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## IL-34 and M-CSF induce macrophages that promote the generation of Th17 cells via membrane IL-1 $\alpha$

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## **IL-34- and M-CSF-induced macrophages switch memory T cells into Th17 cells via membrane IL-1 $\alpha$**

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Abbreviations: M $\phi$ , macrophages; mIL-1 $\alpha$ , membrane IL-1 $\alpha$ ; TAM, tumor-associated macrophages.

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## Abstract

Macrophages (M $\phi$ ) orchestrate the immune response via the polarization of CD4<sup>+</sup> T helper cells. Different subsets of M $\phi$  have been described, with distinct phenotypes and sometimes opposite functions. M-CSF and IL-34 induce the differentiation of monocytes into IL-10<sup>high</sup> IL-12<sup>low</sup> immunoregulatory M $\phi$  which are similar to tumor-associated macrophages (TAM) in ovarian cancer. In this study, we evaluated the capacity of M $\phi$  generated in the presence of M-CSF (M-CSF-M $\phi$ ) or IL-34 (IL-34-M $\phi$ ) and of ovarian cancer TAM, to polarize human CD4<sup>+</sup> T cells. Results showed that, unexpectedly, M-CSF-M $\phi$ , IL-34-M $\phi$  and TAM promote the generation of Th17 cells, contrary to the pro-inflammatory IL-12<sup>high</sup> IL-10<sup>low</sup> GM-CSF-M $\phi$  which favour the generation of Th1 cells. More precisely, these M $\phi$  subsets switch non-Th17 committed memory CD4<sup>+</sup> T cells into conventional CCR4<sup>+</sup> CCR6<sup>+</sup> CD161<sup>+</sup> Th17 cells, expressing or not IFN- $\gamma$ . Finally, the generation of Th17 cells is mediated via membrane IL-1 $\alpha$  which is constitutively expressed by the three M $\phi$  subsets. In conclusion, this study demonstrates that human M-CSF-M $\phi$ , IL-34-M $\phi$  and TAM, initially considered as anti-inflammatory cells, induce in vitro Th17 cells via the constitutive expression of membrane IL-1 $\alpha$ . This process may contribute to maintain locally a restrained and smoldering inflammation required for angiogenesis and metastasis.

## Introduction

Macrophages (M $\phi$ ) have a central role in numerous physiological processes, such as control of infection, wound healing, inflammation and tissue homeostasis. Tissues-resident M $\phi$  (such as microglia, Kupffer cells and osteoclasts) are found in connective tissues and in every organ in the body [1]. Moreover, upon danger signals, monocytes are rapidly recruited locally, where they differentiate into M $\phi$  [1]. Cells of the monocyte-macrophage lineage are characterized by remarkable plasticity and diversity [2, 3]. They rapidly respond to local signals, such as cytokines, by acquiring distinct phenotypes and functions. Accordingly, different subsets of M $\phi$ , with sometimes opposite functions (i.e. anti- versus pro-inflammatory, immuno-stimulatory versus -suppressive), have been described [2]. At the site of injury, M $\phi$ , that exhibit potent phagocytic properties, engulf apoptotic cells and pathogens. Depending on the nature of the effector molecules they express, these professional antigen-presenting cells (APC) instruct immune cells and modulate the nature and intensity of immune responses. M $\phi$  may also contribute to the pathogenesis of severe diseases, such as fibrosis and tumor-induced immunosuppression [2, 4]. Currently, to characterize the factors involved in the generation and acquisition of functional properties by the different M $\phi$  subsets is crucial to determine their respective roles in physiological and pathological conditions.

Although different classifications of M $\phi$  subsets have been proposed [1], the most commonly used is based on the Th1/Th2 dichotomy and describes two states of M $\phi$  polarization (called M1 and M2), which represent extremes of a spectrum of polarization profiles [2]. M1 cells stimulated with IFN $\gamma$ , alone or in combination with microbial moieties, produce high levels of IL-12 (IL-12<sup>high</sup>) and low levels of IL-10 (IL-10<sup>low</sup>). They produce pro-inflammatory cytokines and nitrogen and oxygen intermediates, and have potent anti-microbial and tumoricidal properties. At the opposite, M2 cells, initially described as M $\phi$  exposed to IL-4 or IL-13, exhibit an IL-10<sup>high</sup> IL-12<sup>low</sup> phenotype. IL-10, glucocorticoids, immune complexes, and M-CSF also induce different M2 subsets with phenotypes distinct to the one of IL-4-M $\phi$ . M2 cells have potent phagocytic properties and are involved in physiological (tissue remodelling, immune homeostasis) and pathological (tumor-associated immunosuppression, tumor growth) processes.

In human, three cytokines, GM-CSF, M-CSF, and IL-34, promote monocyte survival and differentiation into M $\phi$  [5]. M $\phi$  generated in the presence of GM-CSF (GM-CSF-M $\phi$ ) produce IL-12, IL-23, and pro-inflammatory cytokines, but low levels of IL-10 [6, 7]. They exhibit T cell costimulatory [6], anti-tumoral and microbicidal properties [2]. GM-CSF-M $\phi$  are usually referred to as M1 cells (even in the absence of IFN $\gamma$  stimulation). IL-34 and M-CSF, which signal via the M-CSF receptor (c-fms or CD115), induce the differentiation of human monocytes into M $\phi$  with closely similar phenotypes [8]. These M $\phi$  subsets are IL-

$10^{\text{high}}$  IL-12 $^{\text{low}}$  M2 cells that do not produce IL-23 and express low levels of MHC-II, CD80 and CD86 [8, 9]. As a consequence, M-CSF-M $\phi$  and IL-34-M $\phi$  exhibit low T cell costimulatory properties and dampen the activity of effector T cells [8, 9].

In solid tumors, tumor-associated macrophages (TAM) are by far the most abundant immunosuppressive leukocyte population in the tumor microenvironment. In numerous cancers, their density is correlated with a poor prognosis [10]. In addition to immunoregulatory properties, TAM favor angiogenesis and promote tumor-cell progression and metastasis via the production of growth and angiogenic factors, proteases, and chemokines [4, 9, 11]. In previous studies, we have shown that IL-34-M $\phi$  and M-CSF-M $\phi$  exhibit most of the phenotypic (i.e. CD14 $^{\text{high}}$  CD163 $^{\text{high}}$  IL-10 $^{\text{high}}$  IL-12 $^{\text{low}}$  CD86 $^{\text{low}}$ ) and functional characteristics (i.e. low T cell-costimulatory properties, inhibition of activated effector T cell functions) of TAM isolated from human ovarian cancer [8, 9].

