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Enhanced Toll-like receptor responses in the absence of DAP12

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ABSTRACT

DAP12 is an immunoreceptor tyrosine-based activation motif (ITAM)-containing signaling adaptor that pairs with receptors on myeloid cells and NK cells. We examined the responses of DAP12-deficient mice to stimulation through Toll-like receptors. Unexpectedly, DAP12-deficient macrophages produced higher concentrations of inflammatory cytokines in response to a variety of pathogenic stimuli. Additionally, macrophages deficient in syk, the tyrosine kinase which signals downstream of DAP12, exhibited an identical phenotype to DAP12-deficient macrophages. DAP12-deficient mice were more susceptible to endotoxic shock and had enhanced resistance to infection with the intracellular bacterium *Listeria monocytogenes*. These data suggest that one or more DAP12-pairing receptors negatively regulate signaling through TLRs.

Toll-like receptors (TLRs) are pattern recognition receptors used by cells of the innate immune system to detect the presence of a wide variety of pathogens. There have been 11 TLRs described in mammals, which allow the innate immune system to respond to a range of pathogen products, including proteins, lipoproteins, polysaccharides and nucleic acids[1]. In macrophages, TLR ligation leads to the production of pro-inflammatory cytokines, including tumor necrosis factor (TNF), interleukin (IL)-6 and IL-12. This cytokine production is critical to a productive innate and adaptive immune response, leading to pathogen clearance. Additionally, when cytokine production is not properly regulated, inflammatory disorders, such as rheumatoid arthritis or septic shock, may result.

The TLR signaling pathway differs somewhat for each TLR[1]. In common are a series of signaling proteins beginning with the adaptor protein MyD88, which associates with TLRs through homotypic interactions of Toll-IL-1 receptor (TIR) domains. When associated with a TLR, MyD88 recruits members of the IL-1 receptor associated kinase (IRAK) family of serine-threonine kinases, which then phosphorylate and activate the ubiquitin ligase TNF receptor associated factor 6 (TRAF6). Activation of TRAF6 leads to activation of the three mitogen-activated protein kinase (MAPK) pathways, p38 MAPK, ERK and JNK, and results in translocation of the transcription factor NF-κB into the nucleus. This occurs through the phosphorylation and degradation of IκBα, a protein that binds and retains NF-κB in the cytoplasm in resting cells. NF-κB is a key transcription factor in the induction of pro-inflammatory cytokines in macrophages, including TNF, IL-6 and IL-12 p40. Some TLRs also use other TIR-containing adaptor

proteins, including TIRAP (also called Mal), TRIF (also called TICAM-1) and TRAM, which contribute to MyD88 signals or activate additional signaling pathways, such as those leading to interferon β production.

The DAP12 signaling adaptor protein is expressed in cells that participate in innate immune responses, including macrophages, granulocytes, and natural killer cells. DAP12 is a disulfide-linked homodimeric transmembrane protein with a minimal extracellular domain, a charged aspartic acid in the transmembrane domain and an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail[2]. DAP12 uses the acidic residue in its transmembrane domain to non-covalently associate with cell surface receptors that have a basic amino acid in their transmembrane region. Neither DAP12 nor many of its associated receptors can efficiently reach the cell surface alone, and DAP12 is required for their signaling abilities. In myeloid cells, several DAP12-associated receptors have been identified. These receptors fall into 2 categories, members of the immunoglobulin (Ig) domain superfamily, such as triggering receptor expressed on myeloid cells (TREM)-1, TREM-2, TREM-3, myeloid-associated immunoglobulin-like receptor (MAIR)-II, CD200RLa, signal regulatory protein (SIRP) β, and paired immunoglobulin-like type 2 receptor (PILR) β, and members of the C-type lectin family, such as myeloid DAP12-associating lectin (MDL)-1 and mouse NKG2D-short[3-14]. In DAP12-deficient mice, dendritic cell abnormalities have been observed[15,16].

The signaling cascade downstream of DAP12 and other ITAM-containing signaling adaptors, such as FcεRIγ and CD3ζ, has been well characterized[17,18]. After ligation of a

DAP12-associated receptor, the tyrosines within the ITAM are phosphorylated, presumably by src family tyrosine kinases. In macrophages, these phosphotyrosines recruit the syk tyrosine kinase, which promotes the recruitment and activation of adaptor complexes leading to the activation of the phospholipase C (PLC) γ, ERK and phosphatidylinositol-3 (PI3) kinase pathways. The best studied of these downstream pathways, that of PLCγ, leads to the activation of the transcription factor NFAT. In macrophages, ligation of DAP12-associated receptors results in secretion of cytokines and chemokines, including TNF, IL-6 and MCP-1[3].

