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CD28 is expressed by macrophages with anti-inflammatory potential and limits their T cell activating capacity

Estrada-Capetillo L. [1*], Aragoneses-Fenoll L. [3*], Domínguez-Soto A. [2], Fuentelsaz-Romero S. [1], Nieto C. [2], Simón-Fuentes M. [2], Alonso B. [2], Portolés. P. [3], Corbí A.L. [2#], Rojo J.M., [2#] Puig-Kröger A [1#].

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[1] Unidad de InmunoMetabolismo e Inflamación, Instituto de Investigación Sanitaria Gregorio Marañón, Hospital General Universitario Gregorio Marañón, Madrid, Spain; [2] Centro de Investigaciones Biológicas Margarita Salas, Madrid, Spain; [3] Unidad de Inmunología Celular, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain.

[*] L.E-C and L.A-F contributed equally to this work.

[#] A.P-K, J. M. R. AND A.L.C. contributed equally to this work.

Corresponding author: **Dr. Amaya Puig-Kröger. Unidad de InmunoMetabolismo e Inflamación, Instituto de Investigación Sanitaria Gregorio Marañón, Hospital General Universitario Gregorio Marañón, Madrid, Spain. Phone: +34-915868750; FAX: +34-91-5868749. e-mail: amaya.puig@iisgm.com**

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ABSTRACT

CD28 expression is generally considered to be T lymphocyte-specific. We have previously shown *CD28* mRNA expression in M-CSF-dependent anti-inflammatory monocyte-derived macrophages (M-MØ), and now demonstrate that CD28cell surface expression is higher in M-MØ than in GM-CSF-dependent macrophages, and that macrophage CD28 expression is regulated by MAFB and Activin A. *In vivo*, CD28

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was found in Tumor-Associated Macrophages and, to a lower extent, in pro-inflammatory synovial fluid macrophages from rheumatoid arthritis patients. Analysis of mouse macrophages confirmed Cd28 expression in bone-marrow derived M-MØ. Indeed, anti-CD28 antibodies triggered ERK1/2 phosphorylation in mouse M-MØ. At the functional level, *Cd28*KO M-MØ exhibited a significantly higher capacity to activate the OVA-specific proliferation of OT-II $CD4^+$ T cells than WT M-MØ, as well as enhanced LPS-induced IL-6 production. Besides, the *Cd28*KO M-MØ transcriptome was significantly different from WT M-MØ regarding the expression IFN response-, Inflammatory response- and TGFβ signaling- related gene sets. Therefore, defective CD28expression in mouse macrophages associates to changes in gene expression profile, what might contribute to the altered functionality displayed by *Cd28*KO M-MØ. Thus, CD28 expression appears as a hallmark of anti-inflammatory macrophages and might be a target for immunotherapy.

INTRODUCTION

CD28 belongs to the group of cell surface proteins involved in the modulation of immune responses because of their capability to inhibit, amplify or foster the intracellular signals triggered upon antigen recognition by T lymphocytes. CD28 is the paradigmatic T cell "co-stimulatory" molecule, because of its ability to act as an efficient co-stimulus for antigen activation of naive T lymphocytes. CD28 was initially identified with monoclonal antibodies that a) recognized T cell and thymocyte surface molecules, and b) enhanced antigen receptor-induced proliferation and/or cytokine secretion by T lymphocytes [1]–[3]. It was thus assumed that CD28 is expressed and is functional mainly, if not exclusively, in T cells, and this vision has prevailed to this day [4],[5].

While CD28 is essential to T cell function and the development of T-dependent adaptive immune responses, growing evidence shows that it can be also expressed by innate immune cells, including NK cells [6] and Innate Lymphoid Cells (ILC) [7]. Interestingly, activated T cells, ILC and NK cells can express both CD28 and its ligands CD80 and CD86 with important functional consequences [8]–[13]. On the other hand, the CD28 protein has been also detected in cells of the myeloid lineage like mouse plasmacytoid dendritic cells (pDC) [14], monocytes [15] and human eosinophils [16],[17]. In fact, gene expression profiling has detected

CD28 mRNA in mouse pDC [14], infected mouse macrophages [18],[19] and primary human monocytes [20].

During the identification of polarization-specific markers for human macrophages we detected the preferential expression of *CD28* mRNA in monocyte-derived macrophages generated in the presence of M-CSF (M-MØ), which exhibit a potent anti-inflammatory potential, whereas its expression was significantly lower in macrophages differentiated in the presence of GM-CSF (pro-inflammatory GM-MØ) [21],[22]. We now demonstrate that the expression of CD28 by human macrophages is mediated by cytokines and factors that determine the acquisition of pro- or anti-inflammatory profiles, namely Activin A and MAFB, and show that CD28 expression associates with a reduced T cell stimulatory ability and an altered gene expression profile in mouse macrophages.

RESULTS

CD28 gene expression in human macrophages in vivo in homeostasis and pathological settings. Determination of the transcriptome of human M-MØ and GM-MØ has previously revealed that *CD28* belongs to the "Anti-inflammatory gene set", whose expression is specific for M-CSF-dependent IL-10-producing M-MØ [21],[22]. Due to the existence of scattered information suggesting the existence of $CD28^+$ myeloid cells [14]–[17], we sought to assess whether *CD28* expression could be also detected in macrophages *in vivo*. To that end, we initially took advantage of the available cell-specific transcriptional information for human kidney [23] and placenta [24]. As shown in **Figure 1A**, *CD28* mRNA was detected in kidney $CD45^+$ cells with a macrophage transcriptome, and its pattern of expression overlapped with that of *CD163*, a macrophage-specific marker, as determined by *t*-SNE [23]. Similarly, *CD28* mRNA was found in placental macrophages and Hoffbauer cells [24], placental macrophages with an anti-inflammatory polarization that promote tolerance and are resistant to inflammatory cues [25]. In line with these findings and our previous observations, Kang et al. detected *CD28* mRNA in primary human monocytes, and found that its expression is significantly reduced in response to IFNγ [20] (**Figure 1B)**. Regarding pathology, *CD28* expression was found to significantly correlate with the expression of the macrophage marker-coding genes *CD163* and *FOLR2* (which also belong to the "Anti-inflammatory gene set") in breast carcinoma

(METABRIC cohort) (**Figure 1C**) and in numerous other tumor types (**Figure 1D**). Indeed, *CD28* expression in tumors correlated with the expression of the *SPI1* gene (**Figure 1C,D**), which codes for the myeloid differentiation master transcription factor PU.1 [26],[27] and interacts with various regions in the vicinity of the *CD28* gene (**Figure 1E**). Altogether, these hints point towards CD28 being expressed in human myeloid cells, and preferentially in macrophages exhibiting an anti-inflammatory polarization.