CD4 $^+$  T cells, commonly referred to as "helper" T (Th) cells, orchestrate immune responses. Upon signals provided by dendritic cells, inexperienced naive CD4 $^+$  T cells become activated and differentiate into various Th cell subsets with distinct cytokine profiles and functions (such as Th1, Th2, Th17 and regulatory T [Treg] cells). There is increasing evidence that memory CD4 $^+$  T cells retain unexpected flexibility and plasticity and can adapt their phenotypes and functions, depending on the signals received [12, 13]. However, the factors that govern memory T cell polarization remain poorly defined. In inflammatory lesions, infiltrating memory T cells interact with resident and newly recruited M $\phi$  which control, locally, their polarization and effector functions [3]. As an example, murine M $\phi$  from the red pulp and from the gut lamina propria favor the generation of Foxp3 $^+$  Treg cells [14, 15]. Although human M1 and M2 subsets differentially produce cytokines which are involved in human T cell polarization (IL-1 $\beta$ , IL-10, IL-12, and IL-23) [7, 16], their role in this process remains poorly studied. In this study, we analyzed the ability of the anti-inflammatory and immunoregulatory M-CSF-M $\phi$  and IL-34-M $\phi$  and of ovarian TAM to modulate the polarization of human memory CD4 $^+$  T cells.

## Results

**IL-34-M $\phi$  and M-CSF-M $\phi$  promote the generation of human Th17 cells.** We analyzed whether M-CSF-M $\phi$  and IL-34-M $\phi$  may modulate the pattern of cytokines expressed by human T cells. Day 7 LPS-stimulated M-CSF-M $\phi$  or IL-34-M $\phi$  were cultured with highly pure memory CD4<sup>+</sup> T cells, in the presence of an anti-CD3 mAb, and in the absence of IL-2 (in order to avoid their polarization into Treg cells [17]). The intracellular expression of IFN $\gamma$ , IL-4, IL-17, IL-10, and FoxP3, reflecting Th1, Th2, Th17, Tr1 cell polarization and Treg cell expansion, respectively, was analyzed by FACS.

Unexpectedly, upon contact with immunoregulatory M-CSF-M $\phi$  and IL-34-M $\phi$ , a huge increase in the frequency (Fig. 1A&B) and absolute number (Fig. 1C) of IL-17-producing T cells was observed in the memory CD4<sup>+</sup> T cell population. IL-34-M $\phi$  and M-CSF-M $\phi$  were as efficient as IL-1 $\beta$  plus IL-23 plus anti-CD28 mAb, a potent human Th17-inducing stimulus [18, 19], in upregulating Th17 cell frequency (Fig. 1B).

Currently, in addition to the conventional pro-inflammatory CCR4<sup>+</sup> CCR6<sup>+</sup> CD161<sup>+</sup> human Th17 cells, immunoregulatory Th17 cells (which express membrane TGF $\beta$  [20], IL-10 [21], CD39 or CD73 [22]), and IL-17-producing FoxP3<sup>+</sup> Treg cells, have been described [20, 21, 23-25]. Because we were expecting that IL-10<sup>+</sup> IL-12<sup>-</sup> M-CSF-M $\phi$  and IL-34-M $\phi$  would have favor T cell polarization into immunoregulatory T cells, we precisely analyzed the phenotype of Th17 cells generated in the presence of these M $\phi$ . Results showed that the Th17 cells exhibited a conventional Th17 profile; they expressed CCR4, CCR6, and CD161, while the expression of membrane TGF $\beta$ , CD39, and CD73, and of intracellular IL-10 and FoxP3, remained undetectable (Table 1).

Two subsets of Th17 cells, expressing or not IFN $\gamma$ , have been described [26]. Both these subsets were induced by M-CSF-M $\phi$  and IL-34-M $\phi$  (Fig. 1A-C). The ratio between these two subsets was similar in Th17 cells induced by M-CSF-M $\phi$ , IL-34-M $\phi$ , or by IL-1 $\beta$  plus IL-23 plus anti-CD28 mAb (Fig. 1A-C).

M-CSF-M $\phi$  and IL-34-M $\phi$  are usually considered as anti-inflammatory [6, 8] while, in contrast, GM-CSF-M $\phi$  exhibit a pro-inflammatory phenotype [6]. We therefore compared the capacity of M-CSF-M $\phi$  and IL-34-M $\phi$  versus GM-CSF-M $\phi$  to induce the generation of Th17 cells. Results showed that GM-CSF-M $\phi$  also promoted Th17 cell generation (increase of Th17 cell frequency and absolute number), although in a lower extent than M-CSF-M $\phi$  and IL-34-M $\phi$  (Fig. 1A-C).

We also observed that (i) unstimulated M-CSF-M $\phi$  and IL-34-M $\phi$  were as efficient as LPS-stimulated M $\phi$  in upregulating Th17 cell frequency (Fig. 1D) and (ii) that the absolute number of Th17 cells was higher with LPS-stimulated than non-stimulated M $\phi$  (Fig. 1E). Consequently, LPS-stimulated M $\phi$  were used in the following experiments, unless otherwise specified.

IL-34-M $\phi$  and M-CSF-M $\phi$  (either stimulated or not with LPS) did not modulate the frequency and absolute number of IFN $\gamma$ <sup>+</sup> IL-17<sup>-</sup> Th1 cells (Fig. 1F&G) while, as expected [3, 6, 16], GM-CSF-M $\phi$  promoted Th1 cell

generation (Fig. 1F&G). They did not induce the generation of IL-4-producing Th2 cells (data not shown) and did not modulate the frequency of IFN $\gamma$ <sup>-</sup> IL-17<sup>-</sup> IL-10-producing Tr1 cells, while they decreased the frequency of FoxP3<sup>+</sup> Treg cells (Supplemental Fig. 1).

Moreover, M-CSF-M $\phi$ , IL-34-M $\phi$ , and GM-CSF-M $\phi$  failed to polarize highly pure naive CD4<sup>+</sup> CD45RA<sup>+</sup> T cells into Th17 cells (data not shown).

Finally, we evaluated whether IL-34-M $\phi$  and M-CSF-M $\phi$  enhanced Th17 cell frequency by polarizing non Th17-committed memory T cells into Th17 cells and/or by increasing the proliferation of pre-existing Th17 cells. We observed that M-CSF-M $\phi$  and IL-34-M $\phi$  switched highly pure non Th17-committed IL-23R<sup>-</sup> CCR6<sup>-</sup> CD161<sup>-</sup> memory CD4<sup>+</sup> T cells [19, 27, 28] into Th17 cells (Fig. 1H). In parallel, M-CSF-M $\phi$  and IL-34-M $\phi$  did not modulate the proliferation of human Th17 cell clones (data not shown).

These results show that, unexpectedly, immunoregulatory IL-34-M $\phi$  and M-CSF-M $\phi$  switch non-committed human memory T cells into Th17 cells.