Two DAP12-pairing receptors, TREM-1 and TREM-2, are specifically implicated in responses to bacteria and bacterial products[4,19]. TREM-1 expression is induced on macrophages and neutrophils in response to lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria and a ligand for TLR4[3]. Additionally, a fusion protein consisting of the TREM-1 extracellular domain and the Fc portion of IgG reduces LPS-induced toxicity in a mouse model of septic shock[4]. Ligation of TREM-1 on monocytes and neutrophils synergizes with signals through several TLRs[4,20,21]. TREM-2, another DAP12-paired receptor, has been implicated as a pattern recognition receptor for a variety of bacteria and fungi[19]. Taken collectively, these data suggest that DAP12 may be involved in the innate immune response to pathogens. We initiated these studies to further characterize the role of DAP12-associated receptors in TLR responses by investigating the responses of DAP12-deficient mice[15] to pathogen products and bacterial infection. Unexpectedly, we found that DAP12-deficient mice exhibit enhanced TLR

responses *in vitro* and *in vivo*, suggesting that certain DAP12-associated receptors may function as negative regulators of TLR responses.

RESULTS

Increased cytokine production by DAP12-deficient macrophages

To investigate the role of macrophage DAP12-associated receptors in the responses to pathogenic stimuli, we generated bone marrow-derived macrophages from wild-type and DAP12-deficient mice[15] and treated them with a variety of pathogen products that signal through different TLRs. Bone marrow macrophages from DAP12-deficient mice secreted higher concentrations of the pro-inflammatory cytokine TNF in response to TLR stimulation when compared to wild-type macrophages (**Fig. 1a**). TNF production was increased in response to all TLR stimuli tested (i.e. LPS-TLR4, synthetic bacterial lipopeptide-TLR2/1, CpG DNA-TLR9, zymosan-TLR2, poly (I:C)-TLR3, and peptidoglycan-TLR2 (**Fig. 1a** and data not shown)). Increased TNF was not observed at all concentrations of TLR stimuli, but was most consistent and pronounced at doses that were below saturating amounts for TNF production. This is particularly evident for LPS stimulation, where concentrations that elicited no TNF from wild-type macrophages, such as 0.5 ng/ml, elicited maximal amounts from the DAP12-deficient macrophages.

We investigated whether enhanced cytokine production in DAP12-deficient macrophages was specific to TNF or whether secretion of other pro-inflammatory cytokines was also elevated. Both IL-6 and IL-12 p40 secretion were higher in DAP12-deficient macrophages compared to wild-type macrophages (**Fig. 1b, c**). Similar to TNF, cytokine production was more pronounced at lower concentrations of the TLR stimulus. Similarly, increased cytokine concentrations were also observed in response to stimulation with whole bacteria, including *Escherichia coli* and *Staphylococcus aureus*

(data not shown). These data suggest that in the absence of DAP12, macrophages are more sensitive to signals through TLRs, and that, in wild-type macrophages, a DAP12 signal limits the TLR-induced response. TNF production was also assessed by intracellular TNF staining to determine whether the elevated TNF secretion was due to an increased percentage of cells producing TNF or increased TNF production on a per cell basis. In response to LPS, CpG DNA and zymosan, both the percentage of cells that produced TNF (**Fig. 2a**) and the amount of TNF staining, as assessed by the mean fluorescent intensity (of the TNF-producing population), were increased in the DAP12-deficient macrophages (**Fig. 2b**). We also investigated the kinetics of the TNF secretion from wild-type and DAP12-deficient macrophages. Increased TNF production was observed as early as 4 h after activation with LPS and zymosan, and the difference between wild-type and DAP12-deficient macrophages increased over the course of the experiment (**Fig. 2c**).