CD28 expression in human macrophages in vitro. Given the above indications, we next sought to analyze the acquisition of CD28 expression by M-CSF-dependent macrophages (M-MØ). *CD28* mRNA was found to be significantly higher in M-MØ derived from $CD14^+$ (classical) monocytes than in their corresponding GM-MØ counterparts (**Figure 2A**). The differential *CD28* mRNA expression between GM-MØ and M-MØ became maximal at day three along the macrophage differentiation process, and was maintained afterwards (**Figure 2B**). In line with this finding, CD28 cell surface expression was acquired only during differentiation of M-MØ but not GM-MØ (**Figure 2C**), although both macrophage subtypes exhibited similar expression of CD80 and CD86 (**Supplementary Figure 1A**). Moreover, *CD28* mRNA and cell surface CD28 were also detected by macrophages generated under the influence of IL-34 (IL34-MØ) (**Figure 2D**), a cytokine that promotes microglial and dermal macrophage differentiation in an M-CSF receptor-dependent manner [28],[29]. These results confirm that CD28 is expressed by *in vitro* generated human monocyte-derived macrophages and suggest that the receptor for M-CSF (CD115) positively controls the acquisition of CD28 expression during M-MØ differentiation. Of note, flow cytometry on monocytes, macrophages and T lymphocytes confirmed that the cell surface expression of CD28 in M-MØ and IL34-MØ is significantly lower than in peripheral blood CD3+ T lymphocytes (**Figure 2E**).

Macrophage CD28 expression is regulated by pro- and anti-inflammatory factors. To dissect the factors controlling the preferential acquisition of CD28 along M-MØ differentiation, we next evaluated whether CD28 expression is regulated by activin A or MAFB, which are critical for the differentiation of GM-MØ or M-MØ, respectively [21],[22]. siRNA-mediated knockdown on the MAFB transcription factor, which determines the acquisition of the "Anti-inflammatory gene set" [30], led to a significant reduction of CD28 mRNA (**Figure 3A**). On the contrary, activin A, a member of the TGFβ family that drives the generation of pro-inflammatory GM-MØ [21],[22] prevented the up-regulation of cell surface CD28 in M-MØ (**Figure**

3B). Therefore, CD28 expression is positively regulated by MAFB and inhibited by activin A, further supporting the preferential expression of CD28 in anti-inflammatory human M-MØ.

The preferential expression of CD28 in M-CSF-conditioned macrophages (M-MØ) [21],[22],[31]–[33] led us to analyze the effect of additional macrophage-polarizing factors. As shown in Figure 3C, IFNγ treatment significantly down-regulated *CD28* mRNA in M-MØ, whereas IL-4 significantly increased *CD28* mRNA only in GM-MØ (**Figure 3C**). Therefore, CD28 expression is positively regulated by cytokines with homeostatic and anti-inflammatory activity (M-CSF, IL-34, IL-4), whereas it is downregulated by pro-inflammatory stimuli (activin A, IFNγ). These results are in agreement with previous data from gene expression profiling on virus-infected mouse macrophages [18],[19].

CD28 expression in human macrophages in vivo. To assess the patho-physiological significance of the above findings, we next analyzed the expression of CD28 in macrophages isolated from inflamed tissues. Specifically, CD28 expression was determined in macrophages from tumor ascites (Tumor-Associated Macrophages, TAM), which resemble anti-inflammatory M-MØ, as well as in macrophages from the synovial fluid of patients with active rheumatoid arthritis (RASF-MØ), whose profile is similar to that of pro-inflammatory GM-MØ [34]. As shown in **Figure 4A**, *CD28* mRNA was significantly higher in TAM than in RASF-MØ. At the protein level, both TAM and RASF-MØ displayed detectable levels of cell surface CD28, although CD28 expression was significantly higher in TAM (**Figure 4B**), whereas TAM and RASF-MØ displayed similar levels of CD86 (**Supplementary Figure 1B**). Altogether, these results confirm that CD28 is expressed by human macrophages both *in vitro* and *in vivo*, and further support the preferential expression of the *CD28* gene in macrophages with known anti-inflammatory capacity.

CD28 expression and function in mouse macrophages. The evaluation of the functional role of CD28 in human M-MØ was compromised by the lack of efficacy of *CD28*-specific siRNA knockdown (*data not shown*). Therefore, we turned to the murine system to assess whether cell surface CD28 is a functional molecule in macrophages. To that end, we initially assessed CD28 expression in bone-marrow derived GM-MØ and M-MØ. *CD28* mRNA expression was detected in both GM-MØ and M-MØ, but its expression was significantly higher in murine M-MØ (**Figure 5A**). Indeed, cell surface expression of CD28 was evident in

M-MØ from WT C57BL/6, whereas CD28 was expressed by only a fraction of WT GM-MØ, and no CD28-specific staining was found on M-MØ derived from the bone marrow of *CD28*KO mice (**Figure 5B**). Therefore, and in line with the data from human cells, CD28 is also detected on bone marrow-derived mouse macrophages, where its expression was found to be roughly similar to that on spleen $CD4^+$ T lymphocytes (**Figure 5B**).

To determine the functionality of Cd28 on mouse macrophages, murine M-MØ were treated with the 37.51 anti-CD28 antibody, which exhibits agonist activity [35]. Ligation of CD28 in M-MØ from WT C57BL/6 mice resulted in ERK1/2 phosphorylation, thus demonstrating the signaling capability of mouse macrophage CD28 (**Figure 5C**, **Supplementary Figure 2**). The specificity of the effect was demonstrated on M-MØ from *Cd28*KO mice, where 37.51 did not induce ERK1/2 activation (**Figure 5C**, **Supplementary Figure 2**). As a control, similar levels of LPS-induced ERK1/2 activation were detected in M-MØ from WT and *Cd28*KO mice (**Figure 5C**), and CD28 ligation did not co-stimulate the LPS-initiated ERK1/2 activation in WT M-MØ macrophages (**Figure 5C**). Therefore, these results show that CD28 on mouse M-MØ has intracellular signaling capability and that its ligation leads to immediate ERK1/2 phosphorylation.

Then, we hypothesized that macrophage CD28 might impair T cell activation by competing the costimulatory signals provided by macrophage CD80/CD86 to CD28 on T lymphocytes. To test this hypothesis, we analyzed the ability of WT and *Cd28*KO M-MØ to activate OVA-specific OT-II CD4+ T cells in the presence of antigenic OVA peptide (OVA323–339) [36]. M-MØ derived from *Cd28*KO mice promoted a significantly higher lymphoproliferation than WT M-MØ (**Figure 5D**), a difference that was observed both before and after macrophage exposure to LPS. In line with this finding, M-MØ from *Cd28*KO showed a significantly higher production of IL-6 upon activation by 100 ng/ml LPS, whereas no difference was seen in LPS-induced IL-6 production between WT and *Cd28*KO GM-MØ (**Figure 5E**). By contrast, the 37.51 anti-CD28 antibody did not significantly modify cytokine production in macrophages from WT or *Cd28*KO mice, either in basal conditions or after 20 ng/ml LPS activation (Supplementary Figure 3). Collectively, these results indicate that CD28 expression is associated with a lower T cell stimulatory ability and reduced production of 100 ng/ml LPS-induced IL-6 in mouse macrophages, in line with the preferential expression of CD28 on macrophages with anti-inflammatory potential.

