



Published in final edited form as:

*J Immunol.* 2013 September 1; 191(5): 2282–2289. doi:10.4049/jimmunol.1300868.

## CD70 is downregulated by interaction with CD27

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### Abstract

Engagement of the receptor CD27 by CD70 affects the magnitude and quality of T cell responses in a variety of infection models, and exaggerated signaling via this pathway results in enhanced immune responses and autoimmunity. One means by which signaling is regulated is tight control of cell surface CD70, which is expressed on dendritic, T, and B cells only upon activation. Here we show that there is a second level of regulation. First, although undetectable on the cell surface by flow cytometry, immature dendritic cells (DC) have a small pool of CD70 that continuously recycles from the plasma membrane. In addition, surface levels of CD70 on DC and T cells were higher in mice deficient in CD27, or on DC for which the interaction between CD70 and CD27 was precluded by blocking antibodies. Binding of CD70 by its receptor resulted in downregulation of CD70 transcription and protein levels, suggesting that CD70-mediated “reverse signals” regulate its own levels. Therefore, the ability of CD70 to trigger costimulation is self-regulated when it binds its complementary receptor.

### Introduction

Interaction between the costimulatory receptor CD27 and its ligand CD70 is required for optimal T cell activation (1–3). Studies using CD27- and CD70-deficient mice or anti-CD70 blocking antibodies have found defects in primary and/or secondary T cell responses in a variety of infectious models (4–8). Furthermore, manipulations that increase CD27-CD70 interactions have been successfully used in experimental vaccination protocols (9, 10). It is notable that a fine line exists between beneficial and deleterious CD70-mediated effects. For example, whereas efficient clearance of acute LCMV strains requires CD27 occupancy by CD70, this interaction precludes clearance of the chronic LCMV strain (7, 8, 11). Therefore the existence of regulatory mechanisms for the CD70-CD27 pathway ensures effective and prevents deleterious immune responses.

Normally, tight control of CD27 and CD70 expression avoids excessive T cell activation. CD27, a member of the TNFR family, is constitutively expressed by T cells as a membrane-bound homodimer, and its surface levels change during T cell activation (3). The baseline level in resting naïve and memory T cells is upregulated during the first days after TCR engagement because of increased transcription (12–14). Notably, surface levels of CD27 are downregulated during T cell effector differentiation by shedding and/or decreased transcription, and some terminally-differentiated effector memory T cells (T<sub>EM</sub>) retain a CD27-negative phenotype (13–15). CD27 can also be reversibly downregulated on memory

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CD8 T cells that enter non-lymphoid organs (16). On the other hand, expression of CD70, a homotrimeric transmembrane member of the TNF family, is much more restricted, and is barely detectable on the cell surface at steady state, and even then only rare cells in the thymic medulla and the lamina propria are CD70<sup>+</sup> (17–20). Transient transcriptional upregulation of CD70 occurs in DC activated via Toll Like Receptor (TLR)- or CD40-mediated stimulation and in antigen-activated T and B cells (6, 20). In DC, where its expression seems to be most relevant, CD70 is transported by the invariant chain to late endocytic structures where it colocalizes with MHC II molecules (21, 22). Upon interaction of activated DC with cognate CD4 T cells, CD70 is co-delivered to the immune synapse with MHC II, ensuring optimal T cell stimulation.

Uncontrolled CD27-CD70 interactions have detrimental effects. In mouse models where CD70 was constitutively expressed on B cells, DC, or T cells, a continuous generation of effector T cells was observed, which in B and DC CD70 transgenics resulted in an autoimmune disease and death (23–25). On the other hand, constitutive CD70 expression on DC was sufficient to break peripheral tolerance and, among other things, generate tumor-specific responses to peptide immunization without the need for adjuvants (24). In addition to these observations made in transgenic mice, the importance of excessive CD27-CD70 interactions has been demonstrated in a chronic LCMV infection model (11). Continuous CD27 engagement, likely mediated by a subset of CD70-expressing B cells, led to T cell cytokine-mediated splenic germinal center and marginal zone destruction, thus precluding the generation of a neutralizing antibody response.

It is generally believed that the downregulation of T cell CD27 levels during persistent stimulation is an activation-intrinsic event. However, there is evidence that it is the interaction with CD70 that results in decreased CD27 levels in the absence of activation. For example, T cell co-culture with B-cell lines expressing CD70 triggered CD27 downregulation, and even naïve T cells in CD70 Tg mice had substantially lower CD27 levels (26, 27). In the course of studying mice deficient in either CD27 or CD70, we made the unexpected observation that in the absence of one the other was upregulated. Here we show by antibody blocking and genetic manipulation that the relationship between CD27 and CD70 expression is reciprocal and mediated by direct protein-protein interactions.

## Materials and Methods

### Mice

C57BL/6 (B6) mice were obtained from Frederick Cancer Research Facility (Frederick, MD). CD70<sup>-/-</sup> mice backcrossed to B6 for 13 generations were described (8). CD27<sup>-/-</sup> mice (4) were a gift from Jannie Borst and were maintained in our facility. The NCI Animal Care and Use Committee approved all animal studies.