We also examined whether DAP12 regulates the production of inflammatory cytokines induced by activating macrophage receptors distinct from TLRs. The low affinity Fc Receptor (FcR) for IgG (FcγRIII or CD16) associates with another ITAM-containing adaptor, the FcεRIγ chain, and crosslinking of FcγRIII on macrophages results in phagocytosis and production of TNF and IL-6. Similar to what was observed for TLR stimulation, crosslinking of FcγRIII resulted in higher TNF and IL-6 secretion from DAP12-deficient macrophages compared to wild-type macrophages (**Supplementary Fig. 1/online**). Because dendritic cells (DCs) are closely related to macrophages and also express several DAP12-associated receptors, we also investigated TLR-induced

inflammatory cytokine production by DAP12-deficient DCs. The DAP12-deficient DCs responded identically to wild-type DCs in terms of TNF and IL-12 p40 production after stimulation with CpG DNA and in the production of IL-12 p40 after stimulation with LPS (**Supplementary Fig. 2/online**). In contrast to DAP12-deficient macrophages, LPS-induced TNF production was actually somewhat decreased in the DAP12-deficient DCs in comparison with wild-type DCs. This suggests that TLR-induced cytokine production is regulated differently in macrophages and DCs.

DAP12 reconstitution reduces TNF production

To confirm the increase in intracellular TNF in response to TLR stimulation in DAP12-deficient macrophages was due to DAP12 deficiency, we used retroviral transduction of macrophages to introduce DAP12 into either wild-type or DAP12-deficient macrophages. After retroviral transduction of a control vector or DAP12-encoding vector, the macrophages were activated with zymosan or CpG DNA, and TNF production was assessed by flow cytometry. DAP12-deficient macrophages transduced with a control vector showed an increased percentage of cells producing TNF in comparison with transduced wild-type macrophages (**Fig. 3**), consistent with previous data (**Fig. 2a**). In contrast, re-introduction of DAP12 into DAP12-deficient macrophages caused a reduction in the percentage of TNF-producing cells to an amount equivalent to wild-type macrophages. Expressing additional DAP12 protein in wild-type macrophages did not change the percent of TNF-expressing cells (**Fig. 3**). This experiment showed that DAP12 deficiency caused the increased TNF production in the DAP12-deficient macrophages.

This reconstitution experiment ruled out the possibility that DAP12 deficiency had caused a developmental defect in the bone marrow precursors used to generate the macrophages, thereby altering their ability to respond to TLR signals. Bone marrow-derived DAP12-deficient macrophages grew similarly to wild-type macrophages and uniformly expressed F4/80 and Mac1 (CD11b) on their surface (data not shown). Additionally, DAP12-deficient macrophages were identical to wild-type macrophages in their surface TLR4-expression as assessed by flow cytometry (data not shown), suggesting that at least this component of the LPS response was not different in DAP12-deficient macrophages.

Enhanced TNF production requires ITAM and syk

We next investigated the signaling mechanism by which the lack of DAP12 resulted in increased cytokine production. Signaling through DAP12 is mediated through its ITAM, which relies on phosphorylation of the two tyrosines within the ITAM for propagation of a signal. To determine whether these tyrosines are required for DAP12-mediated negative regulation of TNF production, a retrovirus encoding a mutant DAP12 protein in which both tyrosines in the ITAM were mutated to phenylalanines was transduced into wild-type and DAP12-deficient bone marrow-derived macrophages[22]. Mutant DAP12 cannot be phosphorylated and activate the ITAM-dependent signaling cascade. The mutant DAP12 was not able to reduce the TNF production in response to stimulation with zymosan or CpG DNA(**Fig. 3b, c**). This ITAM-mutant DAP12 behaved similarly to the empty control retrovirus and contrasted with wild-type DAP12, which caused a reduction

in cytokine production. These data show that ITAM-mediated signaling by DAP12 is required for dampening TLR signaling.

In macrophages, the syk tyrosine kinase is recruited to ITAMs in adaptor proteins after phosphorylation, and syk is required for downstream signaling[18]. We therefore compared the TLR responses of syk-deficient macrophages to DAP12-deficient macrophages. As syk-deficient mice are embryonic lethal[23], we generated bone marrow-derived macrophages from syk-deficient fetal liver chimeras[24]. The cytokine production induced by TLR stimulation was compared in wild-type, syk-deficient and DAP12-deficient bone marrow-derived macrophages. Syk-deficient macrophages behaved similarly to DAP12-deficient macrophages as shown by increased secretion of pro-inflammatory cytokines (TNF, IL-6, and IL-12 p40), in response to LPS, CpG DNA, and synthetic lipopeptide (**Fig. 4**). These data suggest that DAP12 uses the conventional ITAM signaling pathway to regulate TLR signaling.

