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Thymus-derived regulatory T cells restrain pro-inflammatory Th1 responses by downregulating CD70 on dendritic cells

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Abstract

The severity and intensity of autoimmune disease in immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) patients and in scurfy mice emphasize the critical role played by thymus-derived regulatory T cells (tTregs) in maintaining peripheral immune tolerance. However, although tTregs are critical to prevent lethal autoimmunity and excessive inflammatory responses, their suppressive mechanism remains elusive. Here, we demonstrate that tTregs selectively inhibit CD27/CD70-dependent Th1 priming, while leaving the IL-12-dependent pathway unaffected. Immunized mice depleted of tTregs showed an increased response of IFN-γ-secreting CD4^{+} T cells that was strictly reliant on a functional CD27/CD70 pathway. *In vitro* studies revealed that tTregs downregulate CD70 from the plasma membrane of dendritic cells (DCs) in a CD27-dependent manner. CD70 downregulation required contact between Tregs and DCs and resulted in endocytosis of CD27 and CD70 into the DC. These findings reveal a novel mechanism by which tTregs can maintain tolerance or prevent excessive, proinflammatory Th1 responses.

Introduction

Thymus-derived regulatory T cells (tTregs) are critical to prevent autoimmunity and to restrain T-cell responses to foreign antigens (Brunkow *et al*, 2001; Suffia *et al*, 2006; Lahl *et al*, 2007; Shafiani *et al*, 2010). Their ablation in newborn mice (Lahl *et al*, 2007) leads to the development of severe autoimmunity, a disease similar to the one observed in scurfy mouse mutant which harbors a mutated version of the Treg-specific *foxp3* gene (Bennett *et al*, 2001). Although tTregs were first believed to control reactivity to self, it is now clearly established that they also dampen immune responses to foreign antigens, thereby limiting immunopathology (Belkaid *et al*, 2002; Oldenhove *et al*, 2003; Suffia *et al*, 2006; Pacholczyk *et al*, 2007; Shafiani *et al*, 2010).

Despite their critical role in counter-balancing immune responses, the biological basis of tTreg-cell suppression is still elusive. Potential immunosuppressive mechanisms include the inhibitory cytokine IL-35 (Collison *et al*, 2007), TGF-β (Li *et al*, 2006), the perforin–granzyme pathway (Cao *et al*, 2007), the cytokine deprivation-mediated apoptosis (Pandiyan *et al*, 2007), and the intercellular transport of cAMP via gap junctions (Bopp *et al*, 2007). IL-10 seems to be required for Treg-dependent suppression mainly in mucosal tissues (Atarashi *et al*, 2011; Chaudhry *et al*, 2011), while CTLA-4 may act by out-competing CD28 for their common CD80/CD86 ligands (Yokosuka *et al*, 2010), reducing their expression (Qureshi *et al*, 2011) and/or inducing the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (Grohmann *et al*, 2002). Tregs have also been shown to directly (Boissonnas *et al*, 2010) or indirectly (Giroux *et al*, 2007) control the number of DCs, and to prevent effective DC/T interaction (Hugues *et al*, 2004; Tadokoro *et al*, 2006; Tang *et al*, 2006). More recently, a number of reports have provided evidence that expression of specific transcription

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factors may endow Treg cells with the capacity to selectively inhibit the corresponding T-helper subset (Chaudhry et al, 2009; Koch et al, 2009; Zheng et al, 2009). The effector response-specific suppressive mechanism is not known at present, but could be related to induction of chemokine receptors by the transcription factors. In addition, the co-inhibitory molecule TIGIT, expressed on Tregs, has been shown to induce expression of fibrinogen-like protein 2 which promotes Treg suppression of Th1 and Th17 cells while protecting Th2 cells (Joller et al, 2014).

In this study, we sought to determine the molecular mechanism by which tTregs may inhibit Th1-type responses. Indeed, we and others have shown previously that tTregs selectively restricted the development of CD4$^+$ and CD8$^+$ IFN-γ-producing T cells in vivo (Belkaid et al, 2002; Oldenhove et al, 2003; McLachlan et al, 2009), suggesting that factors directing Th1 differentiation could be the target of suppression. It is widely accepted that IL-12 produced by DCs exerts a powerful positive regulatory influence on the development of Th1 cells in vivo (Macatonia et al, 1995; Maldonado-López et al, 1999; Maldonado-Lopez et al, 2001; Pulendran et al, 2001; Mashayekhi et al, 2011). In addition, Soares et al have identified a major IL-12-independent pathway which promoted Th1 priming in vivo. Immunization of IL-12 p40$^{-/-}$ mice using chimeric anti-DEC-205 mAb (expressing LACK from *Leishmania major*) induced the development of IFN-γ-producing cells at a frequency comparable to WT mice (Soares et al, 2007). This pathway required the membrane-associated tumor necrosis factor (TNF) family member CD70, a homotrimeric type II transmembrane glycoprotein expressed on activated DCs, B cells and T cells (Oshima et al, 1998; Tesselaar et al, 2003) and its receptor CD27, a dimeric type I transmembrane glycoprotein expressed on T lymphocytes and NK cells. Here, we demonstrate that tTregs selectively control the CD27/CD70 pathway by regulating the expression of the costimulatory molecule CD70 on DCs in a CD27-dependent manner, leading to impaired Th1-prone costimulation.

Results

Enhanced Th1 priming in the absence of tTregs is dependent on CD70

We have shown previously that tTreg exerted a selective negative feedback mechanism on Th1-type responses in vivo (Oldenhove et al, 2003). Using the same model of Th1 priming with KLH-loaded splenic dendritic cells (DCs) and tTreg depletion with anti-CD25 mAb (Fig 1A), we measured the proportion of IFN-γ-producing cells upon antigenic stimulation in vitro and found a threefold to fourfold increase in the generation of KLH-specific, IFN-γ-producing CD4$^+$ T cells (Fig 1B and Supplementary Fig S1). The selective control of Th1 priming by CD25$^+$ tTregs (Belkaid et al, 2002; Oldenhove et al, 2003; McLachlan et al, 2009) suggested that Th1-prone factors could be the target of suppression. To determine whether the increased Th1 response depended on IL-12 production, WT and IL-12p40-deficient mice were immunized with DCs from syngeneic mice. The analysis of the immune response clearly showed that tTreg depletion increased the antigen-specific IFN-γ response independently of IL-12 (Fig 1C for a summary of three experiments). Therefore, we considered that tTregs may control the CD70-dependent pathway of

Th1 priming that is known to be IL-12 independent (Soares et al, 2007). CD70 is a member of the TNF family that is expressed on activated DCs (Goodwin et al, 1993; Keller et al, 2008) and that promotes Th1 priming in both mouse and human by triggering the costimulatory receptor CD27 on CD4$^+$ T cells (Soares et al, 2007; Van Oosterwijk et al, 2007; Xiao et al, 2008; Pen et al, 2013). We tested the involvement of the CD27/CD70 pathway by antibody blocking and the use of CD27$^-$ or CD70-deficient mice. When mice were treated with a blocking mAb to CD70 (Oshima et al, 1998), tTreg depletion enhanced the antigen-specific IFN-γ response to a lesser extent than when they were treated with a control antibody (Fig 1D). Furthermore, CD27$^{-/-}$ mice (Hendriks et al, 2000) did not show an enhanced antigen-specific IFN-γ response upon tTreg depletion (Fig 1E), nor did CD70-deficient mice (Coquet et al, 2013) that were primed with CD70-deficient DCs (Fig 1F). These data indicate that tTregs control the IL-12-independent pathway for Th1 priming that is driven by CD27/CD70 interaction.