### Reagents

Antibodies to CD27, TCR $\beta$ , B220, CD16/CD32 clone 2.4G2 (Fc block), isotype controls, and fixation and permeabilization buffers were obtained from BD Biosciences. The unconjugated antibody to CD70 (clone FR70), the fluorochrome-conjugated antibodies to CD70 (clone FR70), CD11c and MHC class II, the blocking antibody to CD27 (clone LG. 3A10), the isotype control, and recombinant IFN- $\gamma$  were purchased from eBioscience. LCMV Armstrong 53b (LCMV) was a gift from Rafi Ahmed (Emory University School of Medicine). GP33–41 H-2D<sup>b</sup> (GP33) tetramers were obtained from the NIH Tetramer facility at Emory University. Power SYBR Green was obtained from Applied Bioscience. Cycloheximide (CHX), lipopolysaccharide (LPS), and poly(I:C) were purchased from Sigma-Aldrich. LIVE/DEAD Fixable Dead Cell Stain was obtained from Life Technologies.

B16-FLT3L murine melanoma cells were a gift from Ulrich H. von Andrian, Harvard Medical School.

### Viral growth and LCMV infection

LCMV was grown in our laboratory in baby hamster kidney cells (BHK-21) and viral titers were determined as described (28). Wild type (WT), CD27<sup>-/-</sup>, and CD70<sup>-/-</sup> mice were infected i.p. with  $2 \times 10^5$  plaque-forming units (PFU) of LCMV.

### Cell stimulation and flow cytometry

Splenocytes were cultured at a concentration of  $5 \times 10^6$  cells/ml and stimulated with poly(I:C) (30  $\mu$ g/ml) for 22 hr or primed with IFN- $\gamma$  (50 ng/ml) for 2 hr and then stimulated with LPS (200 ng/ml) for additional 20 hr. In some experiments cells were treated for 15 min with anti-CD27 blocking antibody (5  $\mu$ g/ml) or with isotype control prior to stimulation. In internalization experiments, anti-CD70 PE-conjugated antibody was added to the cultures after stimulation at the indicated time-points, at a concentration of 2  $\mu$ g/ml. Cells were treated or not with CHX (20  $\mu$ g/ml) one hr before adding anti-CD70 PE-conjugated antibody. For staining of surface molecules, CD16/CD32 clone 2.4G2 was added to block Fc-receptor binding prior to the addition of fluorochrome-conjugated antibodies. In some experiments cells were incubated with unconjugated anti-CD70 Ab prior to surface staining. Detection of intracellular molecules was performed after fixation and nuclear permeabilization. Dead cells were excluded using the LIVE/DEAD Fixable Dead Cell Stain, doublet exclusion gating was performed, and splenic DC were gated as CD11c<sup>+</sup>MHC-II<sup>+</sup> cells. Flow cytometry was done with a BD LSRFortessa cytometer using BD FACSDiva software (BD Biosciences).  $\Delta$ MFI was calculated by subtracting the nonspecific background on cells from CD70<sup>-/-</sup> mice (for CD70) or CD27<sup>-/-</sup> mice (for CD27). All flow cytometry data analysis was performed with FlowJo software (Tree Star).

### Expansion of DC in vivo

B16-FLT3L cells were cultured in DMEM containing 10% fetal calf serum and antibiotics. Mice were injected subcutaneously with  $10^6$  B16-FLT3L cells and spleens were analyzed 10–13 days later.

### Cell sorting and purification

Transcriptional analysis of CD70 was performed either on  $10^7$  total splenocytes from mice injected with B16-FLT3L cells, or on DC:T (ratio 1:5) co-cultures. DC and T cells were isolated by flow cytometry-based sorting (Facs Aria II, BD) with a purity >95%.  $6 \times 10^4$  DC were cultured with  $3 \times 10^5$  T cells in the absence or presence of stimulus. In some experiments, DC were isolated with CD11c MicroBeads (Miltenyi Biotec) and T cells were purified with EasySep kit (Stem Cell Technologies). In this case  $3.5 \times 10^5$  DC were cultured with  $1.75 \times 10^6$  T cells in the absence or presence of stimulus.

### RNA isolation and quantitative RT-PCR (qRT-PCR)

RNA isolation from total splenocytes or from sorted cells was performed with the RNeasy Mini Kit or RNeasy Micro Kit (Qiagen) respectively. cDNA was prepared with the SuperScript II Reverse Transcriptase Kit (Life Technologies). Real-time PCR for CD70 was performed with SYBR Green using 7500 Real Time PCR System by Applied Bioscience (Carlsbad, CA), with the following primers: CD70-Forward: TGCTGTTGGTTTCATTGTAGCG CD70-Reverse: ATCCTGGAGTTGTGGTCAAGGG. Housekeeping ribosomal 18S RNA was amplified to normalize RNA content of the lysate and obtain dCT value. Values were then normalized to CD70-deficient cells, that were assigned an arbitrary unit of 1.

## Statistical analysis

Statistical analysis was done with GraphPad Prism software using a Student's two-tailed unpaired t test.