Anti-inflammatory cytokine production

Macrophages produce both inflammatory and anti-inflammatory cytokines in response to ligation of TLRs. Anti-inflammatory cytokine production, particularly that of IL-10, is important in down-regulating secretion of inflammatory cytokines. We hypothesized that DAP12-deficient macrophages may produce less anti-inflammatory cytokines than wild-type macrophages, and this may explain their increased secretion of TNF, IL-6 and IL-12 p40. To test this hypothesis, we treated wild-type and DAP12-deficient macrophages with TLR stimuli that induced TNF production and measured IL-10 concentration in the

supernatants. Rather than producing less IL-10, DAP12-deficient macrophages actually secreted more IL-10 than wild-type macrophages (**Fig. 5a**). To address the production of transforming growth factor (TGF) β or other anti-inflammatory cytokines, we activated wild-type and DAP12-deficient macrophages in a transwell culture dish where these macrophages were separated by a 0.2 μm porous membrane. The production of TNF by the macrophages in the bottom chamber was then assessed by flow cytometry. Neither the wild-type nor the DAP12-deficient macrophages secreted soluble factors that influenced TNF production by the other macrophage population (**Fig. 5b**). This suggests soluble factors produced by the TLR-activated wild-type macrophages are unable to diminish the hyper-responsive production of inflammatory cytokines by DAP12-deficient macrophages.

Enhanced ERK phosphorylation in DAP12-deficient macrophages

To determine where DAP12-initiated signals intersect the TLR signaling pathway, we examined the kinetics of activation of the three MAPKs and the NF-κB pathway, which are all downstream of TLR stimulation[1]. For these experiments, we used 0.5 ng/ml LPS, a concentration which gave us reproducible differences in cytokine secretion between the wild-type and DAP12-deficient macrophages. The kinetics and magnitude of p38 MAPK and JNK phosphorylation were similar in wild-type and DAP12-deficient macrophages (**Fig. 6**). In contrast, p42/44 ERK signaling was enhanced in the DAP12-deficient cells. In wild-type macrophages, no ERK phosphorylation was detected until 40 min after stimulation, whereas in DAP12-deficient macrophages, ERK phosphorylation was clearly detected at 20 min (**Fig. 6**). Additionally, at all time points the magnitude of ERK

phosphorylation in the DAP12-deficient macrophages was considerably greater than in the wild-type macrophages. Although the amount of ERK phosphorylation was different, ERK phosphorylation peaked at 40 min after stimulation and had returned to baseline by 90 min for both wild-type and DAP12-deficient macrophages (**Fig. 6**).

We also measured activation of the NF-κB pathway by examining the degradation of the inhibitor IκB-α, which serves to retain NF-κB in the cytoplasm, thereby preventing transcriptional activation by NF-κB. In both wild-type and DAP12-deficient macrophages, IκB-α was degraded with similar kinetics and was present in similar amounts (data not shown). Therefore, the principal difference in TLR downstream signaling between wild-type and DAP12-deficient macrophages was in ERK phosphorylation.

Increased endotoxic shock in DAP12-deficient mice

Because DAP12-deficient macrophages produced more TNF in response to LPS *in vitro*, we investigated whether this happens *in vivo* as well. We first analyzed a model of endotoxic shock where mice are injected i.p. with a low dose (10 μg) of LPS and D-galactosamine, a hepatotoxic agent that makes mice more sensitive to TNF. In this model, mice usually succumb to shock between 5 and 10 h after injection and death is dependent upon TNF[25]. DAP12-deficient mice were more susceptible to LPS-induced shock than wild-type mice. At 12 h after injection, 40% of the wild-type mice had survived, whereas none of the DAP12-deficient mice had survived (**Fig. 7a**). To determine whether this was due to elevated TNF production, the concentration of this cytokine in plasma was measured in mice injected with a higher dose of LPS (100 μg). A

representative experiment shows that whereas wild-type and DAP12-deficient mice had identical kinetics of TNF induction after LPS injection, with TNF levels peaking at 1 h and returning to baseline by 4 h, DAP12-deficient mice often contained approximately 2-fold more TNF in their plasma at the peak of the response (**Fig. 7b**). The averaged amount of TNF produced one hour after LPS injection from 5 experiments with 25 mice showed DAP12-deficient mice contained significantly more TNF in their plasma compared to wild-type mice ($p<0.03$) (**Fig. 7c**). Additionally, in some experiments, DAP12-deficient mice had increased plasma IL-6 concentrations at 4 h post-injection of LPS (data not shown) and after i.p. injection with 20 mg of zymosan (data not shown). Therefore, DAP12 negatively regulates TLR responses *in vivo* as well as *in vitro*.