Plasma membrane expression of CD70 on DCs is regulated by tTregs

Since the contribution of CD70 to Th1-cell priming is likely to correlate with its expression on the surface of DCs (Keller et al, 2007), we tested whether tTregs controlled CD70 expression on DCs. Based on a previous study showing that CD70 was expressed on the surface of DCs when they were presenting antigen to T cells (Soares et al, 2007), we cultured spleen and lymph node cells from immunized mice with KLH and measured CD70 expression by flow cytometry 24 h later. In vivo, tTreg depletion led to an increase in the frequency of DCs that expressed CD70 in these conditions and to an increase in CD70 expression level at the DC surface (Fig 2A and B). To further confirm the role of tTregs, we used DEREG mice in which DCs upregulated CD70 after diphtheria toxin injection (Supplementary Fig S2A). In addition, anti-CD25 treatment did not deplete activated T lymphocytes in our model (Supplementary Fig S2B). This was not a global effect on DC activation, since the frequency of DCs that expressed the activation marker CD86 remained unchanged upon tTreg depletion while the level of CD86 expression at the DC surface decreased (Fig 2B). We also determined the effect of tTregs on Th1 priming and CD70 expression in vitro. For this purpose, naïve conventional CD4$^+$ CD25$^-$ T cells (Tconvs) were co-cultured with allogeneic DCs in the absence or presence of tTregs. IFN-γ production decreased proportionally to the number of tTregs that were added (Supplementary Fig S3). Culturing Tconvs with allogeneic DCs led to an increase of CD70 levels at the cell surface of DCs, but, in the additional presence of tTregs, CD70 upregulation was impeded (Fig 2C). Addition of tTregs also reduced CD86 levels on DCs, as reported (Onishi et al, 2008), while expression of MHC class II remained unaffected (Fig 2C). Collectively, these observations suggest that tTregs inhibit CD70 expression on the plasma membrane of DCs in vivo and in vitro.

tTregs inhibit CD70 surface expression in a CD27-dependent manner

To test whether tTregs directly inhibited CD70 expression, we co-cultured tTregs with DCs that expressed CD70 following their activation with LPS and anti-CD40 mAb. The tTregs inhibited the
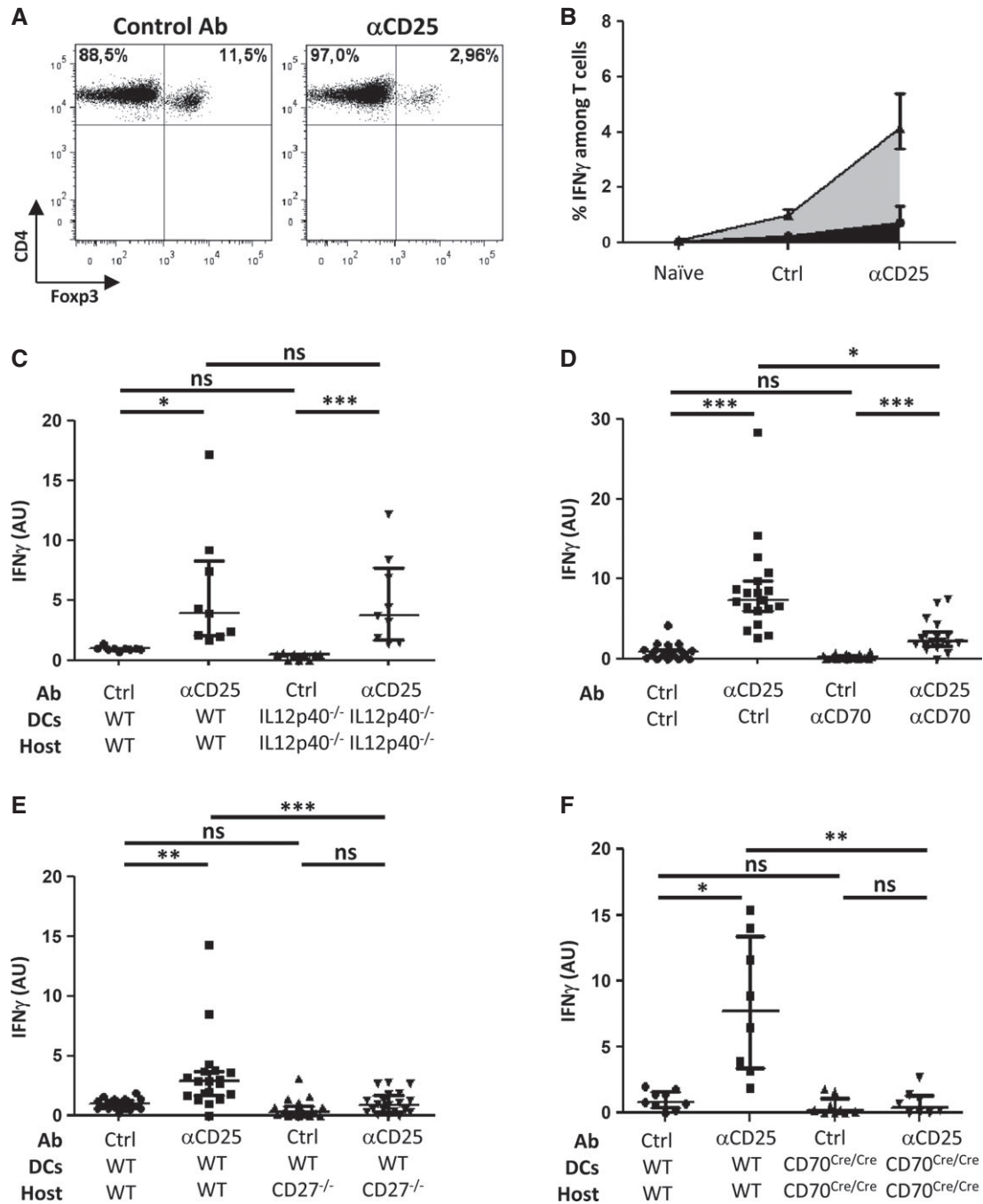
Figure 1. tTregs inhibit the CD70-driven pathway of Th1 priming.

A, B   KLH-pulsed splenic DCs were injected into the footpads of mice that were treated with control or anti-CD25 mAb. The draining LNs of immunized or naive mice were harvested 5 days later, and isolated cells were analyzed by flow cytometry to validate the efficacy of Treg depletion (A) or restimulated *in vitro* with KLH (B). IFN-γ production was analyzed by flow cytometry after 2 days of culture following 4 h incubation with brefeldin A to block IFN-γ secretion. Shown is the proportion of IFN-γ-producing cells among CD4$^+$ (shaded area) and CD8$^+$ (black area) T cells after stimulation with 20 μg/ml KLH. Results are shown as median ± interquartile range from five individual mice per group and are representative of two independent experiments.