## Results

### Cell surface levels of CD70 and CD27 are inversely correlated

Infection with a variety of pathogens induces CD70 expression within a few days, but the levels detected by flow cytometry are relatively low (6). The recent availability of CD70-deficient mice provided an ideal negative staining control, and revealed that CD70 expression during acute LCMV infection is barely detectable (our unpublished observations), despite the fact that CD70-deficient mice exhibited a defective CD8 T cell response and delayed viral clearance (8). Because the relevance of the CD70-CD27 pathway seems to be highly dependent on the timing and levels of expression of these molecules, we sought to determine the kinetics of expression of CD70 and CD27 on immune cells during acute LCMV infection. CD70 was undetectable on resting splenic DC, and only very low levels were detected on day 2 of infection (Fig. 1A and B). Interestingly, DC of mice lacking CD27 had higher levels of CD70 on their surface (Fig. 1A and B). At day 6 levels of CD70 were downregulated in both WT and CD27-deficient mice. Because activated T cells express CD70, we evaluated its levels on antigen-specific T cells at the peak of the immune response and found that CD70 was detectable only in CD27-deficient mice (Fig. 1C). To correlate CD27 levels with those of CD70, the kinetics of CD27 expression on T cells during LCMV infection was assessed. As previously described (13), CD27 levels rose slightly in the first few days and fell to less than resting levels during the effector differentiation phase (Fig. 1C). Notably, the levels of CD27 on days 1 and 2 were clearly higher on T cells from CD70-deficient mice (Fig. 1C and D), whereas during the downregulation phase the difference between the two mice strains progressively narrowed. Taken together, these data suggest that CD70 and CD27 cell surface expression is reciprocally regulated, and raised the possibility that they in fact regulated each other.

### Downregulation of CD70 is mediated by a direct interaction with CD27

To assess whether the CD70 upregulation during LCMV infection was diminished by direct interaction with CD27, we studied CD70 levels on DC from splenocyte cultures stimulated with TLR agonists. In this system, most of the CD27 is provided by T cells, although NK and memory B cells could also participate. In line with the *in vivo* observations, upon stimulation with poly(I:C) CD27-deficient DC consistently displayed higher levels of CD70 than those of control mice (Fig. 2A and B). An important question was whether this was due to lack of the CD70-CD27 interaction itself or was secondary to some other consequence of CD27 loss. To address this, WT splenocytes were treated with isotype control or blocking anti-CD27 antibody and stimulated with either poly(I:C) or IFN- $\gamma$  and LPS. Blockade of the CD27-CD70 interaction resulted in a substantially larger increase in CD70 expression than that observed in isotype-treated cultures (Fig. 2C, middle row). Notably, this was equal to the CD70 increase found in DC from CD27-deficient mice (Fig. 2C, top row). Moreover, blocking antibodies to CD27 in CD27-deficient mice did not result in a further increase of CD70, ruling out non-specific antibody-mediated effects (Fig. 2C, bottom row). These results indicated that the elevated levels of CD70 in CD27-deficient mice were directly caused by a lack of interaction with its receptor, and that engagement of CD70 by CD27 downregulates CD70 levels on DC.

### CD70 constitutively recycles from the cell surface

We considered the possibility that CD70 might be undetectable on the surface of resting cells because of continuous internalization. Notably, most of the CD70 expressed by both resting and stimulated DC was intracellular, as detected by specific intracellular staining after blocking surface CD70 with unlabeled anti-CD70 antibody (Figure 3A). To determine whether CD70 is turning over on the cell surface of resting cells, splenocytes were cultured overnight with fluorescently-labeled antibody to CD70 in the presence or absence of stimulation, and antibody accumulation was measured. The final signal detected accounted for both the accumulation of anti-CD70 and surface CD70 expression. Whereas labeled isotype control was not detectably internalized, the level of anti-CD70 fluorescence in DC after overnight culture was easily detectable (Fig. 3B). When internalization from the cell surface was prevented by incubation on ice, the total cellular level of fluorescent signal was similar to that found with surface staining alone (Figure 3C). Importantly, unlike in DC, internalization of CD70 was not detected in B or T cells, establishing that CD70 is not expressed by resting lymphocytes. Therefore, although undetectable by conventional cell surface staining, there is in fact constitutive expression of CD70 on resting DC (Fig. 3B and D). Accumulation of antibody was even more prominent in stimulated DC (Fig. 3B and D), and increased over time (Fig. 3E). To determine if CD70 recycles to the plasma membrane after internalization, we assessed the accumulation of fluorescently-labeled anti-CD70 antibody under conditions in which new CD70 molecules could not be synthesized. Cells pretreated with the protein synthesis inhibitor cycloheximide (CHX) prior to stimulation failed to upregulate CD70 and CD80, as expected, demonstrating the efficiency of blockade (Supplementary Figure, top panel). CD80 levels on CHX-treated stimulated cells were substantially below the levels on resting cells, suggesting that newly synthesized protein is the major contributor to surface upregulation. This was not the case for CD70, consistent with a slower turnover. Importantly, inhibition of protein synthesis had little effect on surface CD70 levels when added during the last nine hours of stimulation, indicating that after the initial upregulation, CD70 levels are largely maintained in the absence of new protein (Supplementary Figure, bottom panel). Notably, inhibition of de novo protein synthesis did not reduce fluorescent antibody accumulation, indicating that cell surface CD70 cycles from and to the plasma membrane (Fig. 3E). This was true also for the unstimulated splenocytes, providing direct evidence that the small steady-state CD70 pool found in untreated DC was not a result of their maturation during cell culture. Thus, CD70 continuously recycles from the cell membrane in both unstimulated and stimulated DC.