Enhanced clearance of *L. monocytogenes* in DAP12-deficient mice

Because TNF is a critical cytokine in the innate immune response to bacterial infection, we examined whether DAP12-deficient mice have increased protection from infection with the intracellular bacterium *Listeria monocytogenes*. Wild-type and DAP12-deficient mice were infected i.v. with varying doses of *Listeria*, and the total colony forming units (cfu) in the spleens and livers were determined at day 3 post-infection. At this time point, the response to the infection will be predominantly from the innate immune system, including macrophages, as this is before there are detectable T cell responses in this model[26]. At day 3 after infection, DAP12-deficient mice had substantially less bacteria in their spleens and livers compared to wild type mice (**Fig. 8**). In some experiments, this difference could be seen as early as 1 day after infection (data not shown). These data are

consistent with increased inflammatory cytokine production after infection, resulting in an enhanced innate immune response and increased bacterial clearance.

DISCUSSION

Here we report the unexpected finding that inflammatory cytokine production in response to TLR stimulation is increased both *in vitro* and *in vivo* in DAP12-deficient mice. Our findings that TLR-induced cytokine production is increased, rather than decreased, in the absence of DAP12 or syk suggest the outcome of DAP12-mediated signaling may depend on the context of receptor ligation. Signaling through another ITAM-dependent receptor, the T cell antigen receptor (TCR), can have different functional consequences depending on the nature of the ligand. During T cell development, strong TCR ligands cause T cell death (negative selection), whereas weak ligands favor survival (positive selection)[27]. Signaling through the IgA receptor (FcαRI), which pairs with the ITAM-containing FcεRIγ adaptor, can also result either in positive or negative signaling[28]. Ligation of FcαRI with monomeric IgA (low avidity) results in negative regulation, whereas extensive crosslinking of the IgA receptor with multimeric IgA complexes (high avidity) causes cellular activation.

We propose that a similar situation applies to DAP12. DAP12 has previously been shown to induce inflammatory cytokine production[3,7], and we now report that DAP12 can also give an inhibitory signal, thereby negatively regulating the production of these same cytokines in macrophages. As shown for the IgA receptor, our model predicts that a weak signal through one or more DAP12-paired receptors on macrophages results in basal syk phosphorylation and the subsequent activation of a negative regulator of inflammatory cytokine production, thereby dampening ERK1/2 activation. Although we have not identified the critical substrate of syk that functions as a negative regulator of ERK, phosphatases in the MAPK pathway upstream of ERK are candidates.

Furthermore, we predict that the basal DAP12-induced signaling may emanate from the interaction between one or more DAP12-associated myeloid receptors on macrophages with low affinity/avidity ligands that are also present on these macrophages. Although ligands for the DAP12-associated myeloid receptors have not been identified, if this basal DAP12 signaling is indeed ligand-induced then they must be expressed on macrophages themselves because in the *in vitro* experiments, these are the only cells present in the culture. *In vivo*, the ligands may be expressed on macrophages or other cells. In order for these DAP12-associated receptors to convert from negative to positive signaling, we propose that encounters either with higher affinity or higher avidity ligands would be necessary. In other words, only when the DAP12-associated receptors are cross-linked by a high affinity/avidity ligand would this evoke stronger recruitment of syk – with the net balance resulting in positive activation. Experiments showing that cross-linking DAP12-associated receptors on myeloid cells results in potent activation used antibodies against these receptors, i.e. mimicking high affinity/avidity engagement[3,7]. In accordance with our findings, microglial cells lacking the DAP12-associated TREM-2 receptor have been reported to express higher amounts of inflammatory cytokines[29], possibility causing the brain pathology observed in TREM-2-deficient humans[30].