Finally, to further assess the link between CD28 expression and the macrophage inflammatory and immuno-stimulatory potential, we determined whether Cd28 ablation modifies the transcriptional profile of mouse M-MØ. To that end, we compared the gene signatures of bone marrow-derived M-MØ from WT and *Cd28*KO mice through RNAseq (GSE157837) (**Figure 6A**). Analysis of both transcriptomes revealed no significant change in the expression of genes coding for *Cd80*, *Cd86* or other co-stimulatory molecules, a result confirmed by flow cytometry for Cd80 and Cd86 (GSE157837 and data not shown). However, gene ontology analysis using Gene Set Enrichment Analysis (GSEA) showed that the transcriptome of *Cd28*KO M-MØ exhibits a very significant positive enrichment of the gene set "HALLMARK_INTERFERON_ALPHA_RESPONSE" (NES, 2.58; FDRq, 0.090) and a weaker, although significant, negative enrichment of the "HALLMARK_INFLAM MATORY_RESPONSE" data set (NES, -1.45; FDRq, 0.06) (**Figure 6B**). Besides, the transcriptome of *Cd28*KO M-MØ was significantly different from that of WT M-MØ regarding expression of GSEA Hallmark gene sets associated to Oxydative phosphorylation and TGFβ signaling (**Figure 6C**). Altogether, these results indicate that a defective expression of Cd28 in mouse macrophages is associated to an altered gene expression profile, what might contribute to the altered functionality (enhanced T cell stimulatory ability, increased LPS-induced IL-6 production) displayed by *Cd28*KO M-MØ.

DISCUSSION

CD28 is essential to T cell function and the development of T-dependent adaptive immune responses. However, CD28 has been also detected in plasma cells and lymphoid and myeloid innate cells [6],[7],[14],[37]–[39], where its expression often coincides with CD80 and CD86 [9],[10],[13],[39], thus raising the question of the functional role of CD28-CD28 ligand interactions in non-T cells. We now report that CD28 is preferentially expressed by anti-inflammatory human macrophages like M-MØ and TAM, and that its expression depends on MAFB, the transcription factor which determines the acquisition of the "Anti-inflammatory gene set" in M-CSF-primed monocyte-derived macrophages [30]. In the case of macrophages, our results show that CD28 is capable of inducing intracellular signals that might modify macrophage differentiation and functional responses. In fact, murine M-MØ lacking CD28 exhibit an altered gene

profile, as well as enhanced T cell stimulatory and LPS-induced production of IL-6, which is required for full T cell activation [40][41], thus supporting a functional role for CD28 on M-CSF-primed macrophages.

CD28 typically functions as a co-signaling molecule during *trans* interactions of T cell with Antigen-Presenting Cells (APC) expressing the CD28 ligand partners CD80 (B7-1) and/or CD86 (B7-2). The efficacy and final outcome of these activating interactions strongly depends on the binding affinity and their expression levels on the cell surface, which is transcriptionally and post-transcriptionally regulated [42]. Although CD28 levels in M-MØ are lower than those of naïve T cells, CD28 in macrophages could physically and functionally impair interactions between CD80/CD86 on APC and CD28 on T lymphocytes, thus affecting the efficacy of the APC-T lymhocyte interactions. If so, macrophage CD28 expression would constitute a novel additional to the numerous interactions that modify, positively or negatively, the functional outcome of APC-T lymphocyte contacts. Specifically, CD28 on anti-inflammatory macrophages might compete with CD28 in T cells to inhibit APC-mediated activation. This proposed mechanism is compatible with the higher antigen-specific activation of CD4+ T cells displayed by M-MØ from *Cd28*KO. If so, CD28 expression by TAM might be one additional mechanism for suppressing anti-tumor immune responses.

Alternatively, CD28 might be engaged in interactions with other molecules on the macrophage cell surface (in *cis*). These potential CD28-CD80/CD86 interactions on macrophages not only would affect the efficacy of APC-T cell binding by limiting the availability of ligands for T cell CD28 or CTLA-4 on the macrophage membrane, but would also influence macrophage functions because CD80 and CD86 can signal to induce secretion of cytokines and other immunomodulatory molecules in professional APC [43]. These interactions *in cis* are not unprecedented regarding co-stimulatory molecules and, in fact, they are reminiscent of the PD-L1-CD80 interactions that occur in *cis* on the APC membranes [44]–[46], and that disrupt CD80-CD80 dimer formation, and of the in *cis* PD-L1/PD-1 interactions on APC, that prevent PD-1 inhibition of T cell responses [47]. Since human M-MØ and TAM express CD86 and low levels of CD80 (Supplementary Figure 1), CD28 might also affect the inhibitory or costimulatory outcome of APC-T lymphocyte contacts through interactions with other co-stimulatory molecules on the macrophage membrane.

The interferring action of macrophage CD28 on APC-T cell interactions is compatible with the enhanced T cell stimulatory ability of M-MØ from *Cd28*KO mice. However, our results have shown that the lack of CD28 in mouse macrophages appears associated with global transcriptional changes that determine an enrichment of genes involved in the response to IFN type I, as well as a reduction in the expression of genes involved in the inflammatory response. Interestingly, the augmented expression of the "HALLMARK_INTERFERON_ ALPHA_RESPONSE" gene set in *Cd28*KO M-MØ resembles the augmented type I IFN production previously observed in CD28-deficient mouse plasmacytoid dendritic cells, which highly express CD28 [14]. Taken together, all these results support CD28 as a negative regulator of the type I IFN pathway and of innate responses [14], an hypothesis that is also in line with the enhanced production of IL-6 that we have observed in *Cd28*KO M-MØ.

In summary, several features of CD28 expression by macrophages are worth noting: 1) innate cells, particularly M-MØ or TAM, can simultaneously express CD28 and their ligands; 2) Interactions of costimulatory molecules with their ligands can be inhibitory, as exemplified by the limiting effect of CD28 expression on the production of LPS-induced IL-6 (this manuscript), and in the secretion of type I IFN secretion by TLR-activated pDC [14]; 3) Diminished expression of costimulatory molecules by innate immune cells has been linked to dysregulated inflammatory or infectious diseases (asthma and inflammatory bowel disease) [48],[49], and impaired skin injury and anti-viral responses [14]. Given these antecedents, and the complex net of physical and functional interactions between CD28 and B7 family molecules, it is tempting to speculate that macrophage CD28 can contribute to the immunosuppressive activity of anti-inflammatory and tumor-associated macrophages. If so, the potential of macrophage CD28 as a target for immunotherapy should be checked.