### CD70 transcription but not internalization is affected by CD27 binding

Given that CD70 is constantly recycling from the membrane, we considered the possibility that the rate or extent of internalization was increased by its interaction with CD27. In such a case, preventing the interaction with CD27 would result in accumulation on the cell surface. To test this, CD70 internalization was measured in splenocytes cultured in the absence or presence of blocking anti-CD27 antibodies (Fig. 4A and B). No difference in anti-CD70 internalization was detected in resting DC, and the small and not statistically significant increase found in stimulated DC could be accounted for by the increase seen on the cell surface (Fig. 2C). These results suggest that CD70 downregulation by CD27 is not caused by enhanced internalization. Another possibility is that the decrease in cell surface CD70 could be due to redistribution between plasma membrane and cytosolic pools. However, combined intracellular and surface staining for CD70 indicated that the total amount of CD70 in dendritic cells, not just the amount on the cell surface, increased after blockade of CD27 (Fig. 5A). The possibility that CD27 engagement regulates CD70 levels by suppressing its transcription was evaluated. Although several reports have shown that blocking antibodies to CD70 have effects on the proliferation and survival of T cells, there has not been a direct demonstration of the ability of CD70 to perform “reverse signaling”.



To determine if this occurs, splenocyte CD70 mRNA levels were measured *in vitro*. Because the percentage of DC in total splenocytes is very low and CD70 mRNA undetectable, DC were expanded *in vivo* by injection of B16 melanoma cells engineered to express FLT3 ligand (FLT3L) (29). WT mRNA in unmanipulated splenocytes was undetectable (that is, the signal was detected at the same level as in CD70 knockout splenocytes) after 6 hr of culture, and was detected at only very low levels after 24 hr (Fig. 5B). In contrast, when CD27 was blocked there was almost a 300–1000-fold increase in CD70 transcripts even in the absence of activation. Stimulation with poly(I:C) or IFN- $\gamma$  and LPS caused large increases in CD70 mRNA that was further increased by blockade of CD27. The differences between blocked and unblocked splenocytes were greater at 6 hr than 24 hr of stimulation, suggesting that the signal triggered by CD27 engagement plateaus. To exclude the possible contribution of CD70 mRNA from other cell types, DC were purified by flow cytometric sorting or isolated by magnetic beads from naïve mice and co-cultured with WT or CD27-deficient T cells (Figure 5C and D). In the case of sorted DC, CD70 transcripts were detected only in co-cultures with CD27-deficient T cells, confirming that CD70 transcription is enhanced when interactions with CD27 are removed. CD70 mRNA could not be detected in WT DC stimulated in the presence of WT T cells, likely because the low amount of material derived from sorted cells was below the limit of detection (Figure 5C). Indeed, when higher numbers of DC were purified using magnetic beads, we were able to detect CD70 mRNA also in WT DC stimulated in the presence of WT T cells. Substantially higher CD70 levels were observed in DC:CD27-deficient T cells co-cultures, confirming the results obtained with sorted cells (Figure 5D). Taken together, these results indicate that the interaction of CD27 with CD70 suppresses CD70 transcription leading to a tight control of CD70 levels.

## Discussion

Costimulation is a key requirement for efficient activation of T cells, but it needs to be regulated to prevent an overly aggressive immune response. Controlled and tissue-specific expression of costimulatory molecules is often not sufficient to mediate this regulation, and other mechanisms are required. For example, activated T cells costimulated through the B7-CD28 ligand/receptor pair express the co-inhibitory receptor CTLA-4, which competes with CD28 for ligand and suppresses T cell responses (30). In the case of the CD70-CD27 pathway, other competing ligands or receptors have not been described, and CD27 is the only known receptor for CD70. Therefore, other levels of regulation must exist. One mechanism is controlled expression of both the ligand and the receptor. For example, CD27 mRNA and protein is dynamically regulated upon T cell activation, being constitutively expressed on naïve T cells, upregulated in the first few days of activation, and transiently downregulated during the effector phase (2). Whereas CD27 downregulation is thought to be a direct result of activation-induced signaling, it is also possible that it is a consequence of interactions between CD70 and CD27. Indeed, in this report we found that CD70-deficient mice have slightly higher CD27 levels than WT mice at steady state, and this difference increases during the first few days of LCMV infection when CD70 levels on DC rise. Direct CD70-mediated downregulation of CD27 could provide real-time fine-tuning of ongoing T cell activation processes necessary for controlling immune responses. Formal proof of this hypothesis could be provided by studies investigating the consequences of expressing permanently high levels of CD27 on T cells, but at this time such transgenic mice are not available.

Although CD70 is thought to be constitutively expressed in small subsets of non-hematopoietic cells in the thymic medulla and lamina propria, it has not been found on DC of unmanipulated mice (19, 20). Our data indicate that resting DC in fact do express low levels of CD70 that can be detected when one amplifies the fluorescent signal by allowing

labeled anti-CD70 to accumulate within the cell. The possibility that the CD70 detected could be the result of marginal maturation of DC during culture was ruled out by the finding that protein synthesis inhibition did not affect this pool. In addition, CD70 actively recycled from the plasma membrane on resting and activated DC, as detected by the specific internalization of labeled anti-CD70 antibodies. These observations indicate that under steady state conditions CD70 is continuously recycling from the DC surface, where it could interact with T cell CD27. In the absence of antigen, this interaction would downregulate CD27, as evidenced by the higher levels of T cell CD27 in CD70-deficient mice. When DC receive a maturation signal, CD70 is transported to the surface in MHC II-containing vesicles (21). The delivery of these relatively high levels of CD70 to the immune synapse would allow costimulation via CD27. In fact, although we could detect only very low levels of CD70 on DC during LCMV infection, primary responses and viral clearance are substantially compromised in CD70 knockouts (8). Therefore the lack of detectable surface CD70 on resting DC does not mean that they are not engaged in interactions with T cells. A similar phenomenon in which proteins expressed at levels below flow cytometry detection limits are functionally important has been observed with IL-1R (31).