C–F   KLH-pulsed splenic DCs were injected into the footpads of mice that were treated with control or anti-CD25 mAb. At day 5 after immunization, the draining LNs were harvested and cells were incubated with 2 μg/ml KLH to stimulate IFN-γ production from *in vivo* activated antigen-specific CD4$^+$ T cells. (C) DCs from C57BL/6 WT or IL-12p40$^{-/-}$ mice were injected into the footpads of syngeneic hosts. (D) DCs from C57BL/6 mice were injected into the footpads of syngeneic hosts, treated or not with control or anti-CD70 mAb 1 day before and 2 days after immunization. (E) DCs from C57BL/6 mice were injected into the footpads of C57BL/6 WT or CD27$^{-/-}$ hosts. (F) DCs from WT or CD70-deficient (CD70$^{Cre/Cre}$) mice were injected into the footpads of syngeneic hosts. Data are shown as individual mice and pooled from two (F) or at least three (C–E) independent experiments. IFN-γ production is expressed in arbitrary units (AU) and normalized for each experiment to the mean value (ranging from 0.5 to 5 ng/ml) obtained for IFN-γ production by LN cells from WT mice that had been immunized with syngeneic DCs (arbitrarily set at 1). The error bars correspond to median ± interquartile range. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, nonsignificantly different.
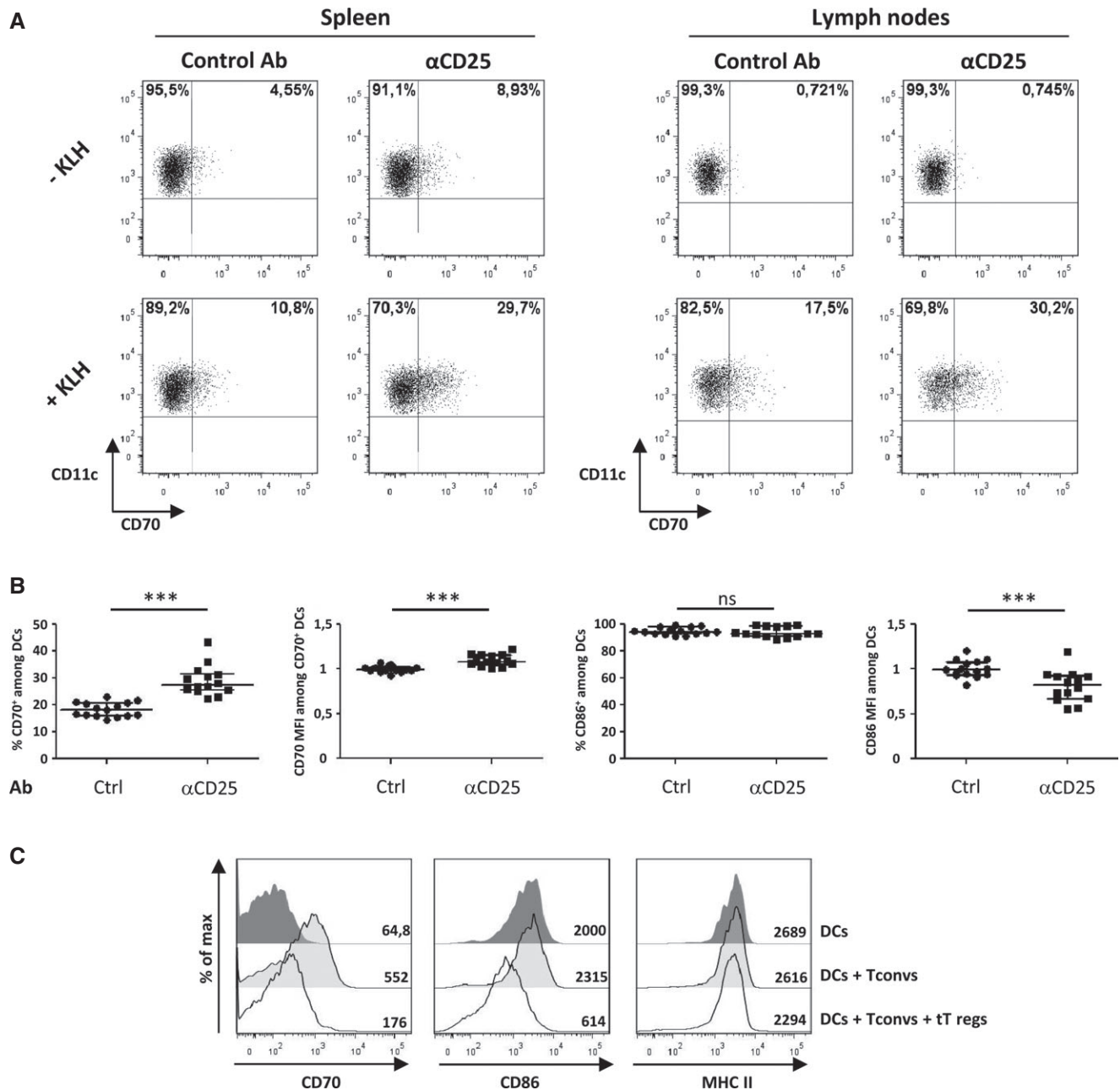
Figure 2. tTregs downregulate CD70 from the cell surface of DCs.

Mice were immunized with KLH-pulsed mature splenic DCs and depleted of Tregs or not. Five days later, LN cells were restimulated or not with 2 μg/ml KLH and tested after 24 h for CD11c, CD70 and CD86 expression.

A   The dot plots depict the percentage of CD70+ cells gated on CD11c+ MHCII+ DCs in cell suspensions from the spleen or draining lymph nodes and are from a mouse representative of at least 15 mice from three experiments.

B   The data are expressed as the percentage of CD70+ or CD86+ cells among DCs (CD11c+ MHCII+), or as the level of expression median of fluorescence intensity (MFI) of CD70 by CD70+ DCs and of CD86 by DCs. Data are from individual mice analyzed in three independent experiments; error bars correspond to median ± interquartile range. MFI is expressed as arbitrary unit normalized for each experiment to the mean MFI from the control group, arbitrarily set at 1. ***P < 0.001; ns, nonsignificantly different.

C   DCs (3 × 10^4) from C57BL/6 mice were cultured with allogeneic CD4+CD25− Tconvs, in the presence or absence of allogeneic CD4+CD25+ tTregs (3 × 10^5 each) from BALB/c mice. After 48 h, the expression of CD70, CD86 and MHCII on CD11c+ cells (DCs) was analyzed by flow cytometry. Numbers indicate the level of expression (MFI) for each condition. Data are from one experiment representative of at least 4.