MATERIALS AND METHODS

Mice. Mice used were 8-16 weeks old C57BL/6J (WT), *Cd28*$^{-/-}$ (*CD28*KO; B6.129S2-*Cd28*^{tm1Mak}/J), and OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J). They were bred and housed at the animal care facility of the Centro de Investigaciones Biológicas Margarita Salas (CSIC) under specific pathogen free conditions with sterilized food, water, bedding and environmental enrichment. The experimental procedures were approved by the

CSIC Ethics and Animal Welfare Committee, and conducted according to institutional, national and European Union guidelines under licenses PROEX 181/15 and PROEX 049/18 (to JMR).

Generation of human monocyte-derived macrophages and murine bone marrow-derived macrophages. Buffy coats were obtained from healthy blood donors, and anonymously provided by the Comunidad de Madrid blood Bank. Human PBMCs were isolated from buffy coats over a Lymphoprep™ gradient (Axis-Shield PoC AS) according to standard procedures. Monocytes (95% $CD14^+$ cells) were purified from PBMCs by magnetic cell sorting using human CD14 microbeads (Miltenyi Biotec), cultured at 0.5 x 10^6 cells/ml for 7 days in RPMI 1640 (Gibco) supplemented with 10% inactivated fetal calf serum (FCS, Biowest) (complete medium) at 37°C in a humidified atmosphere with 5% CO_2, and containing 1000 U/ml human GM-CSF, 10 ng/ml human M-CSF (Immunotools GmbH) or 10 ng/ml IL-34, to generate GM-MØ, M-MØ or IL34-MØ, respectively. Cytokines were added every 2 days. Where indicated, macrophages were treated with Ultra-pure *E. coli* 0111:B4 strain LPS (10 ng/ml, Invivogen), IFNγ (500 U/ml, R&D) or IL-4 (1000 U/ml, R&D) for 48 h. Recombinant human activin A (25 ng/mL; Miltenyi Biotec) was added together with M-CSF every 48h. For MAFB knockdown, M-MØ (10^6 cell/ml) were transfected with a MAFB-specific small interfering RNA (siMAFB, 50 nM) (Life Technologies) using Hiperfect (Qiagen) [30]. As a control, cells were transfected with a non-specific siRNA (siControl, Life Technologies). When required, human $CD16^+$ monocytes were isolated by positive selection using magnetic separation systems (MACS, Miltenyi Biotec) [21],[22]. Bone marrow–derived murine GM-MØ or M-MØ were obtained by flushing the femurs of 8-10 wk-old C57BL/6 or *Cd28*KO mice, and culturing cells during 7 days in DMEM supplemented with 10% FCS and 50 μM 2-ME, containing either murine GM-CSF (1000 U/ml) (PreProtech) or human M-CSF (25 ng/ml), respectively [50][51]. Cytokines were added every 2 days. Where indicated cells were exposed to *E. coli* 0111:B4 strain LPS (20-100 ng/ml, Invivogen), anti-CD28 (37.51 antibody, 2.5 μg/ml) or control IgG (2.5 μg/ml). Mouse IL-6, TNF and IL10 in culture supernatants were determined by ELISA (eBioscience for IL-6 and TNF, Biolegend for IL-10).

***Isolation of human macrophages*.** Synovial fluids were obtained from knee joints of RA patients during therapeutic arthrocentesis. Patients were selected by the presence of active knee arthritis, confirmed by a highly cellular synovial fluid, and were heterogeneous regarding demographic and disease characteristics, and previous RA therapy. Tumor-Associated Macrophages (TAM) were isolated from the ascitic fluid of cancer patients bearing tumors of different histological origins (ovarian cancer, breast adenocarcinoma, gastric carcinoma, metastatic melanoma), as described [52]. Patients received informed consent, and the study was approved by the ethics committees of Hospital General Universitario Gregorio Marañón, Hospital La Princesa and Hospital Doce de Octubre.

Flow cytometry. Flow cytometry was performed according to previously described guidelines [53]. Mouse monoclonal antibodies specific for human CD28 (PE labeled anti-human CD28, #302908, Biolegend), CD3 (FITC-labeled anti-human CD3, clone SK3, BD Biosciences), CD14 (Alexa Fluor-647-labeled anti-human CD14, #301818, clone M5E2, Biolegend) and human CD163 (PerCP-labeled anti-human CD163, #333625, clone GHI/61, Biolegend) were used. Isotype-matched labeled antibodies were included as negative controls. For mouse CD28 detection, monoclonal anti-mouse CD28 syrian hamster antibody (37.51, #553295, BD Pharmingen) and isotype control antibody (Ha4/8, #553962, BD Pharmingen) were used. Flow cytometry analysis of surface was performed using a FC-500 flow cytometer (Beckman Coulter, Brea, California) and the FlowLogic software **(**Miltenyi Biotec**).**

***Quantitative real time RT-PCR*.** Quantitative real-time PCR (qRT-PCR) was performed using the Universal Probe Library system (Roche Diagnostics) as described [21],[22]. All experiments were performed in triplicate wells for each condition, and results were normalized according to the expression levels of *TBP* mRNA. Results were expressed using the ΔΔCT (cycle threshold) method for quantification.

***Western blot assay*.** Early activation of Erk1/2 was determined in WT or *Cd28*KO M-MØ. M-MØ were seeded at 10^6 cells/well in 24-well culture plates in 1 ml of culture supernatant plus 1 ml culture medium. After overnight incubation at 37ºC, 1 ml of supernatant was removed and *E. coli* LPS (20 ng/ml) and anti-CD28 (37.51) or control antibody (2.5 μg/ml) were added. Cells were incubated for further 15 min at 37ºC

and set on ice. After removing the medium, the cells were carefully washed with 2 ml of ice-cold 0.2 mM Na_3VO_4 in PBS. Then, PBS was removed and the cells lysed with 60 µl/well of cold lysis buffer (1% Triton X-100 in 50 mM Tris/HCl, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_3VO_4, pH 7.7 plus protease inhibitors cocktail (Selleckchem)). After 30 min on ice, the lysates were centrifuged, the protein content in the supernatants determined and frozen at -20 ºC until use. Cell lysates were analyzed for Erk phosphorylation by immunoblot as described [54]. Primary antibodies used were rabbit antibody specific for dually phosphorylated Erk (Anti-Active MAPK, V6671, Promega) and monoclonal Anti-Vinculin antibody hVlN-1 (V913, Sigma). Secondary antibodies were anti-Rabbit IgG-HRP (Cell signaling, 7074P2) and anti-mouse IgG-HRP (Bionova, A90-137P). Quantification of OD in blots was performed with ImageJ 1.51j8 public software (NIH). Values were normalized, and relative normalized OD was calculated for each lane as the ratio of pERK to Vinculin normalized OD.