Based on current knowledge, the rate-limiting step of DC CD70 expression is transcription triggered by innate stimuli. This study has identified an additional mechanism that occurs after initial upregulation in which CD70 is downregulated by engagement with CD27. Interruption of this interaction with blocking antibodies, or deletion of CD27, resulted in increased CD70 transcription cell surface levels. This finding implies that CD70 itself has the capability to transmit a signal (reverse signaling), as has been previously suggested for CD70 and other TNF family members (23, 32–34). In one case (23), activation of PI3K and MAP kinases has been implicated in CD70 “reverse signaling”. An important question is whether such reverse signaling has functional repercussions other than regulation of the CD70-CD27 pathway. A model in which the intracellular tail of CD70 is mutated or deleted would perhaps give some insights on roles of CD70 other than that of a costimulatory ligand for CD27. In any case, the findings in this study identify another level of regulation of the CD70-CD27 pathway in which CD70 is actively regulated itself. Given the known functional consequences of fixed CD70 expression (24, 25, 35), it likely that reciprocal regulation of CD70 and CD27 expression plays an important role in the optimal function of this costimulatory couple.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health.

We thank Ehydel Castro for assistance with animal experiments and Atef Allam for LCMV preparation. We also are grateful to Rafi Ahmed for providing LCMV Armstrong, the NIH Tetramer facility at Emory University for their generous supply of tetramers, and Ulrich von Andrian for the B16-FLT3L cell line.