expression of CD70 on DCs (Fig 3A), indicating that the CD70 downregulation was exerted directly by the tTregs and not via Tconvs. Consistently, CD70 was also downregulated from *Cd70*-transgenic DCs in which CD70 expression is driven by the CD11c promoter (Keller *et al*, 2008) (Supplementary Fig S4A), suggesting that tTregs downregulated CD70 at the protein level,
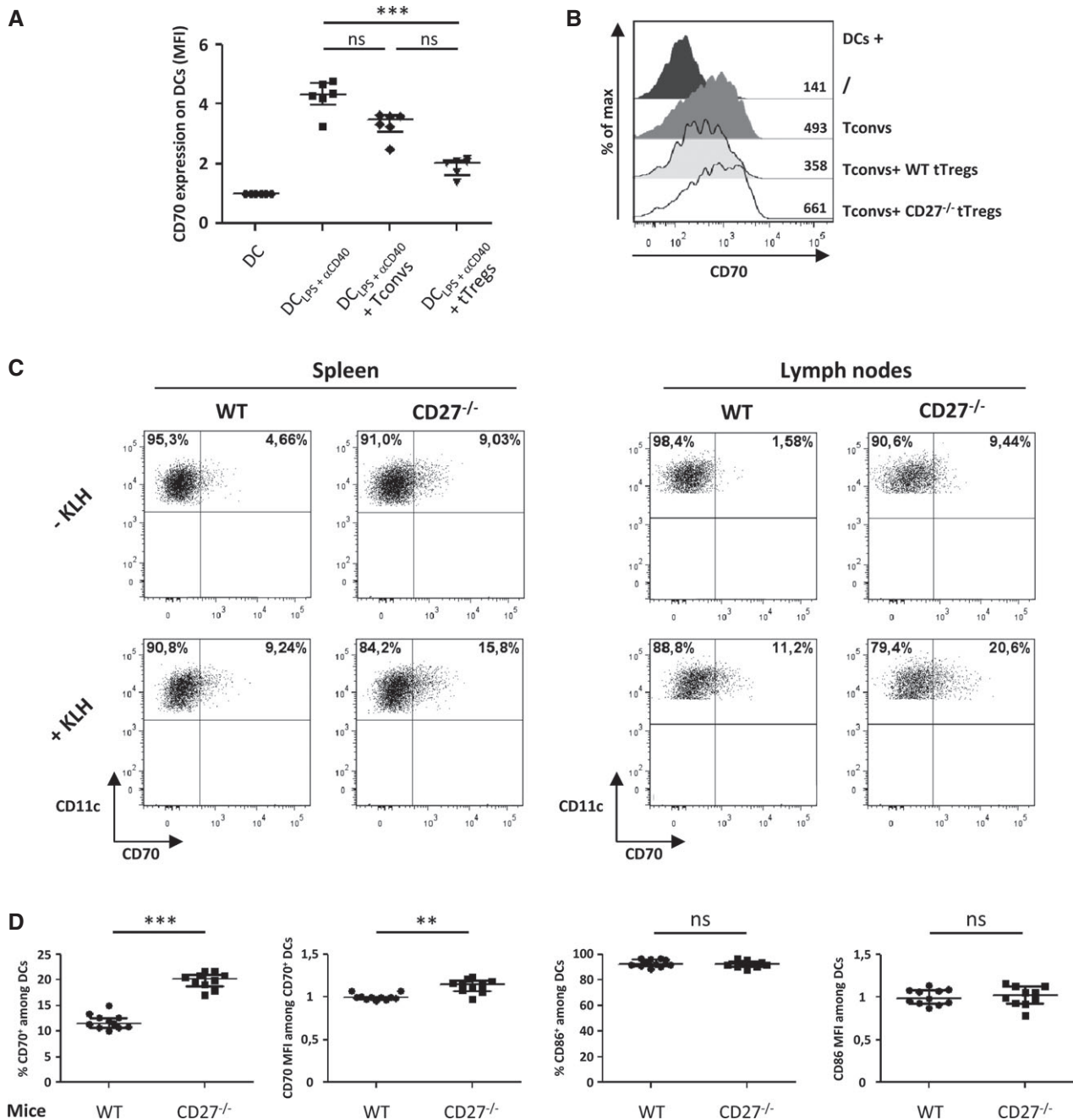
Figure 3.   tTregs directly downregulate CD70 from the plasma membrane of DCs in a CD27-dependent manner.

A   Splenic DCs from C57BL/6 mice were pre-activated *in vitro* with LPS (5 μg/ml) and anti-CD40 mAb (10 μg/ml), and 3×10^4 cells were cultured with 3×10^5 CD4^{+} CD25^{+} tTregs and/or CD4^{+} CD25^{−} Tconvs from BALB/c mice as indicated. The expression level of CD70 (MFI) on viable CD11c^{+} DCs was analyzed by flow cytometry 48 h later. For each experiment, MFI values were normalized to immature DCs. Data are from five independent experiments; error bars correspond to median ± interquartile range.

B   Splenic DCs from BALB/c mice were cultured at 3×10^4 cells with 3×10^5 Tconvs supplemented or not with 3×10^5 Tregs from WT or CD27-deficient C57BL/6 mice. CD70 expression on DCs was analyzed by flow cytometry 48 h later. Data are from one experiment representative of at least 5. Numbers represent the MFI value for each condition.

C, D   WT or CD27-deficient C57BL/6 mice were immunized with KLH-pulsed splenic DCs from WT mice. Five days later, LN cells were restimulated or not with 2 μg/ml KLH and tested after 24 h for CD11c, CD70 and CD86 expression. (C) The dot plots depict the percentage of CD70^{+} cells gated on CD11c^{+} MHCII^{+} DCs in cell suspensions from the spleen and draining lymph nodes and are from a mouse representative of at least 10 mice. (D) The data are expressed as the percentage of CD70^{+} or CD86^{+} cells among DCs (CD11c^{+} MHCII^{+}), or as the level of expression (MFI) of CD70 by CD70^{+} DCs and of CD86 by DCs. Data are from individual mice analyzed in two independent experiments; error bars correspond to median ± interquartile range. MFI is expressed as arbitrary unit normalized for each experiment to the mean MFI from the control group, arbitrarily set at 1.

Data information: **$P < 0.01$; ***$P < 0.001$; ns, nonsignificantly different.

rather than at the transcriptional level. Transwell experiments with separated Tconvs and tTregs, both activated with allogeneic DCs, showed that tTregs downregulated CD70 from the DC cell surface by direct contact rather than via a soluble factor (Supplementary Fig S4B). We therefore considered the possibility that tTregs downregulated CD70 through CD27 via a direct receptor–ligand interaction, as suggested recently (Kuka *et al*, 2013). Indeed, tTregs from CD27$^{-/-}$ mice failed to downregulate CD70 on allogeneic DCs (Fig 3B). Similarly, CD27 regulated the expression of CD70 *in vivo*: After immunization with KLH-pulsed WT DCs, the frequency of CD70$^+$ DCs and the expression level of CD70 on DCs were higher in CD27$^{-/-}$ mice than in WT mice (Fig 3C and D). Interestingly, CD70 was detected on the plasma membrane of DCs in lymph nodes from CD27-deficient but not competent mice, even without KLH restimulation, confirming the regulatory role of CD27. In contrast, the expression of CD86 on DCs was the same in immunized WT and CD27$^{-/-}$ mice (Fig 3D). Thus, tTregs control the expression of CD70 on the plasma membrane of DCs in a CD27-dependent manner.

The CD27 receptor controls surface expression of its ligand CD70

We next examined whether the capacity to downregulate CD70 from the cell surface of DCs was a specific property of tTregs or related to the CD27 expression level. Indeed, tTregs express higher levels of CD27 than naive Tconvs in steady state conditions (Fig 4A). Tconvs that had been stimulated with anti-CD3 and anti-CD28 mAbs displayed similar CD27 expression as tTregs (Fig 4B, upper left panel) and gained the capacity to inhibit CD70 upregulation on allogeneic DCs (Fig 4B, upper right panel). Also, Tconvs that over-expressed an exogenous HA-tagged CD27-GFP fusion protein could do so (Fig 4B, upper panels). Surprisingly, we found that CD27, HA and GFP could be detected on DCs in the culture with allogeneic T cells over-expressing HA-CD27-GFP, suggesting that the intact membrane-anchored CD27 protein from the T cells was taken up by the DCs (Fig 4B, lower panels). The inhibition of CD70 by HA-CD27-GFP T cells was rapid, as T cells over-expressing HA-CD27-GFP downregulated CD70 from the cell surface of LPS/anti-CD40 mAb-activated DCs and acquired GFP within 4 h of co-culture in the absence of antigen (Fig 4C, upper panels). Consistent with the rapid kinetics, CD70 was downregulated at the protein level and not at the transcriptional level, since *Cd70* mRNA expression in the DCs remained unchanged (Fig 4C, lower panel). Taken together, these observations establish a correlation between the level of CD27 expression and the capacity of T cells to inhibit CD70 on the plasma membrane of DCs.