In vitro T cell proliferation assay. $CD4^+$ T cells from the spleen of OT-II mice were isolated using the $CD4^+$ T cell isolation kit II (130-090-860, Miltenyi Biotech) and suspended in DMEM supplemented with 10% FCS and 50 µM 2-ME. Bone marrow-derived mouse M-MØ in the same medium ($2x10^4$ cells per well) were seeded onto the bottom of 96-well V-bottom tissue culture plates (Corning) and cocultured with OT-II CD4 T cells ($2x10^4$ cells per well) in the presence of OVA peptide (20 µg/ml, OVA323–339). After 6-7 days of coculture, T cell proliferation was measured by addition of [^{3}H] thymidine (1 µCi/well) during the last 24 hours of culture.

Statistical analysis. Statistical analyses was done using GraphPad Prism, using paired or unpaired Student "t" test, as appropriate, and one-way ANOVA test coupled with Tukey´s post-hoc test where indicated. $p< 0.05$ was considered significant (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

RNA-sequencing and data analysis. RNA was isolated from M-MØ generated from C57BL/6J (WT) or *CD28*KO mice, and subjected to sequencing on a BGISEQ-500 platform (https://www.bgitechsolutions.com). RNAseq data were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession GSE157837. Clean reads were mapped to the reference

genome (mm10) using Bowtie2 [55]. Gene expression levels were calculated by using the RSEM software package [56], and differential gene expression was assessed by using the R-package DESeq2 algorithms using the parameters Fold Change>2 and adjusted p value <0.05. For gene set enrichment analysis (GSEA) (http://software.broadinstitute.org/gsea/index.jsp) [57], the Hallmark gene set database (v7.2) available at the website was used.

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AUTHORSHIP CONTRIBUTIONS

PP, ALC, JMR and APK designed research and analyzed data; LEC, LAF, ADS, SFR, CB, MSF, and BA performed research and analyzed data; ALC, JMR and APK conceived the study, designed research and wrote the paper. All authors had final approval of the version.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no commercial or financial conflict of interest

Data availibility statement

The data that support the findings of this study are openly available in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession GSE157837.

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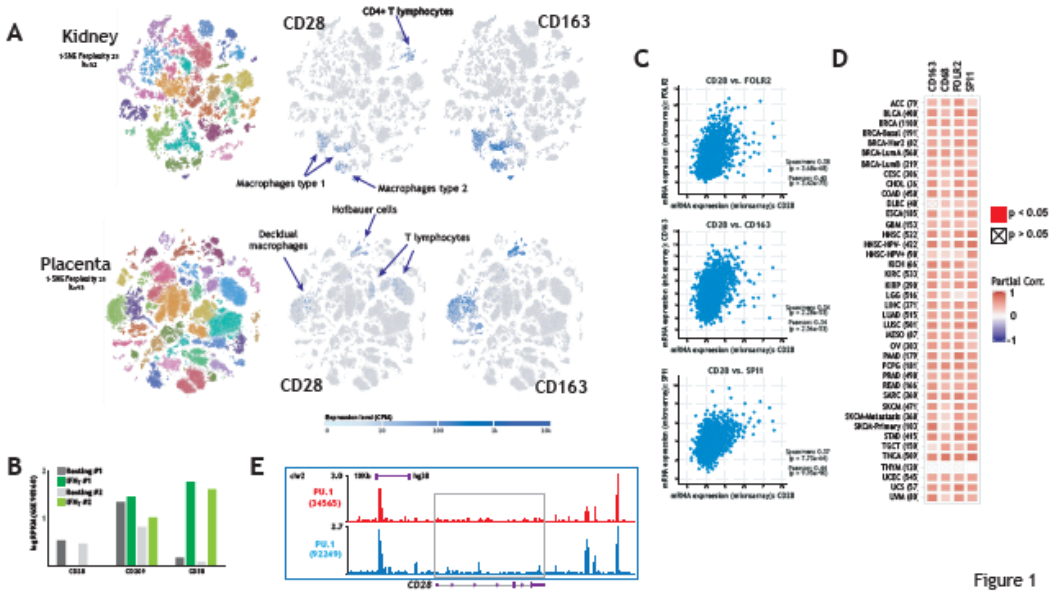


Figure 1.- ***CD28*** **expression and correlation with macrophage-specific gene expression in tissue-resident macrophages and tumor-associated macrophages. A.** *t*-distributed stochastic neighbour embedding (*t*-SNE) plots illustrating *CD28* gene expression in $CD45^+$ $CD163^+$ human kidney cells (upper panels) and in $CD163^+$ human placenta cells and Hoffbauer cells (lower panels). Clusters containing T lymphocytes are also indicated. The "Expression level" bar indicates the expression level in each case (CPM, counts per million). **B**. *CD28* mRNA expression in resting and IFN-γ-treated primary human monocytes (GSE98368,[20]). For control purposes, the expression of *CD209* and *CD38* is shown. Expression levels derive from two independent replicates of resting and IFN-γ-treated monocytes and are normalyzed by means of fragments per kilobase of transcript per million mapped reads (FPKM) [20]. **C.** Correlation of the expression of *CD28* with the expression of macrophage-specific genes of the "Anti-inflammatory gene set" (*CD163*, *FOLR2*) and the PU.1-encoding gene *SPI1* in breast carcinoma (METABRIC cohort). **D**. Partial correlation of *CD28* expression with the expression of the macrophage-specific genes *CD163*, *FOLR2* and *CD68*, and the PU.1-encoding gene *SPI1* in the indicated tumors. **E.** Identification of PU.1-binding sites in the *CD28* gene (data obtained from CistromeDB 34565 and 92249), with indication of the respective scores. CistromeDB 34565 data derive from two distinct samples from three independent donors (GSE31621) [58], while CistromeDB 92249 is from a single experiment on two different samples (GSE103477) [59].