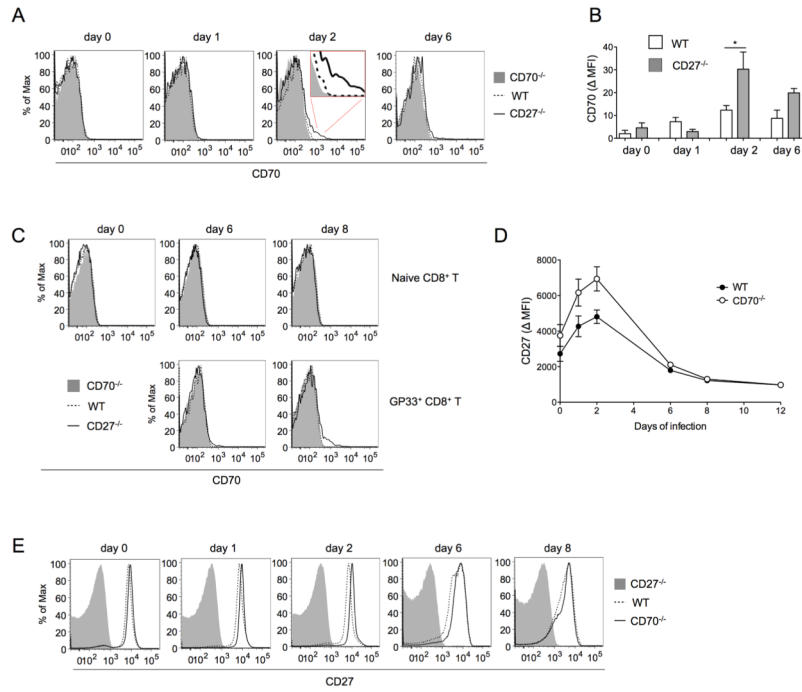
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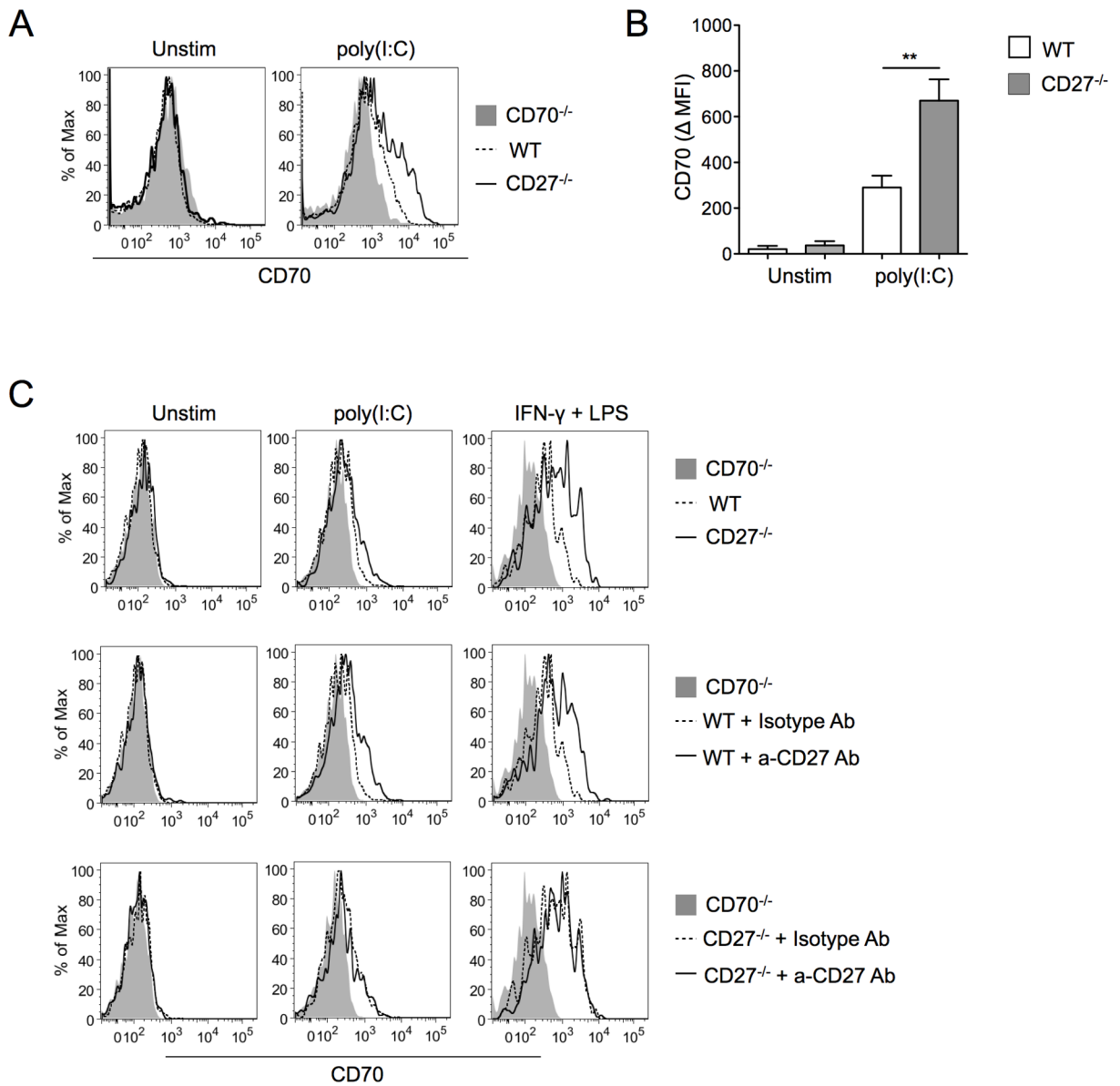
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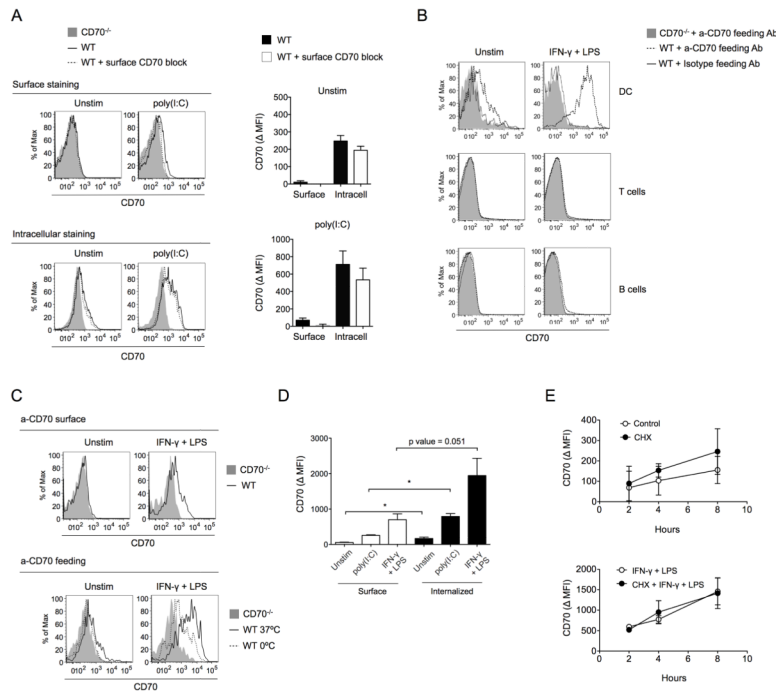


**Figure 1.** CD70 and CD27 levels inversely correlate during LCMV infection. (A–E) WT, CD70<sup>-/-</sup>, and CD27<sup>-/-</sup> mice were infected i.p. with  $2 \times 10^5$  plaque-forming units (PFU) of LCMV Armstrong, and analyzed at the indicated days. CD70 levels detected ex vivo by surface staining on splenic DC (CD11c<sup>+</sup>MHC-II<sup>+</sup>) are shown as one representative experiment (A) or as mean  $\pm$  SEM of 3–7 mice (B). (C) CD70 levels on splenic T naïve CD8<sup>+</sup>T cells (top) or in GP33-specific T cells (bottom) are shown from one representative experiment. (D–E) CD27 levels detected ex vivo on splenic T cells are shown as mean  $\pm$  SEM of 3–9 mice (D) or as one representative staining (E).  $\Delta$ MFI of CD70 and of CD27 was calculated by subtraction of the background signal in CD70<sup>-/-</sup> and CD27<sup>-/-</sup> cells, respectively. \* p value < 0.05.