The CD27 receptor is co-internalized with CD70 in splenic DCs

We next examined the mechanism of CD27 transfer and CD70 downregulation. First, we analyzed the role of CD70 in the acquisition of tagged CD27 molecules by DCs. We co-cultured T cells expressing HA-CD27-GFP (or GFP alone) on their plasma membrane (Supplementary Fig S5) with splenic DCs from WT or CD70$^{Cre/Cre}$ mice, pre-activated with LPS/anti-CD40 mAb (Fig 5A). The GFP signal was only detected when fused to CD27 and on DCs expressing CD70 (and correlated with decreased CD70 expression),

indicating that CD27/CD70 interaction was required for CD27 acquisition by DCs. The tenfold higher proportion of GFP$^+$ DCs, as compared to HA$^+$ DCs, would be compatible with the internalization of the tagged CD27. In favor of this hypothesis, treatment with lysosome inhibitor bafilomycin resulted in increased GFP signal in DCs, suggesting that CD27 was located (and probably degraded) in the lysosome (Fig 5B).

To better understand the mechanism of CD70 inhibition by its receptor, we analyzed the trafficking of CD27/CD70 molecules in DCs. Indeed, recent reports suggested that CD80/CD86 expression was downregulated by tTregs by a mechanism involving CTLA-4-dependent endocytosis of the ligand/receptor pair (Qureshi *et al*, 2011). We co-cultured HA-CD27-GFP T cells with BMDCs expressing CD70 tagged with mCherry at its cytoplasmic tail (Fig 6). Confocal laser scanning microscopy (CLSM) revealed that, within 60 min of co-culture, mCherry-CD70 and CD27-GFP co-clustered at the immune synapse and partially co-localized in an intracellular compartment of BMDCs. Real-time imaging of the cultures showed that the clustering of CD27-GFP and mCherry-CD70 at the contact site was followed by internalization of both molecules within a few minutes (Supplementary Fig S6). The imaging data suggest that membrane exchange may occur at the immune synapse (Fig 6B and Supplementary Movie S1) and via tunneling nanotubes extending from BMDCs (Fig 6C and Supplementary Movie S2). Of note, we made similar observations in co-culture of splenic DCs and T lymphocytes (co-clustering of CD27 and CD70, partial co-localization in DCs and presence of tunneling nanotubes), which may suggest a similar process (Supplementary Fig S7). We conclude that CD27 on T cells may limit CD70 expression on the plasma membrane of DCs by inducing its internalization and probably its degradation in the lysosome.

Discussion

The main finding of this work is that tTregs control inflammatory responses *in vivo* by suppressing the CD70/CD27 costimulatory pathway that is important for Th1 priming. tTregs downregulate CD70 protein levels at the plasma membrane of DCs by direct contact. Our data suggest that endocytosis and degradation of CD70 are enhanced as a result of its contact with CD27 on the surface of tTregs. This effect of Tregs on DC function is compatible with their capacity to sequester DCs (Hugues *et al*, 2004; Tadokoro *et al*, 2006; Tang *et al*, 2006; Onishi *et al*, 2008) and their higher expression of CD27 on the plasma membrane at steady state. Live imaging suggests that the transfer of the CD27 receptor from T cells to DCs may occur by previously described mechanisms, such as the elusive trogocytosis at the level of the immune synapse (Joly & Hudrisier, 2003; Nakayama *et al*, 2011; Somanchi *et al*, 2012) or tunneling nanotubes which allow physical connections of the plasma membranes between remote cells (Watkins & Salter, 2005; Chauveau *et al*, 2010).

Consistent with our observations of intercellular transfer of CD27, a recent report demonstrates that TCR-enriched microvesicles can be released by T cells at the level of the immunological synapse upon contact disruption. Interestingly, these microvesicles mediated TCR transfer to B lymphocytes and delivered transcellular signals (Choudhuri *et al*, 2014). Experiments are underway to test whether
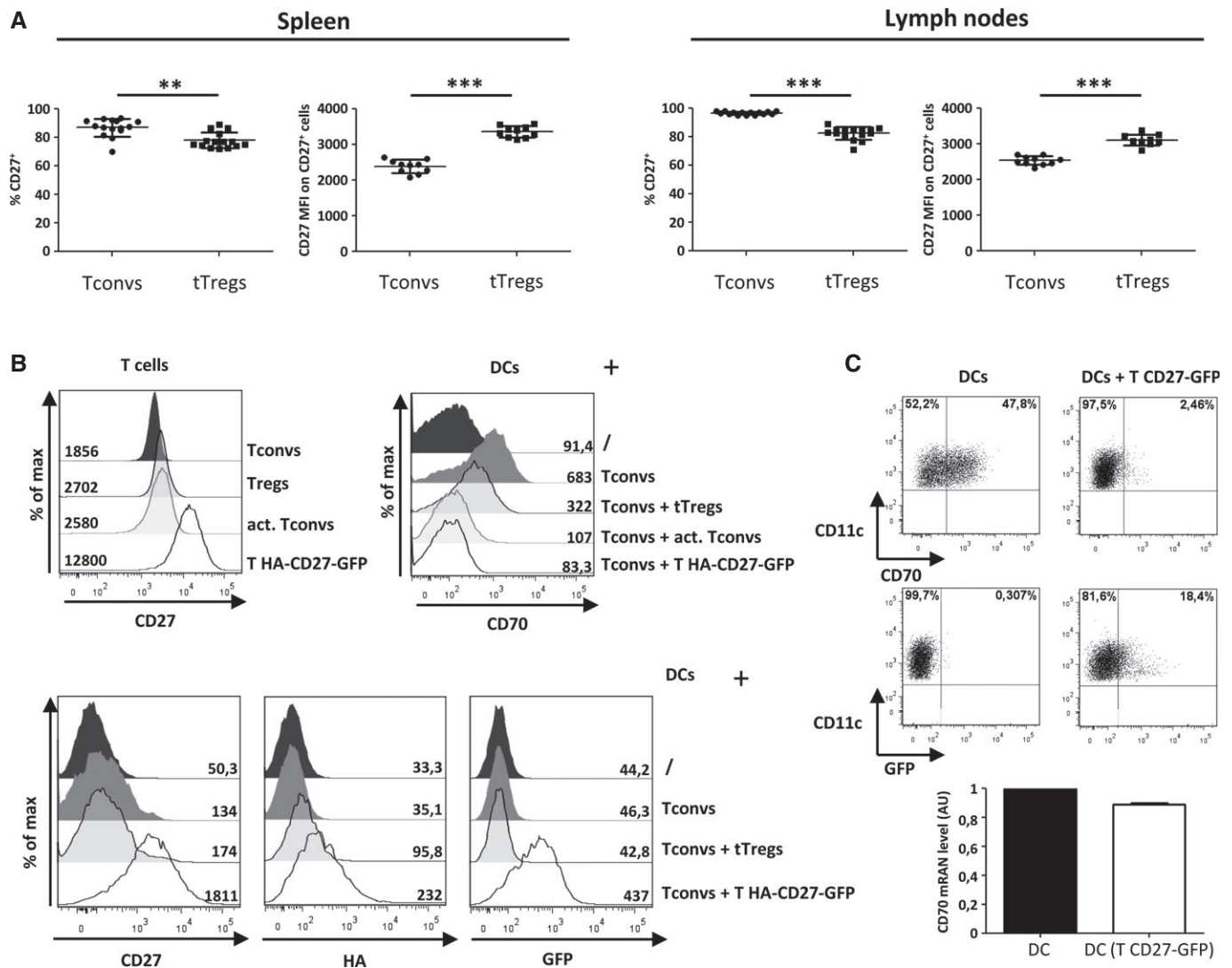
Figure 4. Role of CD27 in the regulation of CD70 expression on DCs.