**M-CSF-M $\phi$  and IL-34-M $\phi$ , but not GM-CSF-M $\phi$ , constitutively express membrane IL-1 $\alpha$ .** We thus analyzed the expression of the human Th17-inducing factors IL-1 $\beta$ , IL-1 $\alpha$ , IL-23, and IL-6 by M-CSF-M $\phi$  and IL-34-M $\phi$ . As previously reported [6, 7, 16], IL-1 $\beta$  (Fig. 2A), IL-1 $\alpha$  (Fig. 2B), IL-23 (Fig. 2C), and IL-6 (Fig. 2D) remained low or undetectable in the supernatants of non-stimulated and LPS-stimulated IL-34-M $\phi$  and M-CSF-M $\phi$ . In support, the supernatants of IL-34-M $\phi$  and M-CSF-M $\phi$  failed to induce Th17 cell generation (data not shown). As expected [7, 29], these cytokines were produced by LPS-stimulated GM-CSF-M $\phi$  (Fig. 2A-D). Interestingly, PFA-fixed IL-34-M $\phi$  and M-CSF-M $\phi$  remained as efficient as non-fixed cells in inducing Th17 cells (Fig. 2E), suggesting that IL-34-M $\phi$  and M-CSF-M $\phi$  express membrane-associated Th17-inducing factor(s).

In contrast to IL-1 $\beta$ , functional IL-1 $\alpha$  can be expressed at the cell surface [30, 31]. Flow cytometry evidenced the constitutive expression of membrane IL-1 $\alpha$  (mIL-1 $\alpha$ ) on IL-34-M $\phi$  and M-CSF-M $\phi$  (Fig. 3A&B). This expression was not modulated by LPS (Fig. 3A). As expected [32, 33], mIL-1 $\alpha$  was poorly detectable on freshly isolated monocytes and was upregulated by LPS (Fig. 3A). IL-34 and M-CSF upregulated, in a time-dependent manner, mIL-1 $\alpha$  expression during the differentiation of monocytes into M $\phi$ , with a maximum at day 6 (Fig. 3C and data not shown). In contrast, mIL-1 $\alpha$  was poorly detectable on GM-CSF-M $\phi$  (Fig. 3A-C), supporting that fixation with PFA greatly reduced the ability of GM-CSF-M $\phi$  to induce Th17 cells (Fig. 2E).

**IL-34-M $\phi$  and M-CSF-M $\phi$  induce Th17 cells through mIL-1 $\alpha$ .** IL-1 has a central role in the generation of human Th17 cells [19, 34, 35]. We observed that IL-1RA and a neutralizing anti-IL-1 $\alpha$  antibody prevented the generation of Th17 cells induced by M-CSF-M $\phi$  or IL-34-M $\phi$  (Fig. 3D).



Given that (i) IL-34-M $\phi$  and M-CSF-M $\phi$  express membrane IL-18 which is shed into a functional soluble form upon stimulation [36, 37] (Supplemental Fig. 2) and (ii) that IL-18 induces murine Th17 cells [38, 39], we had to evaluate whether IL-18 may be also involved in the IL-34-M $\phi$ - and M-CSF-M $\phi$ -induced Th17 cell generation. Results showed that a neutralizing anti-IL-18 mAb did not prevent IL-34-M $\phi$ - and M-CSF-M $\phi$ -induced Th17 cells (Fig. 3D). In support, recombinant IL-18, either alone or in combination with IL-1 $\beta$ , did not induce Th17 cells (data not shown). It has been suggested that IL-6 may also favor IL-1 $\beta$ -induced Th17 cell generation [19, 40]. Supporting that IL-34-M $\phi$  and M-CSF-M $\phi$  produce low levels of IL-6, their ability to induce Th17 cells was unaffected by a neutralizing anti-IL-6 mAb (data not shown). In parallel, IL-1RA, but not a neutralizing anti-IL-1 $\alpha$  mAb, significantly prevented GM-CSF-M $\phi$ -induced Th17 cells (Fig. 3D). Together, these results show that mIL-1 $\alpha$ , constitutively, expressed by IL-34-M $\phi$  and M-CSF-M $\phi$ , is involved in Th17 cell generation.

**TAM from ovarian cancer induce the generation of Th17 cells via mIL-1 $\alpha$ .** We have previously reported that IL-34-M $\phi$  and M-CSF-M $\phi$  exhibit a phenotype similar to the one of TAM isolated from ovarian cancer [8, 9]. We thus analyzed the effect of TAM on memory T cell polarization. Results showed that, as M-CSF-M $\phi$  and IL-34-M $\phi$ , TAM constitutively expressed mIL-1 $\alpha$  (Fig. 3A&B), did not produce detectable levels of IL-1 $\alpha$ , IL-1 $\beta$ , and IL-23, even after stimulation (Fig. 2A-C), and polarized memory T cells into Th17 cells (Fig. 1). Co-cultures of memory T cells with PFA-fixed TAM, in the presence of IL-1RA or a neutralizing anti-IL-1 $\alpha$  antibody, confirmed the involvement of mIL-1 $\alpha$  in TAM-induced Th17 cell generation (Fig. 2E&3D). Although LPS-stimulated TAM produced IL-6 (Fig. 2D), a neutralizing anti-IL-6 mAb did not prevent the generation of Th17 cells (data not shown). Moreover, TAM did not upregulate the percentage and absolute number of Th1 cells (Fig. 1B-E). Finally, Th17 cells generated by TAM exhibited a classical Th17 phenotype (expression of CCR6, CCR4 and CD161 but not of IL-10, FoxP3, and TGF- $\beta$ ) (Table 1).

These results show that TAM from ovarian cancer ascites induce, as M-CSF-M $\phi$  and IL-34-M $\phi$ , the generation of classical human Th17 cells via mIL-1 $\alpha$ .

## Discussion

We demonstrate here that M-CSF and IL-34 give rise to human M $\phi$  that constitutively express mIL-1 $\alpha$  in a similar manner than human TAM isolated from ovarian cancer ascites. Membrane IL-1 $\alpha$  is involved in the acquisition, by non-committed memory T cells, of a conventional Th17 cell phenotype. These results evidence an ambivalent role of these M $\phi$ , usually considered as immunosuppressive, which may also contribute to maintain a smoldering inflammation via Th17 cell generation.

Membrane IL-1 $\alpha$  expression has been reported on different cell types, including myeloid (human monocytes and dendritic cells, murine M $\phi$ ) and non-immune cells (senescent fibroblasts, endothelial cells) [32, 33, 41-44]. Its expression on myeloid cells is upregulated upon stimulation with LPS or bacteria [32, 33, 42]. Surprisingly, in the absence of stimulation, the macrophage differentiation factors M-CSF and IL-34, but not GM-CSF, give rise to human M $\phi$  that express mIL-1 $\alpha$ . The membrane expression of IL-18, another member of the IL-1 family, is concomitantly observed on M-CSF-M $\phi$  and IL-34-M $\phi$ , but not on GM-CSF-M $\phi$  [36, 37], suggesting a common regulation of membrane IL-1 $\alpha$  and IL-18 expression. The insertion of IL-1 $\alpha$  into the membrane requires myristoylation of the IL-1 $\alpha$  precursor [45]. A potential myristoylation site in the IL-18 precursor has been suspected, but whether it accounts for its insertion into the membrane remains unclear [36, 37]. However, the outcome of the membrane forms of these two cytokines seems to differ. While a stimulation with LPS induces the shedding of membrane IL-18 into a soluble functional form [36], LPS did not affect mIL-1 $\alpha$  expression nor induced soluble IL-1 $\alpha$  on M-CSF-M $\phi$  and IL-34-M $\phi$ .