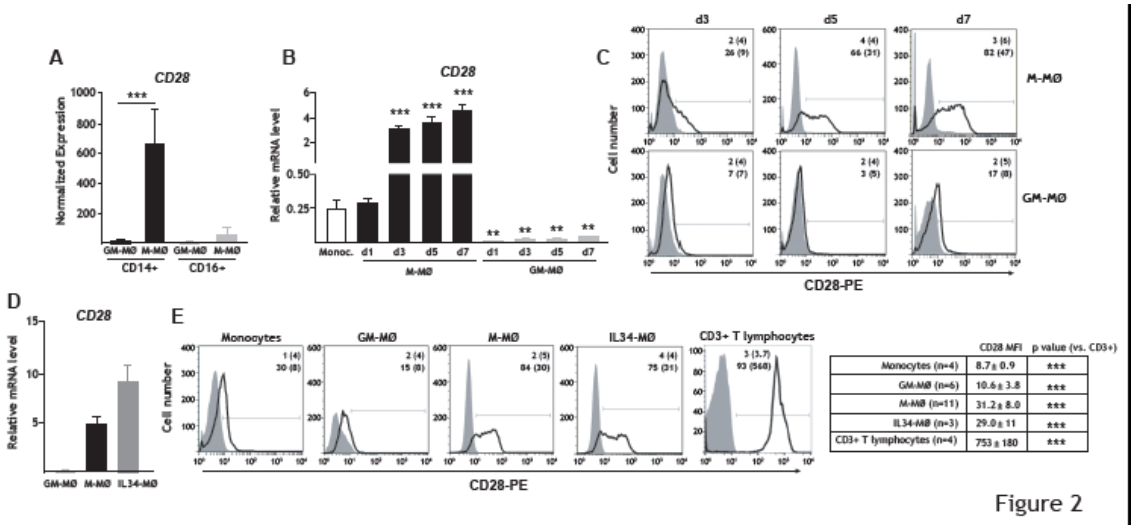


Figure 2.- ***CD28*** **expression in monocyte-derived macrophages generated in the presence of M-CSF (M-MØ) or GM-CSF (GM-MØ). A.** Normalized *CD28* gene expression in M-MØ and GM-MØ generated from three independent $CD14^{++}/CD16^{-}$ ($CD14^{+}$) or $CD14^{+}/CD16^{+}$ ($CD16^{+}$) monocyte prepararions, as indicated in [21], as measured by qRT-PCR using *TBP* mRNA for normalization purposes. **B.** *CD28* gene expression along the differentiation of three independent M-MØ and GM-MØ samples (days 1-7), as measured by qRT-PCR, and with indication of its expression in untreated $CD14^{+}$ monocytes. Relative mRNA level indicates the level of *CD28* mRNA after normalization with *TBP* mRNA levels in each sample. Mean ± SEM are shown. Groups were compared by applying one-way repeated measures ANOVA with Tukey´s post-hoc test (**, $p<0.01$; ***, $p<0.001$, comparison *vs* monocytes). **C.** Cell surface expression of CD28 along the differentiation of M-MØ and GM-MØ, as determined by flow cytometry after gating on live cells (solid lines, days 3-7), with indication of the fluorescence levels produced by an isotype-matched antibody (grey profiles). Mean Fluorescence Intensity (in brackets) and percentage of positive cells are indicated in each case. The experiment was done on three independent donors, and one representative experiment is shown. **D.** Relative *CD28* mRNA levels (after normalization with *TBP* mRNA) in M-MØ, GM-MØ and IL34-MØ generated from $CD14^{+}$ monocytes, as determined by qRT-PCR. The experiment was done on two independent donors and mean ± SEM are shown. **E.** Cell surface expression of CD28 (solid lines) in monocytes, M-MØ, GM-MØ, IL34-MØ and CD3+ T lymphocytes, as determined by flow cytometry after gating on live cells (*Left panel*). Gating strategy for CD3+ T lymphocytes is indicated in Supplementary Figure 4. Fluorescence levels produced by an isotype-matched antibody are indicated (grey

profiles), and the percentage of positive cells and Mean Fluorescence Intensity (MFI, in brackets) are shown in each case (bold case for CD28). One representative experiment is shown. The average CD28 MFI±SD for the different cell types indicated cells is shown in the *Right panel*. Groups were compared by applying one-way ANOVA (with Tukey´s post-hoc test, ***, $p<0.001$). All data are from two to three independent experiments with one donor per experiment.

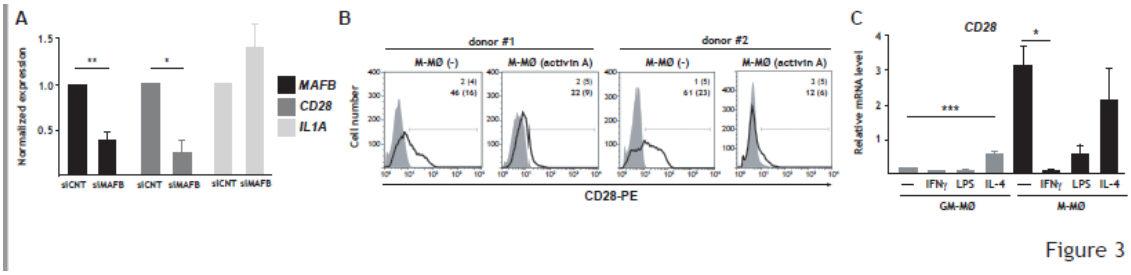


Figure 3.- CD28 expression in macrophages is dependent on MAFB, and is regulated by Activin A and macrophage-activating factors. A. Normalized gene expression of *MAFB, CD28* and *IL1A* in four independent preparations of M-MØ transfected with a MAFB-specific siRNA (siMAFB) or a control siRNA (siCNT), as determined by quantitative RT-PCR. Mean ± SEM are shown (*, $p<0.05$; **, $p<0.01$). Results are expressed as the expression of each gene in siMAFB-transfected cells (and after normalization with *TBP* mRNA) relative to its expression in cells transfected with non-specific siRNA (siCNT). **B.** Cell surface expression of CD28 in untreated M-MØ [M-MØ (-)] or M-MØ treated with activin A [M-MØ (Activin A)], as determined by flow cytometry after gating on live cells (solid lines). The fluorescence levels produced by an isotype-matched antibody is shown (grey profiles), and Mean Fluorescence Intensity (in brackets) and percentage of positive cells are indicated in each case. Results from two independent M-MØ preparations are shown. **C.** *CD28* gene expression in three independent M-MØ and GM-MØ preparations, either untreated (-), or treated with LPS, IFN-γ or IL-4 for 48 hours, as determined by quantitative RT-PCR. Relative mRNA level indicates the level of *CD28* mRNA normalized to *TBP* mRNA levels in each sample. Mean ± SEM are shown. Groups were compared by applying one-way ANOVA (with Tukey´s post-hoc test, *, $p<0.05$; ***, $p<0.001$). All data are from two to three independent experiments with one donor per experiment.