A CD27 expression on CD4$^+$ Foxp3$^-$ Tconvs and CD4$^+$ Foxp3$^+$ tTregs in the spleen and LNs of naive mice. The data are expressed as percentage of CD27$^+$ cells among CD4$^+$ T cells and the level of CD27 expression (MFI) on CD27$^+$ T cells. Data are from individual mice analyzed in three independent experiments, and bars represent median ± interquartile range. **$P < 0.01$; ***$P < 0.001$.

B Allo-MLR assay. Upper left panel: analysis by flow cytometry of CD27 cell surface expression on freshly purified CD4$^+$CD25$^-$ Tconvs or CD4$^+$CD25$^+$ tTregs, activated Tconvs and Tconvs retrovirally transduced to express a HA-CD27-GFP fusion protein. All T cells are derived from C57BL/6 mice. Upper right and lower panels: flow cytometric detection of CD70, CD27, HA and GFP on CD11c$^+$ DCs from BALB/c mice cultured for 48 h with the same T-cell populations. Numbers correspond to the (MFI). The results are from one experiment representative of four.

C Splenic DCs from C57BL/6 mice were pre-activated *in vitro* with LPS (5 µg/ml) and anti-CD40 mAb (10 µg/ml), cultured for 4 h with or without T cells expressing the HA-CD27-GFP fusion protein and analyzed by flow cytometry. The dot plots represent the percentage of CD70$^+$ and GFP$^+$ DCs (viable CD11c$^+$ cells). (Lower panel) PCR-based detection of CD70 mRNA expression by CD11c$^+$ DCs sorted from the same cultures. The results are of one experiment representative of three.

the delivery of CD27 may involve a similar molecular mechanism, depending on ESCRT (endosomal sorting complex required for transport) proteins. Other reports have underlined the role of intercellular transfer as a mechanism of immune regulation, more frequently from APCs to T cells (Huang, 1999; Hwang *et al*, 2000; Romagnoli *et al*, 2013; Dhainaut & Moser, 2014). In particular, CTLA-4 on T lymphocytes has been shown to capture its ligands from opposing cells by trans-endocytosis (Qureshi *et al*, 2011), whereas CD40 on APCs appears to induce CD40L endocytosis in T lymphocytes (Yellin *et al*, 1994).

A strict control of the CD27/CD70 pathway is required to restrain excessive inflammatory responses. In particular, the sole expression of CD70 on steady state DCs can break tolerance and induce immunity to foreign antigen, without the need of DC activation (Keller *et al*, 2008). In this model, constitutive CD27/CD70 interactions drive a spontaneous Th1-type immune activation in response to environmental antigens. In agreement with this unique capacity to break tolerance, a number of reports have suggested that the CD70/CD27 interaction may be involved in the development of autoimmunity (Oflazoglu *et al*, 2009) and inflammatory diseases (Manocha
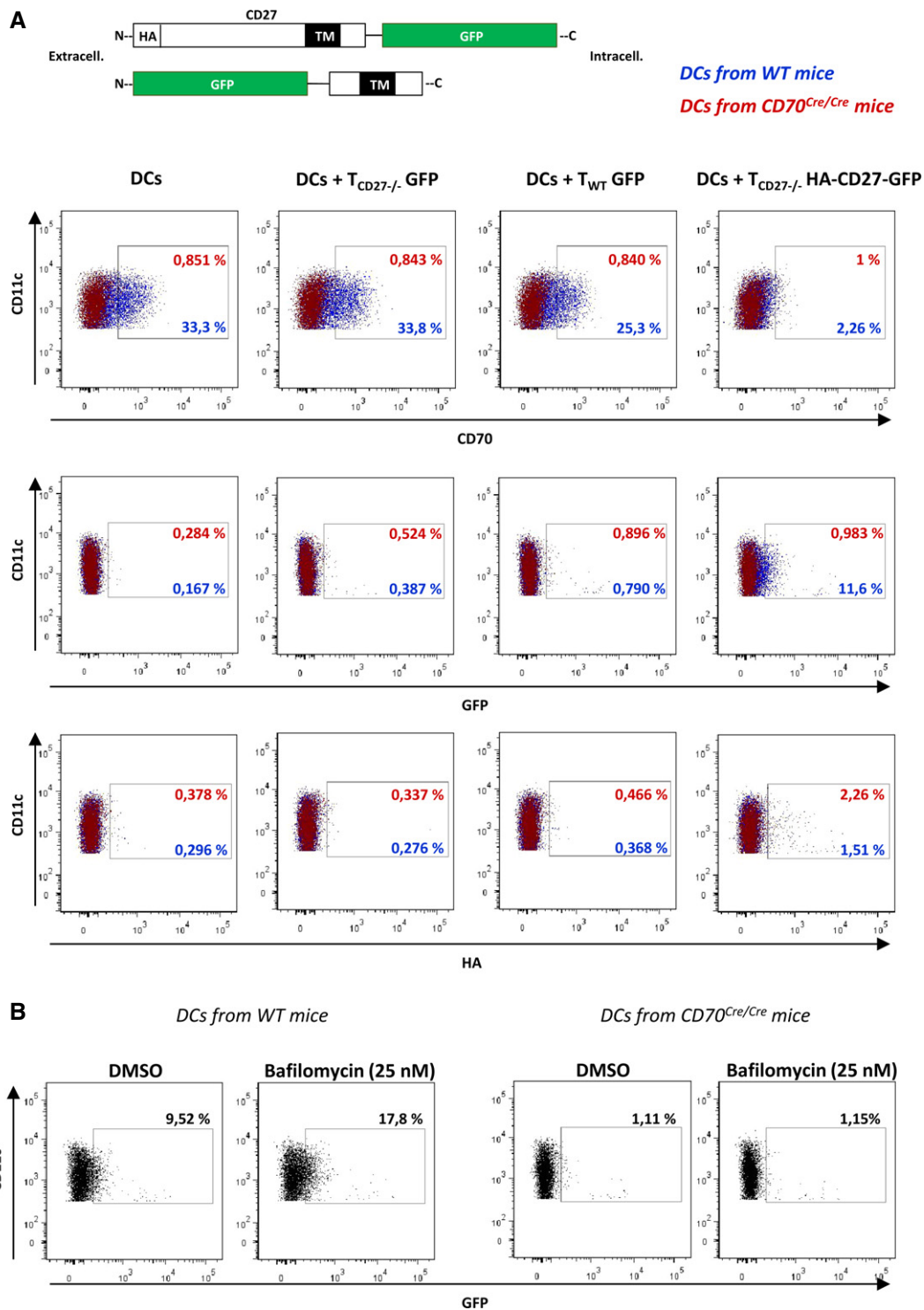
Figure 5. CD27-GFP acquisition by DCs requires CD27/CD70 interactions.

A   Splenic DCs from WT or CD70-deficient (CD70^Cre/Cre) mice were pre-activated *in vitro* with LPS (5 μg/ml) and anti-CD40 mAb (10 μg/ml), cultured for 4 h either alone or with T cells from WT or CD27-deficient mice expressing the HA-CD27-GFP fusion protein or a control protein (in which the extracellular domain of CD27 has been replaced by GFP) and analyzed by flow cytometry. Cells were stained extracellularly for CD11c, TCR, CD70 and HA and analyzed by flow cytometry. Data are expressed as superimposed dot plots (blue, DCs from WT mice; red, DCs from CD70^Cre/Cre mice). Numbers correspond to the percentage of CD70+, GFP+ and HA+ cells among DCs (defined as CD11c+ TCR−). Data are from one experiment representative of three.