In agreement with previous studies, our results showed that mIL-1 $\alpha$  on immunoregulatory M $\phi$  is functional. The first demonstration of a functional membrane form of IL-1 $\alpha$  on stimulated murine M $\phi$  has been done in the 80's [30, 46]. Then, it has been highlighted that mIL-1 $\alpha$  is involved in paracrine cellular interactions and participates to inflammation and immune responses. Membrane IL-1 $\alpha$  triggers senescence-associated IL-6/IL-8 secretion [43], stimulates endothelial cells [33], causes inflammation [47], and stimulates anti-tumor immunity when expressed by tumor cells [48]. In this study, we revisit the impact of mIL-1 $\alpha$  expressed by APC on T cells and show that it promotes Th17 cell generation. Numerous studies have underlined the pivotal role of IL-1 in the generation of human Th17 cells [reviewed in 19, 34]. IL-1 $\beta$  and IL-1 $\alpha$ , which signal via the same receptor, exhibit similar biological activities and both recombinant soluble IL-1 $\alpha$  and IL-1 $\beta$  switch non Th17-committed human memory T cells into Th17 cells, with the same efficacy ([49] and personal observation). On the contrary, IL-1 does not switch naive T cells into Th17 cells [27, 28, 50, 51]. We also failed in detecting an effect of immunoregulatory M $\phi$  on naive T cell polarization into Th17 cells. Although a role of IL-18 in murine Th17 cell generation has been reported [38, 39], we did not observe any effect of

soluble and membrane IL-18 on human Th17 cell generation (personal observation). IL-23, in combination with IL-1 $\beta$ , induces Th17 cell survival and expansion [35, 52]. Although M-CSF-M $\phi$ , IL-34-M $\phi$  and ovarian TAM do not produce detectable levels of IL-23, even after LPS stimulation, they induced Th17 cell generation when fixed with PFA, suggesting that membrane molecules, other than IL-23, may contribute to Th17 cell survival. A recent study reported that a mouse M $\phi$  subset exhibiting M2 characteristics (IL-10<sup>high</sup>, CD163<sup>high</sup>), even though immunosuppressive, also induce in vivo the generation of Th17 cells [53]. In addition to underlining their role in T cell polarization, these results show that the biology of M $\phi$  is more complicated than previously thought and that one M $\phi$  subset may exhibit opposite functions, i.e. suppression of T cell mitosis and generation of pro-inflammatory Th17 cells.

Moreover, given that M-CSF-M $\phi$ , IL-34-M $\phi$  and ovarian TAM exhibit immunoregulatory functions, we suspected that they could induce the generation of Treg cells, unlike Th17 cells. One study showed an induction of Treg cells by M2 cells [54]. However, and supporting the implication of IL-2 and IL-15, which favor Treg cell generation and dampen Th17 cell induction [17, 55], this study showed that the generation of Treg cells in the presence of M2 cells required the presence of exogenous IL-2 and IL-15 [54].

IL-1 has pro-inflammatory and pro-angiogenic properties, and increases tumor growth and metastasis [48, 56, 57]. The neutralization of IL-1 activity currently appears as a promising strategy to treat cancer [58, 59]. Blocking IL-1 activity reduces metastasis and angiogenesis in preclinical tumor models [56, 57, 60]. In numerous cancers, the expression of IL-1 is elevated and patients with IL-1-producing tumors have generally bad prognosis [48, 57, 61]. In the tumor microenvironment, it is thought that the IL-1 activity is mediated by IL-1 $\beta$  secreted by activated cells and by IL-1 $\alpha$  released from dying cells. TAM, the largest APC subset in tumor microenvironment, favor angiogenesis and metastasis [11, 62-64]. The constitutive expression of functional mIL-1 $\alpha$  by ovarian cancer TAM may thus contribute to the chronic inflammation associated to angiogenesis and metastasis. Based on this observation, neutralizing IL-1R could be more efficient than neutralizing IL-1 $\beta$  in cancer treatment.

Interestingly, ovarian cancer TAM also induce Th17 cells via mIL-1 $\alpha$ . The role and prognostic significance of Th17 cells in human cancer remain controversial [49, 65-67]. In established ovarian cancers, Th17 cells may support anti-tumor immunity [68, 69]. Among APC present in ovarian tumor microenvironment, TAM are the most efficient in inducing Th17 cells [68]. However, to date, the respective role of IL-1 $\alpha$  and IL-1 $\beta$  in TAM-associated Th17 cell responses remain unexplored. Given (i) that TAM express IL-1 $\beta$  mRNA, (ii) that IL-1 $\alpha$  was poorly detectable in ovarian ascitis, and (iii) that an anti-IL-1R Ab inhibits TAM-induced Th17 cells, it has been suggested that TAM induce Th17 cells via IL-1 $\beta$  [68]. Our results evidence that mIL-1 $\alpha$ , rather than

IL-1 $\beta$ , has a central role in TAM-induced Th17 cells. Finally, we also report that TAM-induced Th17 cells have a classical phenotype, similar to Th17 cells isolated from ovarian ascitis [68].

In conclusion, this study demonstrates that, although producing low levels of pro-inflammatory cytokines and exhibiting immunosuppressive functions [6, 8, 9], immunoregulatory M $\phi$  may participate locally, in a mIL-1 $\alpha$ -dependent manner, through paracrine M $\phi$ -lymphoid cell interactions, to smoldering inflammation by polarizing memory CD4<sup>+</sup> T cells into Th17 cells.

## Materials and Methods

**Ethics Statement.** Peripheral blood mononuclear cells (PBMCs) were obtained from healthy human volunteers (Blood collection center Angers, France; agreement # ANG-2003-2). Ovarian cancer ascites were obtained with written informed consent, in accordance with the Angers University Hospital ethics committee requirements (N° 2013/38).

