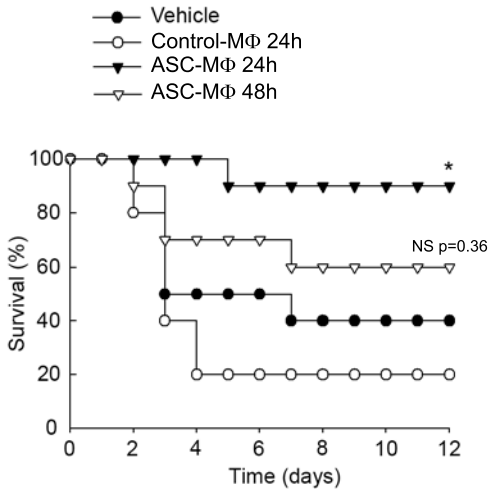
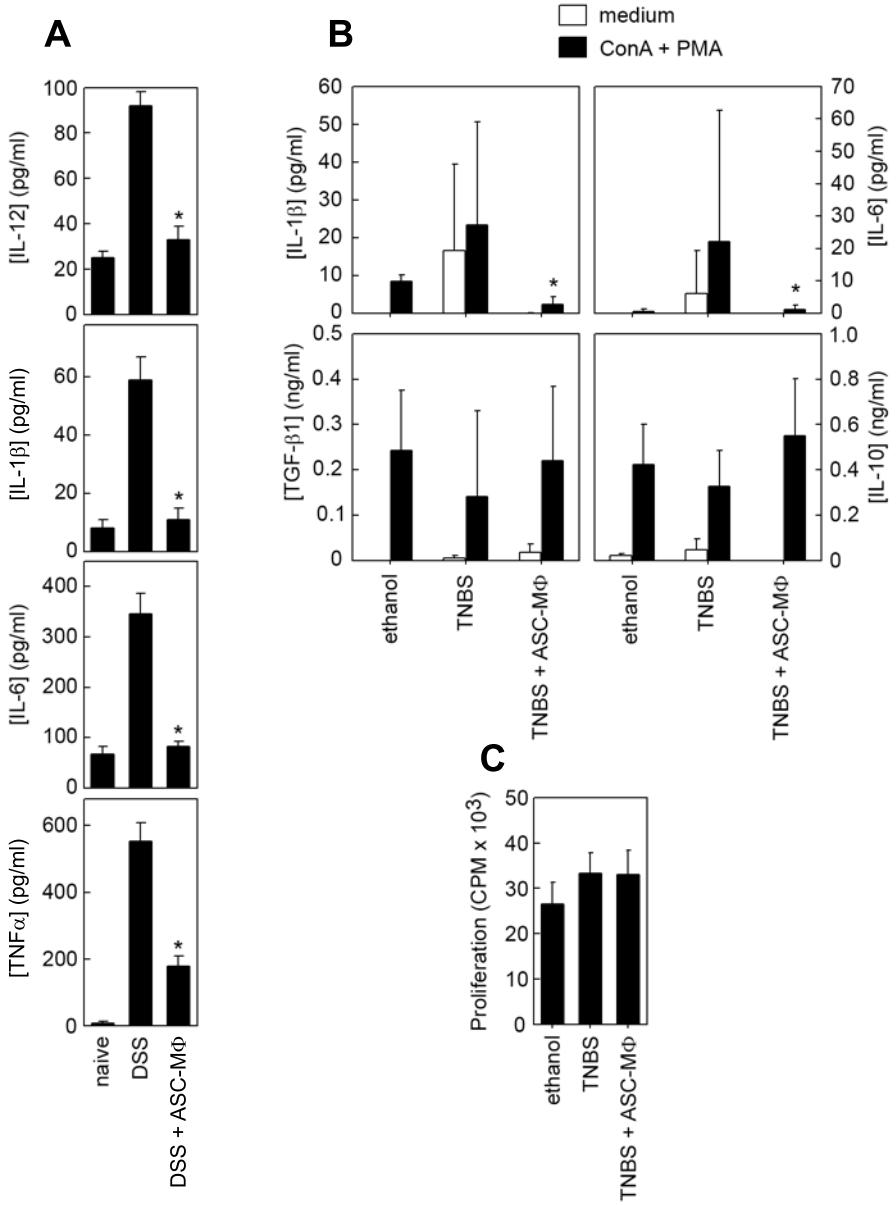
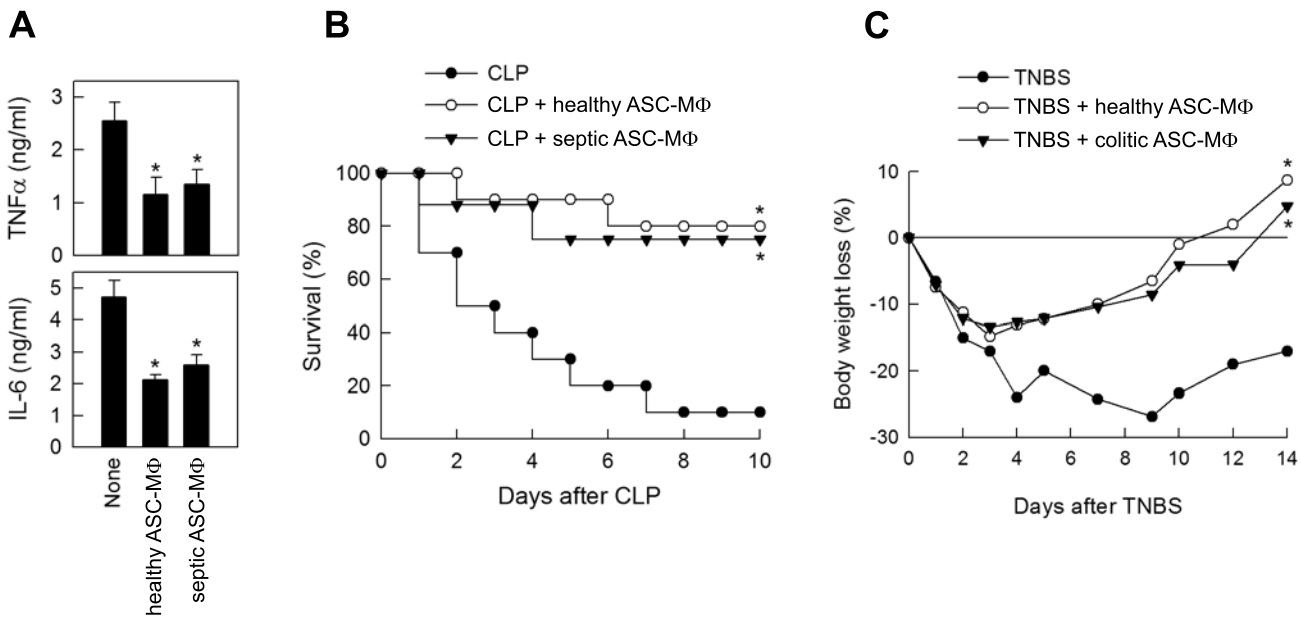
THP-1



Supplementary fig. 5



Supplementary fig. 6



Supplementary fig. 7