B   Same as in (A), but DCs were pre-incubated for 1 h with either DMSO or bafilomycin A (25 nM) and cultured for 4 h with T cells from CD27−/− mice expressing the HA-CD27-GFP fusion protein in the presence of the same compound. Numbers correspond to the percentage of GFP+ cells among DCs. Data are from one experiment representative of three.
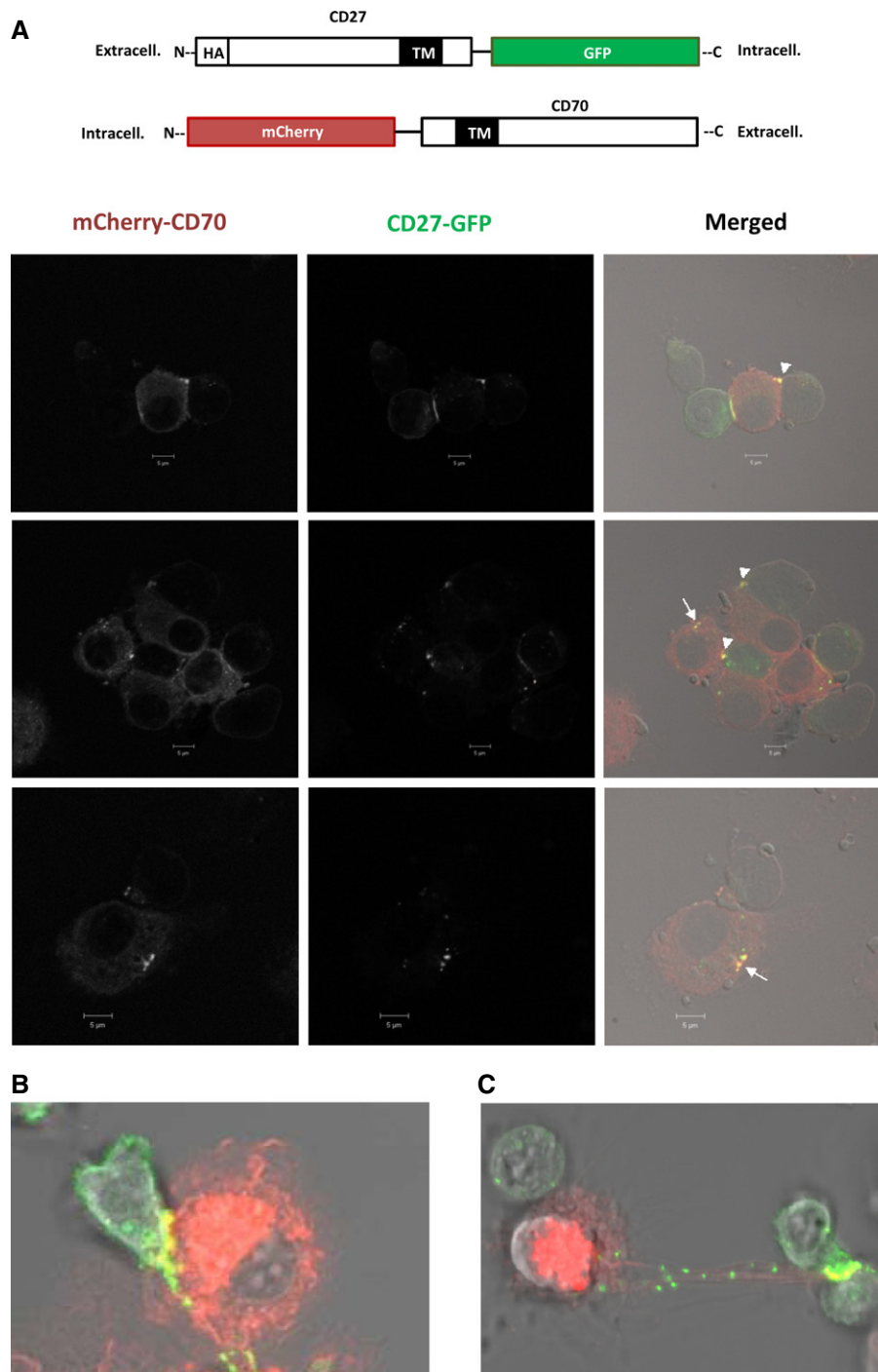
Figure 6. CD27 and CD70 are cointernalized in DCs.

A–C   BMDCs from C57BL/6 mice were retrovirally transduced to express a plasmid coding for mCherry-tagged CD70 (red), co-cultured with T cells expressing HA-CD27-GFP (green) for 1 h, fixed in 4% PFA (A) and imaged by CLSM. Co-localization was revealed in the merged images that include the light microscopic differential interference contrast to visualize the outline of the T cell and the DC. GFP partially co-localized with mCherry at the surface of BMDCs (arrowheads) or inside the cell (arrows). Snapshots of live imaging of the cultures, highlighting transfer of CD27-GFP at the level of the immune synapse (B) or through tunneling nanotubes (C). Data are from one experiment representative of three.

et al, 2009). During viral infection, this pathway may be beneficial to the host by driving *in vivo* expansion of influenza NP-specific CD8^{+} T cells (Ballesteros-Tato et al, 2010) or deleterious by leading to destruction of splenic architecture and immunodeficiency in case of infection with the persistent LCMV strain Docile (Matter et al, 2006).

Our data complete reports showing that tTregs actively inhibit the activation of DCs *in vitro*. Sakaguchi and colleagues have shown

that tTregs initially formed LFA-1-dependent aggregates on DCs and subsequently exerted LFA-1- and CTLA-4-dependent down-modulation of CD80/CD86 (Onishi *et al*, 2008; Wing *et al*, 2008). Similarly, we show that addition of Tregs in culture downregulates CD86 (and CD70) on DCs, but, importantly, we did not notice any modulation of CD86 after *in vivo* immunization in the absence of Tregs. Whether the capacity to inhibit various costimulatory molecules differs according to the nature of the Treg subset and/or environmental factors is an interesting question that deserves further investigation. Of note, Keller *et al* (2007) have reported that, in maturing DCs, newly synthesized CD70 (which contains no classical sorting motif) was specifically directed to MHC class II compartments, resulting in synchronized transport of CD70 and MHC class II toward the immunological synapse. Our data suggest that Tregs may alter the regulated trafficking to the cell surface, for example, by enhancing CD27-mediated endocytosis and degradation of CD70. This process would result in long-lasting defect in CD70 expression as mature DCs display stabilized MHC II expression on the plasma membrane (Chow *et al*, 2002).

It can be envisioned that tTregs restrain T-cell priming by decreasing CD70 cell surface expression by DCs in steady state and at the early phase of immune responses, thereby enhancing the threshold of activation of inflammatory responses. At a later stage, activated Tconvs would contribute to the termination of the response and inhibit CD70 expression on antigen-presenting DCs, which would prevent activation of naive circulating T cells and disrupt DC/T conjugates, allowing T cells to move freely and gain entry to the circulation. The role of the CD27/CD70 pathway may be exacerbated in inflammatory settings where Tregs are destabilized (Oldenhove *et al*, 2009; Sharma *et al*, 2009; Tsuji *et al*, 2009; Zhou *et al*, 2009; Shafiani *et al*, 2013). It is interesting that pathogen-specific Tregs appear to expand early during pulmonary infection with *Mycobacterium tuberculosis* but are subsequently eliminated in response to IL-12 (Shafiani *et al*, 2013), suggesting that the CD27/CD70 pathway may play a dominant role in persisting infection and act as safeguard. The interference with the CD27/CD70 pathway may therefore open new perspectives in the treatment of auto-immune diseases, inflammatory disorders and malignancies.