**Cell isolation.** PBMCs and cells from ascite fluids were isolated with Lymphocyte Separation Medium (Eurobio, Courtaboeuf, France). CD14<sup>+</sup> monocytes and CD14<sup>+</sup> TAM were isolated by positive magnetic sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4<sup>+</sup> T cells were sorted by negative depletion (Miltenyi Biotec) and T cell subpopulations were then sorted by flow cytometry (FACS Aria; BD Biosciences, San Jose, CA). Memory cells and naive CD4<sup>+</sup> T cells were sorted from CD4<sup>+</sup> T cells using the combination of PerCP anti-CD4 (clone VIT4), APC anti-CD45RO (clone UCHL1) and APC-Vio770 anti-CD45RA (clone T6D11) Abs (all from Miltenyi Biotec). Non-Th17 committed T cells were sorted from memory CD4<sup>+</sup> T cells using the combination of PE anti-IL-23R (R&D Systems, Friesoythe, Germany, clone 218213), PerCP-Cy5.5 anti-CCR6 (clone 11A9) and APC anti-CD161 (clone DX12) Abs (both from BD Biosciences). Cell sorting strategy is schematized in Supplemental Fig. 3.

**Macrophage subset generation.** Monocytes were cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (PAA Laboratories, Pashing, Austria), and antibiotics (all from Lonza). To generate M-CSF-M $\phi$  and GM-CSF-M $\phi$ , monocytes ( $1 \times 10^6$  cells/ml) were exposed for 6 days to 50 ng/ml M-CSF (ORF Genetics, Kopavogur, Iceland) or 20 ng/ml GM-CSF (CellGenix, Freiburg, Germany), respectively. In some experiments, day-6 M $\phi$  were fixed with PBS containing 4% paraformaldehyde (PFA) during 15 minutes. In other, M $\phi$  were activated with 200 ng/ml LPS (*E. coli* serotype O111:B4; Sigma-Aldrich, St Louis, MO) for 24 h or 48 h.

**Coculture experiments.** Naive, memory or non-Th17-committed memory CD4<sup>+</sup> T cells ( $1 \times 10^5$ ) were cultured in X-VIVO-20 medium (Lonza) in 96-well flat-bottomed plates, previously coated with 1  $\mu$ g/ml anti-CD3 mAb (clone OKT3; BD Biosciences), without or with  $1 \times 10^4$  allogeneic M-CSF-M $\phi$ , IL-34-M $\phi$  GM-CSF-M $\phi$  or TAM for 7 days. In some experiments, 1  $\mu$ g/mL IL-1RA, 10  $\mu$ g/mL anti-IL-1 $\alpha$  polyclonal antibody or anti-IL-18 monoclonal antibody (all from R&D Systems) were added in the culture. The culture of memory T cells with 1  $\mu$ g/ml soluble anti-CD28 mAb (BD Biosciences) supplemented with 50 ng/mL IL-23 (R&D Systems), 50 ng/mL IL-1 $\beta$  (Miltenyi Biotec) or IL-1 $\alpha$  (Peprotech, Princeton, NJ) (to favor the generation of Th17 cells; [19, 70]), or supplemented with 50 ng/mL IL-2 (Miltenyi Biotec) and IL-10 (Immunotools, Friesoythe,

Germany) (to favor the generation of Tr1 cells; [17, 21, 71]) were used as positive controls of memory T cell differentiation. In some experiments, 200 ng/ml LPS was added directly in the co-culture of M $\phi$  and memory T cells.

**Cytokine quantification.** IL-1 $\beta$ , IL-1 $\alpha$  and IL-6 were quantified using ELISA from Diaclone (Dijon, France). ELISA for IL-23 was from eBioscience (San Diego, CA).

**Flow cytometry analysis.** The intracellular expression of IL-17, IFN $\gamma$ , IL-4, IL-10 and FoxP3 was evaluated by FACS on day 7 T cells, after a 5 h stimulation with 10 ng/mL PMA plus 1 nM ionomycin, in the presence of 10  $\mu$ g/mL of brefeldin A (all from Sigma-Aldrich). After fixation with PFA, cells were stained with the following conjugated antibodies: PE-Cy7 anti-CCR4 (clone 1G1), PerCP-Cy5.5 anti-CCR6 (clone 11A9), BV421 anti-CD161 (clone DX12), FITC anti-IFN $\gamma$  (clone B27) (all from BD Biosciences), AlexaFluor647 anti-IL-17A Abs (eBiosciences, clone eBio64DEC17). Data were acquired using a FACSCantoII flow cytometer (BD Biosciences) and analyzed with the FlowJo software, version 10.0.6 (Tree Star, Ashland, OR). Results are expressed as mean fluorescence intensity (MFI) after subtraction of the value obtained with the isotype control mAb, in percentage or in absolute number of T cell subsets. The percentage of decrease of Th17 cell frequency was calculated as follows: (% Th17 cells with isotype/control – % of Th17 cells with inhibitor) / % of Th17 cells with isotype/control.

**Statistical analysis.** Data were analyzed by the Wilcoxon or the two-way ANOVA test, with  $p < 0.05$  being considered significant.

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## **Author contributions**

Conceived and designed the experiments: EDF, SB YD, and PJ. Performed the experiments: EDF, SB and LP. Wrote the paper: EDF, SB YD, and PJ.

## **Conflict of interest**

The authors who have taken part in this study declare that they do not have anything to declare regarding funding from industry or conflict of interest with respect to this manuscript.

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## Figure legends.

**Figure 1: M-CSF-M $\phi$ , IL-34-M $\phi$  and TAM promote the generation of Th17 cells.** (A-G) After 7 days of coculture with LPS-stimulated (unless otherwise stated in D and E) GM-CSF-M $\phi$ , M-CSF-M $\phi$ , IL-34-M $\phi$  or TAM, intracellular expression of IL-17 and IFN $\gamma$  was analyzed by flow cytometry in memory CD4<sup>+</sup> T cells. Representative dot plots (A) of one out of five separate experiments are shown. The percentages (B, D, F) and the total numbers (C, E, G) of Th17 and Th1 cells are shown. (H) Non-Th17 memory T cells (IL-23R<sup>-</sup> CCR6<sup>-</sup> CD161<sup>-</sup>) were sorted by FACS and cultured as described above, before analysis of the percentage of Th17 cells. B-H, data are shown as mean  $\pm$  SD, (n=5); \*p<0.005, Wilcoxon test.

**Figure 2: M-CSF-M $\phi$ , IL-34-M $\phi$  and TAM induce Th17 cell generation via membrane-associated factor(s).** (A-D) GM-CSF-M $\phi$ , M-CSF-M $\phi$ , IL-34-M $\phi$  and TAM were stimulated or not with LPS for 24 hours. IL-1 $\beta$  (A), IL-1 $\alpha$  (B), IL-23 (C) and IL-6 (D) were quantified by ELISA in cell culture supernatants. (E) Memory CD4<sup>+</sup> T cells were cultured for 7 days with non-fixed or PFA-fixed GM-CSF-M $\phi$ , M-CSF-M $\phi$  or TAM. The frequency of Th17 cells was determined at day 7. Results are expressed in pg/mL (A and B), in ng/mL (C and D) or as a percentage of Th17 cells (E). Data are shown as mean  $\pm$  SD, (n=6); \*p<0.005, Wilcoxon test.