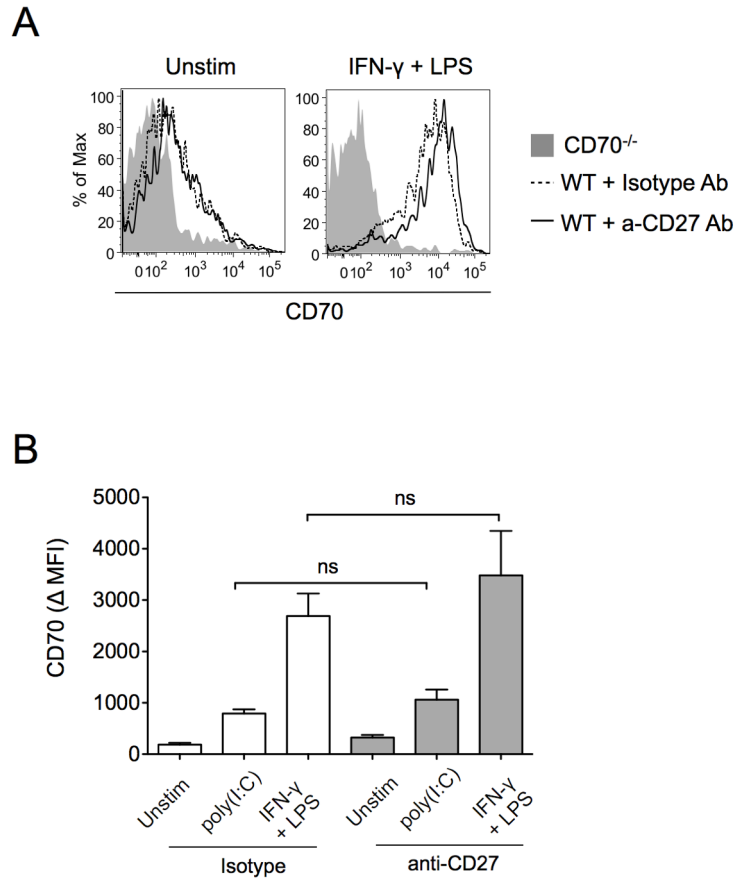


**Figure 2.**

CD70 levels are downregulated upon interaction with CD27. (A–B) WT, CD70<sup>-/-</sup>, and CD27<sup>-/-</sup> splenocytes were stimulated for 20–22 hrs with poly(I:C). Surface CD70 levels on DC are shown in one representative experiment (A) or as mean ± SEM of 15 mice (B). (C) CD70 levels on splenocytes pretreated with an anti-CD27 blocking antibody (thick line) or with an isotype control (dashed line) and stimulated with TLR agonists for 22 hours are depicted from one representative experiment. \*\* p value < 0.01.