Materials and Methods

Mice

BALB/c and C57BL/6 mice were from Harlan Laboratories (Horst, the Netherlands). IL-12p40$^{-/-}$ BALB/c mice were provided by Dr. J. Magram (Hoffmann-LaRoche, Nutley, NJ). CD27$^{-/-}$, CD70$^{Cre/Cre}$ and CD11c-CD70tg mice were from Dr. J. Borst (NKI, Amsterdam). DEREG mice were from Dr. Tim Sparwasser (Institute of Infection Immunology, Hannover). Experiments used sex- and age-matched mice of 8–16 weeks and were performed in compliance with the relevant laws and institutional guidelines.

Cell purification

CD4^{+} cells were purified from naive animals by magnetic depletion of B cells, macrophages, DCs, NK cells, granulocytes and CD8^{+} cells using a cocktail of biotinylated antibodies (see Supplementary

Materials and Methods) and anti-biotin Microbeads (Miltenyi Biotec). CD4^{+} CD25^{+} tTregs were purified from the CD4^{+} cell fraction by positive selection of CD25^{+} cells or sorted by FACS. When indicated, CD4^{+}CD25^{-} Tconvs were activated for 48 h with coated anti-CD3 mAb (5 μg/ml) and soluble anti-CD28 mAb (1 μg/ml) in the presence of IL-2 (50 ng/ml). T cells were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS) and additives (penicillin, streptomycin, glutamine, nonessential amino acids, 1 mM sodium pyruvate and 0.05 mM 2-mercaptoethanol).

CD11c^{+} cells were enriched from low-density spleen cells by magnetic positive selection using anti-CD11c (N418) Microbeads (Miltenyi Biotec). When indicated, DCs were incubated overnight with keyhole limpet hemocyanin (KLH; 30 μg/ml; Calbiochem) or lipopolysaccharide (LPS; 5 μg/ml) and anti-CD40 mAb (10 μg/ml). DCs were cultured in RPMI-1640 medium, supplemented with 2% FCS (and additives).

Differentiation of BMDCs and *in vitro* stimulation

Bone marrow cells were collected from naive mice and cultured for 8 days in DMEM, supplemented with 10% FCS, additives and GM-CSF (20 ng/ml). Cells were stimulated with LPS (5 μg/ml) and anti-CD40 mAb (10 μg/ml) on day 8 and were collected on day 9.

Immunization and T-cell readout *ex vivo*

KLH-pulsed DCs (5×10^5) were suspended in 200 μl PBS and injected into the hind and fore footpads. When indicated, mice received one i.p. injection of 500 μg anti-CD25 mAb (PC61; BioX-Cell) 5 days before immunization, two i.p. injections of 500 ng of diphtheria toxin (Merck/Calbiochem) 2 and 3 days before immunization and/or two i.p. injections with 250 μg anti-CD70 mAb (FR70) 1 day before and 2 days after immunization. IgG from rat serum (> 98% purity, Sigma-Aldrich) was used as control antibody. Draining LNs and spleen were harvested 5 days after immunization, and cells isolated from these organs were plated at a density of 5×10^5 per well in 96-well round-bottom plates in Click's medium (Sigma-Aldrich), supplemented with 0.5% heat-inactivated mouse serum and additives. tTreg depletion was systematically assessed by measuring the percentage of CD4^{+}Foxp3^{+} T cells in the spleen and LNs by flow cytometry (see Supplementary Materials and Methods for antibodies list). Cells were restimulated with the antigen and analyzed by flow cytometry, either after 24 h of culture for CD11c and CD70 expression or after 48 h for IFN-γ production following 4 h incubation with brefeldin A. Alternatively, IFN-γ production was measured by ELISA in the supernatants from cells restimulated for 96 h with KLH. Mice of same age and sex were randomly assigned to each group.

In vitro assays

For the allogeneic mixed lymphocyte reaction (alloMLR), 3×10^4 purified splenic CD11c^{+} DCs were cultured with 3×10^5 CD4^{+} Tconvs and/or tTregs in RPMI supplemented with 2% FCS and GM-CSF (20 ng/ml). DCs from BALB/c mice were cultured with T cells from C57BL/6 mice or vice versa. Cells were analyzed by flow cytometry after 48 h of culture, or IFN-γ concentrations in the supernatants were measured by ELISA after 96 h of culture.

For DC–T-cell co-cultures, BMDCs or splenic DCs [that had been stimulated overnight with LPS (5 μg/ml) and anti-CD40 mAb (10 μg/ml)] were cultured with syngeneic T cells in a 1:1 ratio, in the presence or not of bafilomycin A (Sigma-Aldrich). After 1 or 4 h, cells were analyzed by flow cytometry. When indicated, DCs were sorted from the cultures and lysed in TRIzol reagent (Life Technologies) for RNA extraction.

Quantitative RT–PCR

Total cellular RNA was extracted from cell lysates by the use of TRIzol reagent, and reverse transcription of mRNA was carried out using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed using a StepOne Plus system (Applied Biosystems, Foster City, CA) with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA). Quantification (with RPL32 and GAPDH as endogenous housekeeping genes) was done using standard curves using the following primers: CD70 forward (GGTTTCATTGT AGCGGACTACTC) and reverse (GTCCTTCCGAGGAACTGTGA), RPL32 forward (GGCACCAGTCAGACCGATA) and reverse (CAGGATCTGGCCCTTCAAC), and GAPDH forward (CCGCATCTTC TTGTGCAGT) and reverse (AATACGGCCAAATCCGTTC). Levels of CD70 expression were normalized to GAPDH in each sample.

Retroviral gene transduction

Plasmids used for transduction were constructed as detailed in the Supplementary Materials and Methods. Retroviruses were produced in the supernatant of PlatE cells (kindly provided by Dr. T. Kitamura, University of Tokyo) following transfection, in a mycoplasm-free environment. CD4$^+$ T cells were stimulated for 24 h with anti-CD3 mAb (5 μg/ml, coated), anti-CD28 mAb (1 μg/ml) and IL-2 (50 ng/ml). Up to 1.4 ml of retroviral supernatant supplemented with polybrene (2 μl/ml) was added to 10^6 activated T cells. The cells were transduced following centrifugation (90 min, 6,000 *g*, 25°C) and cultured for 2 days with anti-CD3 mAb (5 μg/ml, coated), anti-CD28 mAb (1 μg/ml) and IL-2 (50 ng/ml). Cells were then flow cytometrically sorted based on GFP expression.

Confocal imaging

For live cell analysis, DCs and T cells were seeded on μ-Slide 8 well (IBIDI, Germany). For analysis of fixed cells, cells were cultured for 1 h on the slides, then fixed with 4% w/v paraformaldehyde for 5 min, washed three times in Tris-buffered saline and once with water and directly mounted using Fluorescent Mounting Medium (DakoCytomation). Alternatively, cells were stained for CD70, CD27, CD11c or TCR before fixation, permeabilized for 5 min with BD Perm/Wash Buffer (BD Biosciences) and stained intracellularly for CD27 and CD70 in the same buffer before being mounted. Images were acquired using a Zeiss LSM710 confocal scanning microscope with a 63 × 1.4NA oil immersion objective.

Statistical analysis

Statistical significance was determined with the Mann–Whitney test for two-tailed data or the Kruskal–Wallis test followed by selected comparison by Dunn's multiple comparison tests. All the performed comparisons are depicted. Data are shown as median ± interquartile range. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, nonsignificantly different.

Author contributions

MD, CC and MM designed the study and analyzed data; MD, CC and VA performed most experiments with a contribution from SU, JD, GO and AG; TS, KT, HY and JB provided reagents; and EP and HY supervised some aspects of the work. MM and MD supervised the study and wrote the paper, with a contribution from JB.

Conflict of interest

The authors declare that they have no conflict of interest.

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