**Figure 3: The generation of M-CSF-M $\phi$ - and TAM-induced Th17 cells is dependent on mIL-1 $\alpha$ .** (A and B) The expression of mIL-1 $\alpha$  was analyzed by flow cytometry on GM-CSF-M $\phi$ , M-CSF-M $\phi$ , IL-34-M $\phi$  and TAM, stimulated or not with LPS for 24 h (A). Representative dot plots (B) of one out of five separate experiments are shown. (C) The expression of mIL-1 $\alpha$  was analyzed by flow cytometry during the 6-day differentiation of GM-CSF-M $\phi$  and M-CSF-M $\phi$ . (D) Memory CD4<sup>+</sup> T cells were cultured for 7 days with non-fixed or PFA-fixed GM-CSF-M $\phi$ , M-CSF-M $\phi$  or TAM in the presence of IL-1RA, polyclonal anti-IL-1 $\alpha$  Ab or monoclonal anti-IL-18 Ab. The frequency of Th17 cells was determined at day 7. Results are expressed as mean fluorescence intensity (MFI) after subtraction of the MFI value obtained with the isotype control mAb (A and C), or are expressed as a percentage of decrease of Th17 cell frequency, as described in the Material and Methods section (D). Data are shown as mean  $\pm$  SD, (n=5); \*p<0.005, Wilcoxon test, A or two-way ANOVA, C. †, means dead cells at day 7 (D).

**Table 1: Analysis of cell surface markers expressed by Th17 cell subsets**

Markers	Th17 (M-CSF-M $\phi$ )	Th17 (IL-34-M $\phi$ )	Th17 (GM-CSF-M $\phi$ )	Th17 (TAM)	Th17 (IL-1 $\beta$ , IL-23)	Tr1 (IL-2+IL-10)	Treg
CCR4	+	+	+	+	+	nd	nd
CCR6	+	+	+	+	+	nd	nd
CD161	+	+	+	+	+	nd	nd
IL-10*	-	-	-	-	-	+	+
FoxP3*	-	-	-	-	-	nd	+

The expression of the indicated markers was analyzed by FACS on human memory CD4<sup>+</sup> CD45RO<sup>+</sup> T cells induced by culture for 7 days with GM-CSF-M $\phi$ , M-CSF-M $\phi$  or TAM. CCR4, CCR6 and CD161 were analyzed on Th17 cells (\*, intracellular expression). Their phenotype was compared to the one exhibited by Th17 cells generated by anti-CD28 mAb plus IL-1 $\beta$  plus IL-23 [19, 70], Tr1 cells generated by anti-CD28 mAb plus IL-2 plus IL-10 [17, 21, 71], and of circulating natural Treg cells (present in PBMC from healthy subjects). Results are representative of 5 independent experiments. +, expression of the marker; -, no expression of the marker; nd, not done.