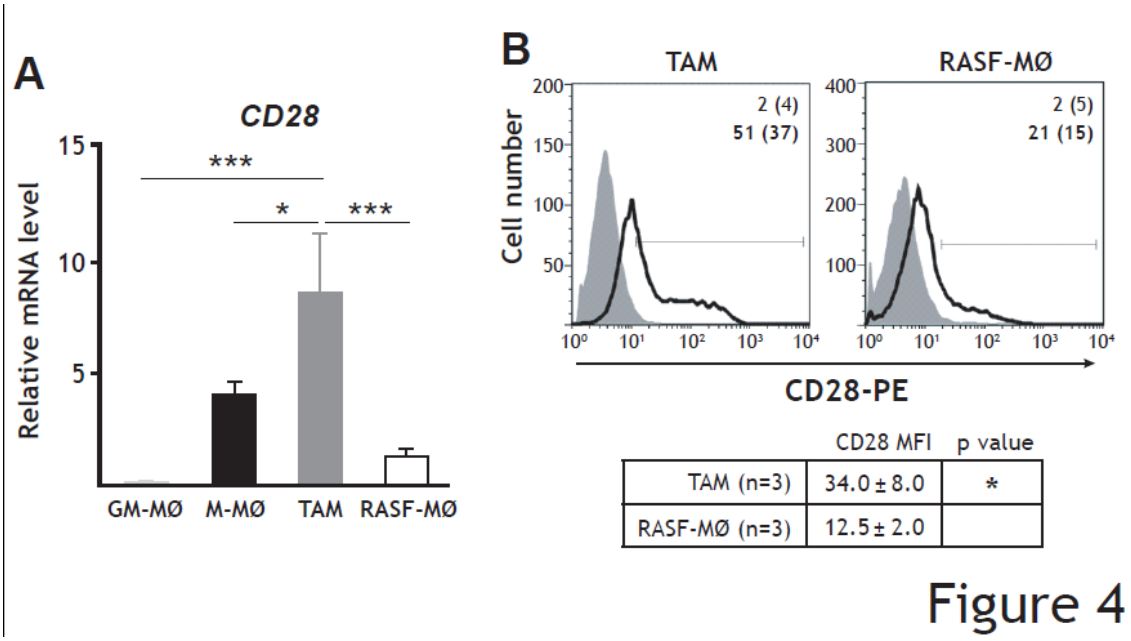


Figure 4

Figure 4.- CD28 expression in macrophages isolated from inflamed tissues. A. *CD28* gene expression in M-MØ (10 independent samples), GM-MØ (10 independent samples), Tumor-Associated macrophages (TAM, 5 independent samples) and macrophages isolated from the synovial fluid of RA patients (RASF-MØ, 16 independent samples), as determined by quantitative RT-PCR. Relative mRNA level indicates the level of *CD28* mRNA in each sample after normalization with *TBP* mRNA (one sample per experiment). Mean ± SEM are shown. Groups were compared by applying one-way ANOVA (with Tukey´s post-hoc test, *, $p<0.05$; ***, $p<0.001$). **B.** Cell surface expression of CD28 in Tumor-Associated macrophages (TAM, 3 independent samples, one sample per experiment) and macrophages isolated from the synovial fluid of RA patients (RASF-MØ, 3 independent samples, one sample per experiment), as determined by flow cytometry after gating on live cells (solid lines). The fluorescence levels produced by an isotype-matched antibody is shown (grey profiles), and the percentage of positive cells and Mean Fluorescence Intensity (MFI, in brackets) are shown in each case (bold case for CD28). Results from one representative experiment on each cell type is shown. The average CD28 MFI±SD in TAM and RASF-MØ is shown in the *Right panel*. Groups were compared by applying unpaired t-test (*, $p<0.05$).

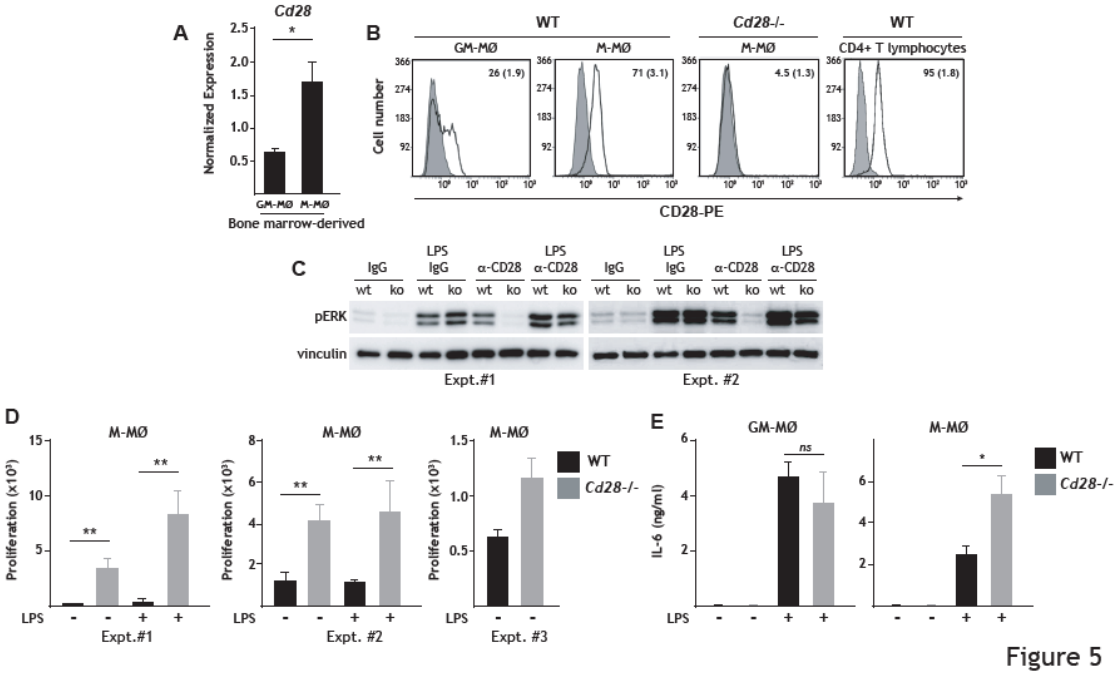


Figure 5

Figure 5.- Signaling and functional capability of macrophage CD28. A. *Cd28* gene expression in four independent M-MØ and GM-MØ preparations (one sample per experiment) generated from murine bone marrow-derived precursor cells, as determined by quantitative RT-PCR (each M-MØ and GM-MØ preparation was obtained from a single mice). Relative mRNA level indicates the level of *Cd28* mRNA normalized to *Tbp* mRNA levels in each sample. Mean ± SEM are shown. Groups were compared by applying unpaired t-test (*, $p<0.05$). **B.** Cell surface expression of CD28 in mouse M-MØ and GM-MØ from WT mice, M-MØ from *Cd28*KO mice, and spleen CD4+ T lymphocytes from WT mice, as determined by flow cytometry after gating on live cells. The fluorescence levels produced by an isotype-matched antibody are shown (grey profiles). One representative experiment is shown from three experiments with one mice per experiment. **C.** Immunoblot analysis of pERK in lysates of mouse M-MØ from WT and *Cd28*KO mice subjected to the indicated treatments for 15 min. Two independent experiments are shown. For loading control purposes, the level of vinculin was determined in parallel. IgG, control antibody; α-CD28, 37.51 anti-CD28 monoclonal antibody; LPS, *E. coli* LPS. **D.** Antigen (OVA323–339)-specific proliferation of OT-II $CD4^+$ T lymphocytes cultured with untreated or LPS-treated murine M-MØ from WT or *Cd28*KO mice. After a 6-day coculture, ^{3}H-thymidine incorporation was determined as a readout for T cell proliferation. Three independent experiments are shown with one mouse per experiment. Six technical replicates were