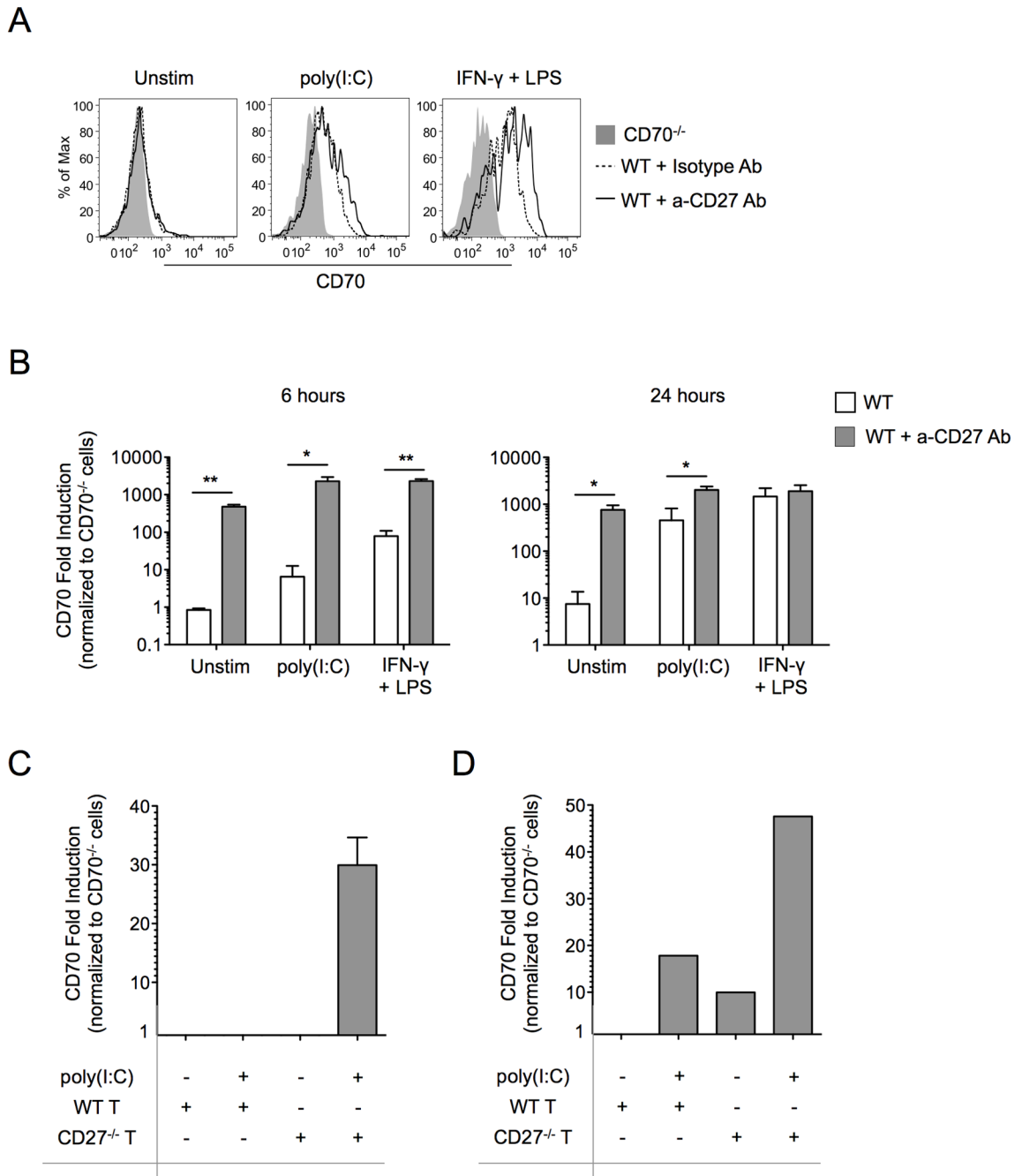
**Figure 3.**

CD70 is internalized and recycles in DC. (A) Surface and intracellular CD70 levels on untreated or poly(I:C)-stimulated WT or CD70<sup>-/-</sup> splenocytes are shown in one representative experiment (left) and as the mean  $\pm$  SD of two independent experiments (right). Staining was performed with (dashed line) or without (black line) blocking of surface CD70 with an unconjugated ant-CD70 antibody. (B) WT or CD70<sup>-/-</sup> splenocytes were stimulated and cultured overnight in the presence of anti-CD70 PE-conjugated antibody (dashed line) or of the isotype control (thin line). Levels of the accumulated anti-CD70 antibody are shown in DC, T, or B cells in one representative experiment. (C) CD70 levels on DC surface or after 4 hours of culture with anti-CD70-PE are depicted as mean  $\pm$  SEM of 2–4 independent experiments. \* p value < 0.05. (D) WT or CD70<sup>-/-</sup> splenocytes were stimulated for 22 hr and cultured in the absence (top row) or presence (bottom row) of anti-CD70 PE-conjugated antibody during the last 8 hr. Cells were incubated on ice (dashed line) or at 37°C (black line). (E) WT or CD70<sup>-/-</sup> splenocytes were stimulated for 22 hr (bottom graph) or left untreated (top graph) and cultured for the last 2, 4 or 8 hours in the presence of anti-CD70 PE-conjugated antibody (open circles). One hour before adding the antibody, some cells were pretreated with CHX (filled circles). As a control for CHX treatment, splenocytes were pre-treated with CHX prior to stimulation. Upregulation of CD80 and CD70 were completely abrogated following such pre-treatment demonstrating that CHX worked (data not shown).  $\Delta$ MFI of CD70 was calculated by subtracting the background signal on CD70<sup>-/-</sup> DC, as well as the levels of CD70 on parallel samples stained only for surface expression at each time point. Mean  $\pm$  SD of 2 independent experiments is shown.



**Figure 4.** The higher levels of CD70 upon blocking of CD27 can not be explained by different rates of internalization. (**A–B**) WT or CD70<sup>-/-</sup> splenocytes were stimulated and cultured overnight in the presence of anti-CD70 PE-conjugated antibody. Cells were pretreated with anti-CD27 blocking antibody (thick line) or with the isotype control (dashed line). Levels of the accumulated anti-CD70 antibody are shown one representative experiment (**A**) or as mean  $\pm$  SEM of 2–5 independent experiments (**B**).





**Figure 5.**

CD70 is downregulated at the transcriptional level by interaction with CD27. (A) WT or CD70<sup>-/-</sup> splenocytes were pretreated with anti-CD27 blocking antibody (thick line) or with the isotype control (dashed line) and stimulated overnight. Intracellular CD70 levels in DC are shown in one representative experiment. (B) Splenocytes of WT or CD70<sup>-/-</sup> mice injected 10–13 days before with B16-FLT3L tumors were pretreated with anti-CD27 blocking antibody (grey bars) or with the isotype control (white bars) and stimulated for 6 or 24 hours. CD70 mRNA was quantified by qRT-PCR, normalizing the Ct values to the 18s housekeeping gene and to the values of CD70<sup>-/-</sup> cells (arbitrary set to 1). Mean ± SEM of 3

independent experiments is shown. \*\* p value < 0.01, \* p value < 0.05. **(C)** DC sorted by flow cytometry (>95% purity) from WT or CD70<sup>-/-</sup> mice were cultured with sorted T cells from WT or CD27<sup>-/-</sup> mice and stimulated for 6 hr as indicated. CD70 mRNA was quantified as in B. Mean ± SD of 2 independent experiments is shown. **(D)** DC isolated with magnetic beads from six pooled WT or CD70<sup>-/-</sup> spleens were cultured with purified T cells from WT or CD27<sup>-/-</sup> mice and stimulated for 6 hr as indicated. CD70 mRNA was quantified as in B.