Figure 1

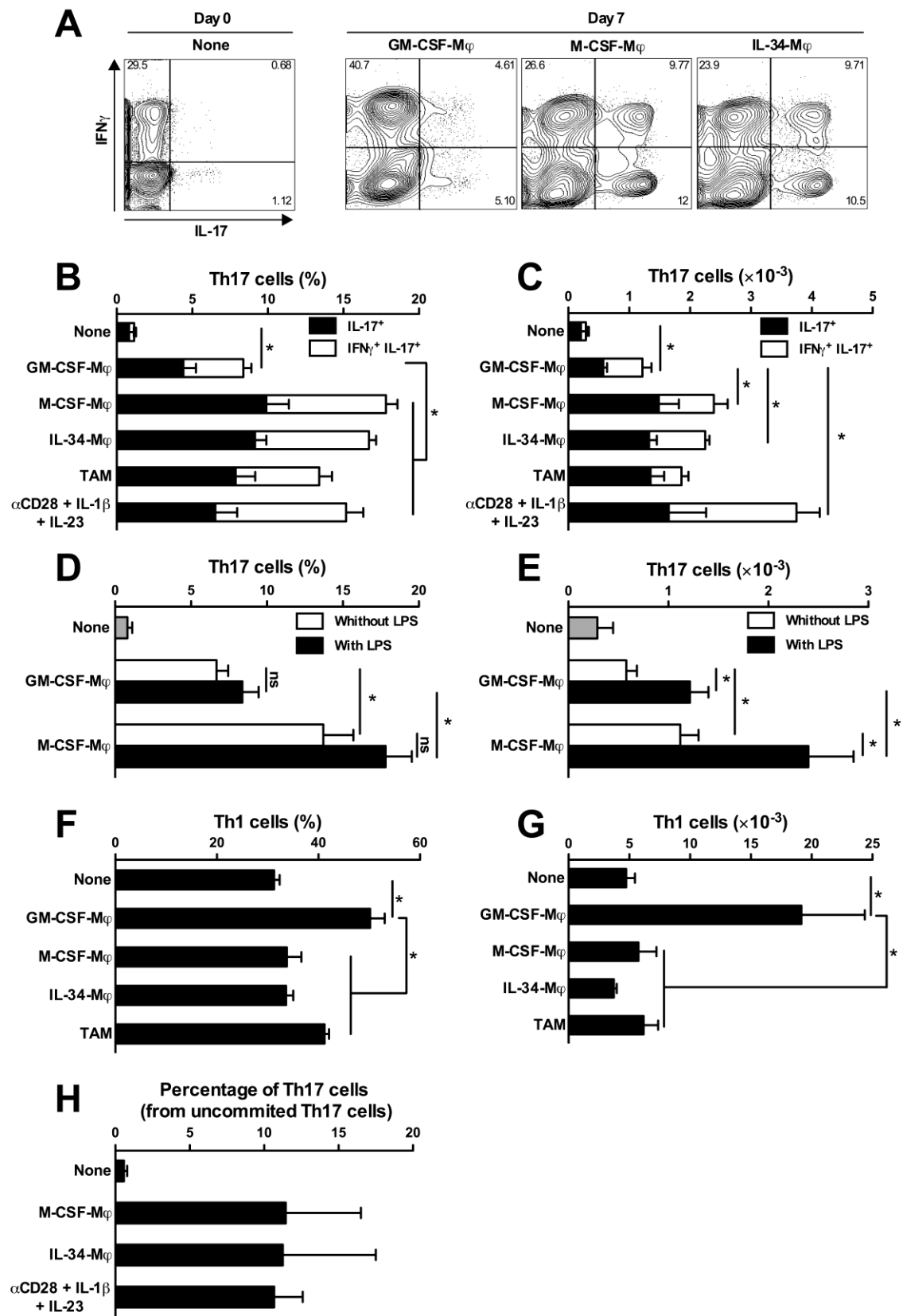


Figure 2

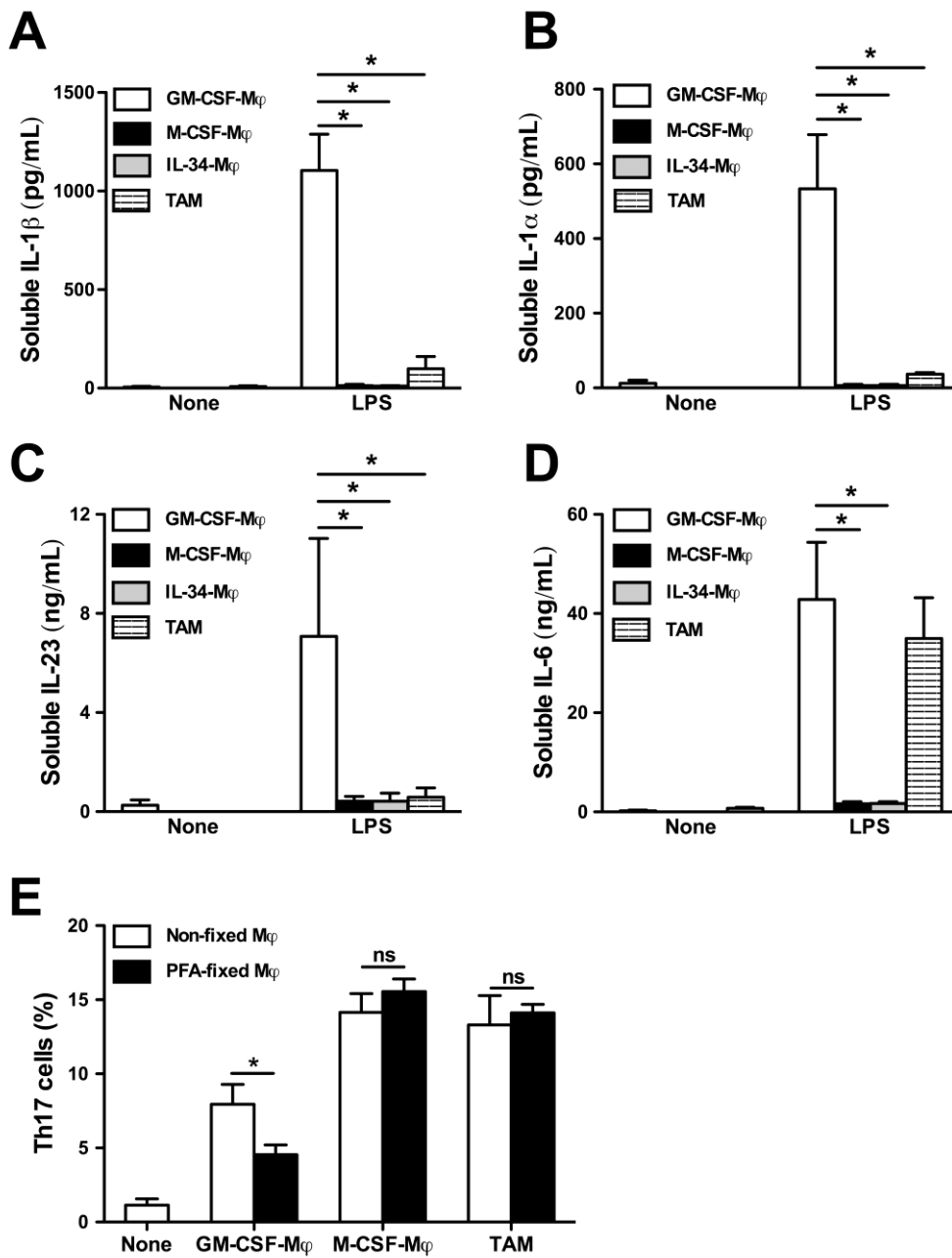
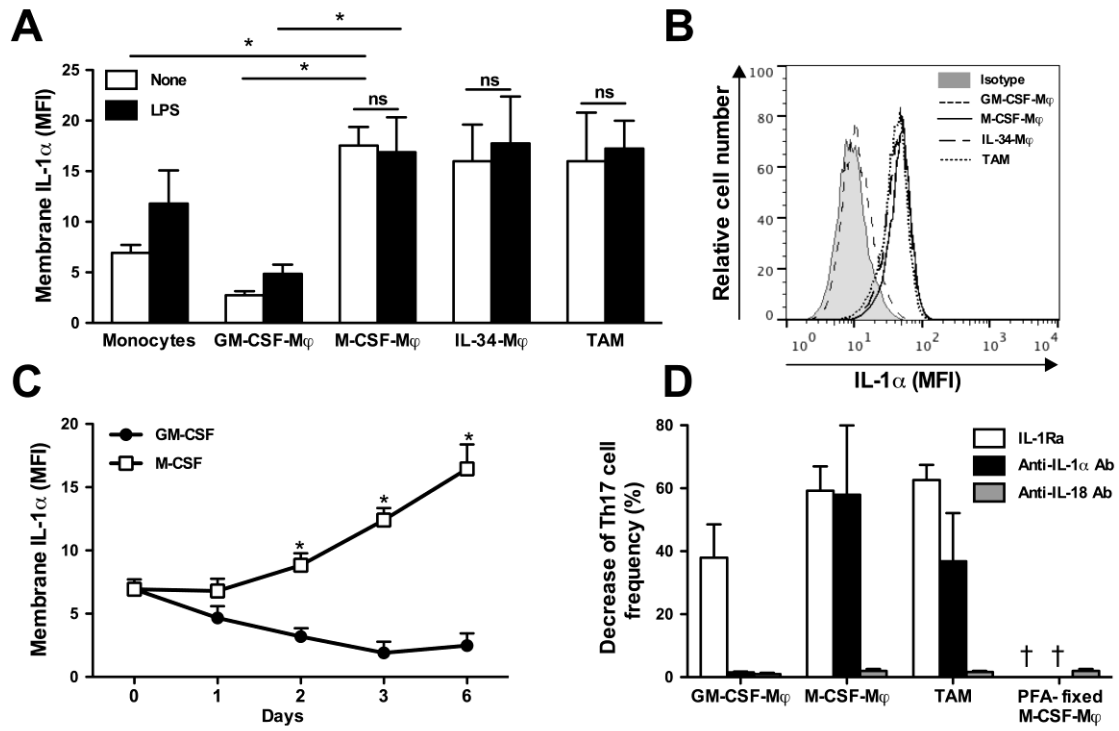
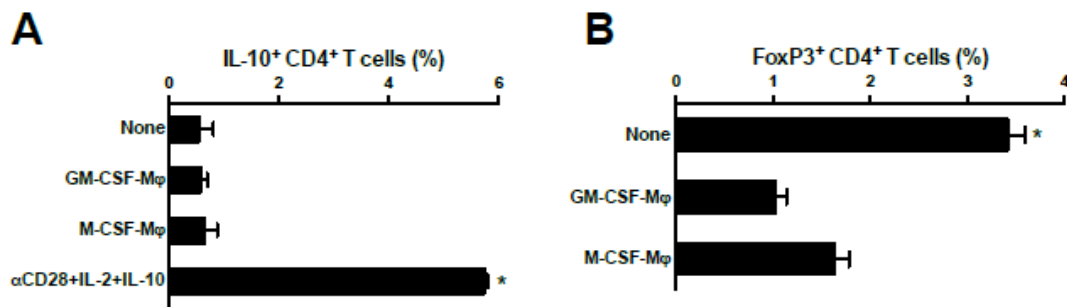