done for each experimental condition, and Mean ± SEM of ^{3}H-thymidine incorporation is shown. Groups were compared by applying unpaired t-test (*, $p<0.05$: **, $p<0.01$). **E**. IL-6 production by murine GM-MØ and M-MØ from WT and *Cd28*KO mice in basal conditions and in response to 100 ng/ml LPS, as determined by ELISA. GM-MØ and M-MØ were generated from WT mice (n= 5) and *Cd28*KO mice (n= 5), seeded at 10^6 cells/well in 24-well culture plates and left untreated or exposed to *E. coli* LPS (100 ng/ml). After overnight incubation at 37ºC, supernatants were collected and assayed for IL-6. Mean ±SE of IL-6 production for each condition is shown and data are from five experiments with one mouse per experiment. Groups were compared by applying unpaired t-test (*, $p<0.05$).

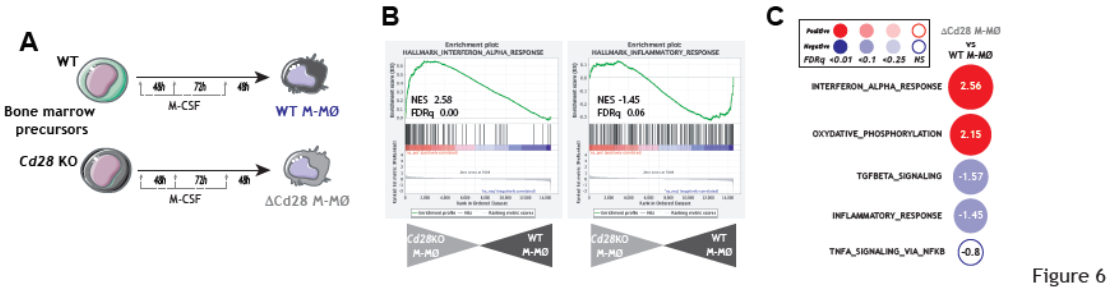
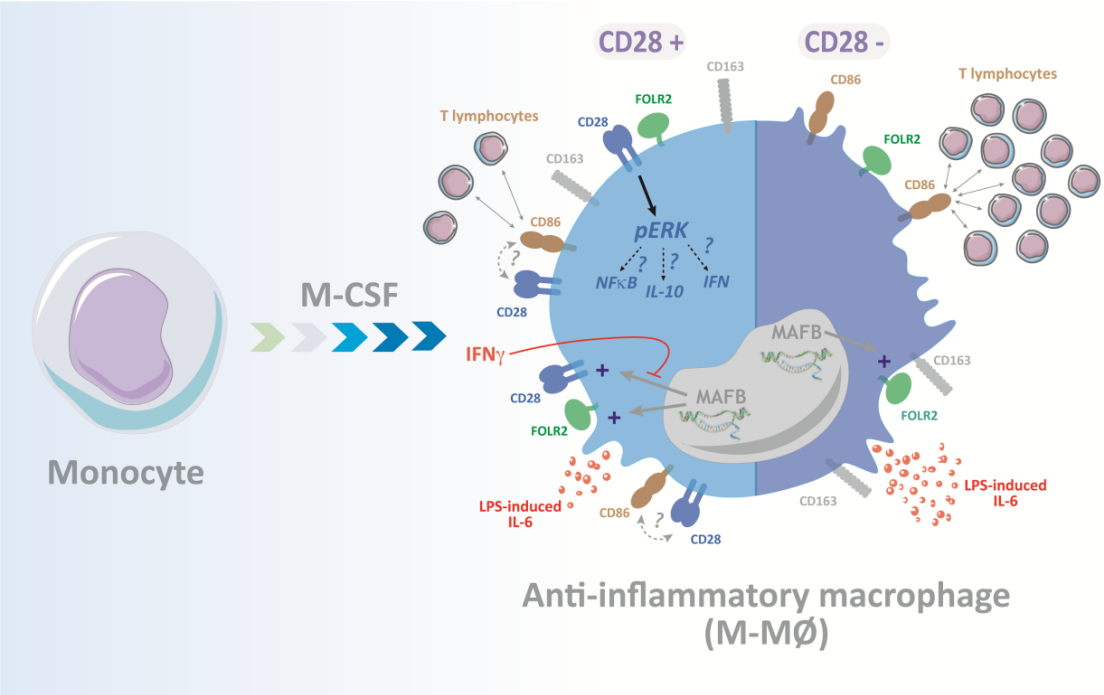


Figure 6

Figure 6.- Transcriptional consequences of *Cd28* ablation in murine M-MØ. A. Schematic representation of the *in vitro* generation of bone marrow-derived M-MØ from WT (WT M-MØ) and *Cd28*KO mice (ΔCd28 M-MØ) before RNA isolation and RNA-sequencing (GSE157837). **B**. GSEA of the "HALLMARK_INTERFERON_ALPHA_RESPONSE" and "HALLMARK_INFLAMMATORY_RESPONSE" gene sets on the ranked comparison of the transcriptomes of M-MØ from *Cd28*KO and WT mice. Normalized Enrichment Score (NES) and False Discovery Rate (FDR) q value are shown in each case. **C** Summary of GSEA with the Hallmark gene set database (v7.2) on the ranked comparison of the transcriptomes of M-MØ from *Cd28*KO and WT mice. The color of the circles illustrate the type of enrichment of each comparison (positive, red; negative, blue). The area of each circle is proportional to the Normalized Enrichment Scores of each comparison, which is also indicated.

Graphical abstract



CD28 was found on anti-inflammatory M-CSF dependent macrophages, and detected on tissue-resident macrophages under homeostatic and pathological conditions. Macrophage CD28 expression is dependent on MAFB and inhibited by IFN-γ and modifies the functional and gene profile of bone-marrow derived murine macrophages. Thus, CD28 appears as a hallmark of anti-inflammatory macrophages.