Figure 3

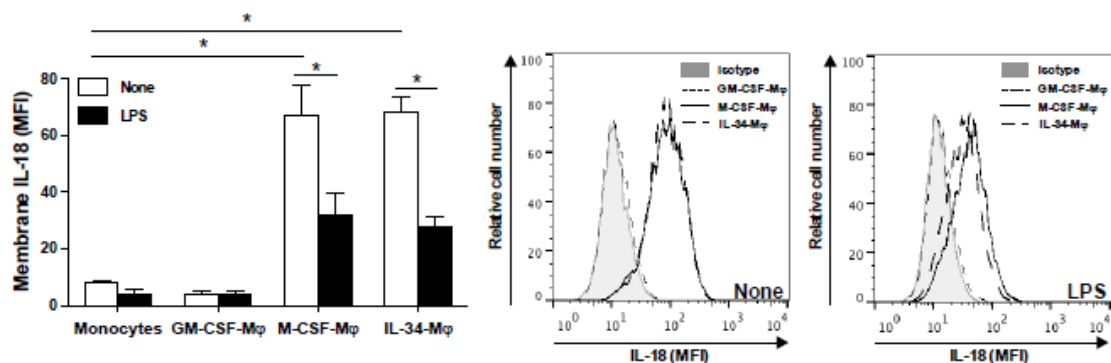




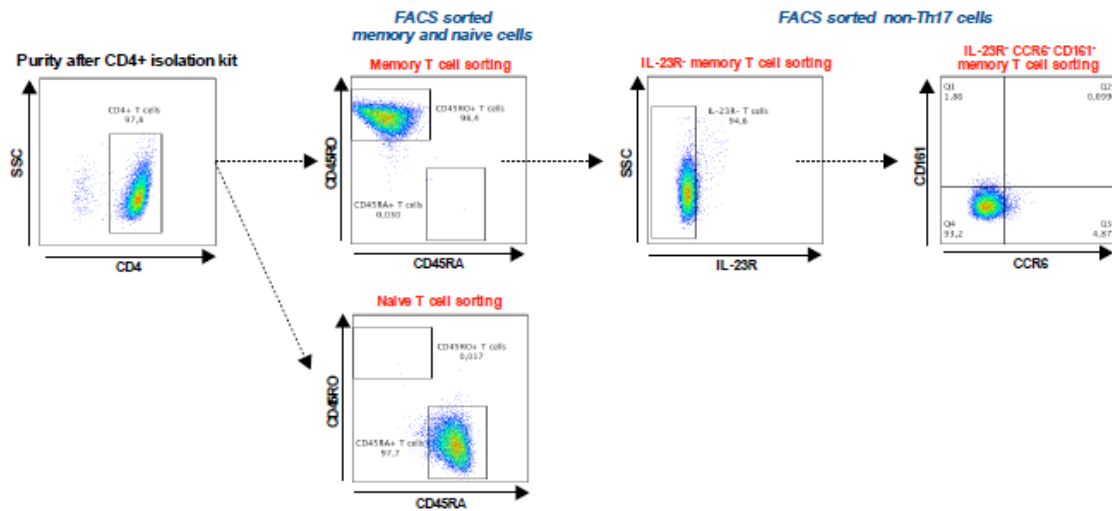
Supplemental figures



**Supplemental Figure 1: M-CSF-M $\phi$  do not induce the generation of immunoregulatory T cells.** After 7 days of coculture with GM-CSF-M $\phi$  or M-CSF-M $\phi$ , the intracellular expression of IL-10 (using PE anti-IL-10 mAb; clone JES3-907, eBiosciences) (A) and FoxP3 (using PE anti-FoxP3 mAb; clone PCH101, eBiosciences) (B) were analyzed in memory CD4<sup>+</sup> T cells by flow cytometry. Their expression was compared to the one exhibited by T cells maintained in culture without M $\phi$  (none) and by in vitro generated Tr1 cells (using anti-CD28 mAb plus IL-2 plus IL-10; Banchereau et al. *Nat Immunol* 2012. 13:925; Horwitz et al. *J Leukoc Biol* 2003. 74:471) (A), and to the frequency of natural Treg present in human memory CD4<sup>+</sup> T cells from healthy subjects (B). Data are shown as mean  $\pm$  SD, (n=3); \*p<0.005, Wilcoxon test.



**Supplemental Figure 2: M-CSF-M $\phi$  and IL-34-M $\phi$  constitutively express membrane IL-18.** Left panel, monocytes, GM-CSF-M $\phi$ , M-CSF-M $\phi$  and IL-34-M $\phi$  were stimulated or not with LPS for 24 h and the expression of membrane IL-18 was analyzed by flow cytometry. Data are shown as mean  $\pm$  SD, (n=5); \*p<0.005, Wilcoxon test. Right panels, representative histograms of one out of five separate experiments.



**Supplemental Figure 3: FACS sorting strategy to purify T cell subsets.** Human CD4<sup>+</sup> T cells were isolated from PBMC using a CD4 isolation kit (Miltenyi Biotech). CD45RO<sup>+</sup> CD45RA<sup>-</sup> memory T cells and CD45RO<sup>-</sup> CD45RA<sup>+</sup> naive T cells were then isolated from CD4<sup>+</sup> T cells by FACS sorting. Non Th17-committed cells are characterized by the absence or low expression of IL-23R, CD161 and CCR6 (Liu et al. *J Immunol* 2008. 180:7948-7957; Cosmi et al. *J Exp Med* 2008. 205:1903-1916.). These cells were isolated from CD45RO<sup>+</sup> CD45RA<sup>-</sup> memory T cells by a two-step FACS sorting: isolation of IL-23R<sup>-</sup> T cells followed by isolation of CCR6<sup>-</sup> CD161<sup>-</sup> T cells (among IL-23R<sup>-</sup> cells). This strategy allowed isolating highly pure IL-23R<sup>-</sup> CCR6<sup>-</sup> CD161<sup>-</sup> CD45RO<sup>+</sup> CD4<sup>+</sup> T cells (routinely >95% pure).