DAP12 negatively regulates signaling for macrophage inflammatory cytokine production by another ITAM-containing receptor, the low affinity IgG receptor, FcγRIII or CD16. Perhaps any receptor that can induce ERK1/2 activation, such as TLRs or FcγRIII, can be inhibited by DAP12. Indeed, signaling through FcαRI was shown to inhibit the ITAM-associated IgG Fc receptors and IgE Fc receptor (FcεRI)[28]. DAP12-deficient DCs did not show increased inflammatory cytokine production to the TLR

stimuli LPS and CpG DNA. Although this may indicate that DAP12 cannot inhibit DC cytokine production, we favor an alternative hypothesis--that the DCs used in these experiments do not express ligands for their DAP12-associated receptors; therefore, there is no basal DAP12 signaling in these cells.

In macrophages, antibody-mediated crosslinking of the DAP12-associated TREM-1[3] and MAIR-II[7] receptors leads to inflammatory cytokine production, demonstrating that a DAP12 signal alone can result in activation. Crosslinking of TREM-1 also can synergize with LPS for TNF and IL-1-β production[4,20]. Treatment of mice with a soluble TREM-1 fusion protein protects mice from LPS-induced shock[4]. Monocyte/macrophages produce a soluble form of TREM-1 in response to LPS treatment *in vitro* and *in vivo*, and a peptide mimic of this soluble receptor can protect mice from LPS-induced death[31,32]. Although it is proposed that the soluble TREM-1 protein or peptide acts as a TREM-1 receptor antagonist, an alternative possibility is that the soluble protein binds to an as yet undefined inhibitory receptor, which would also suppress an immune response. Indeed, many of the activating DAP12-paired receptors have related inhibitory receptors[33].

Although the signaling pathways leading from activation through TLRs to inflammatory cytokine production have been well characterized, there is a growing literature on how these signals are dampened to prevent pathological consequences, such as septic shock and inflammatory disorders[1]. The negative regulators of TLR signaling include diverse proteins that intersect the TLR signaling pathways at different stages. Those expressed in macrophages include the cell surface receptors ST2 and TRAIL-R, the signaling molecules SOCS1, IRAK-M, PI3kinase, MyD88s, SHIP and Tollip, and the

ubiquitin-interacting proteins Triad3A and A20[34-44]. The mechanisms by which these proteins regulate TLR signaling are quite varied and most affect all TLRs, as does DAP12, whereas others, such as Triad3A and ST2, only affect a subset of the TLRs[34,42]. Triad3A acts at the level of receptor expression, ubiquitinating and therefore inducing the downregulation of TLR4 and TLR9[42]. ST2 interferes with the association of TIR-domain containing adaptors with TLRs[34]. Further downstream, IRAK-M interacts with the IRAK-1/4 complex, preventing its release from MyD88 and therefore its binding to TRAF6[44]. MyD88s also acts at this level, preventing the phosphorylation of IRAK-1[39]. A20 acts directly upon TRAF6, de-ubiquitinating it and thereby reducing activation of NFκB[43]. How many of the other negative regulators lead to inhibition of TLR responses is unclear, but deficiencies in these proteins result in a similar phenotype to the DAP12-deficient cells. However, none of these previously described negative regulators appear to work at the same level as DAP12.

Cells deficient in several of the described negative regulators have enhanced TLR signaling, as determined by increased activation of MAPK pathways after LPS stimulation. SOCS-1-deficient macrophages have increased p38 and JNK phosphorylation[36], PI3kinase-deficient DCs have increased p38 phosphorylation[38], and IRAK-M-deficient macrophages exhibit increased phosphorylation of p38, JNK and ERK[44]. DAP12-deficient macrophages exhibited increased ERK1/2 phosphorylation, whereas p38 and JNK phosphorylation was similar to wild-type macrophages. Although the amounts of ERK phosphorylation were increased at all time points after LPS stimulation, the kinetics were similar between DAP12-deficient and wild-type macrophages. DAP12 is known to specifically activate ERK[45-47]. Upstream of ERK

activation in TLR signaling is the MAPKKK, Tpl2, and macrophages lacking Tpl2 secrete less TNF in response to LPS[48]. If DAP12 links to ERK through Tpl2, then Tpl2 activation may also be increased in DAP12-deficient cells.

Collectively, our data support a model whereby basal signaling through one or more DAP12-associated receptors causes a reduction in the amount of inflammatory cytokines produced by macrophages. This leads to an increase in the susceptibility of DAP12-deficient mice to endotoxic shock, and an enhanced resistance of these mice to infection with certain intracellular bacteria. Although other studies have suggested that DAP12 may induce pro-inflammatory signals that augment responses to bacterial infections, it is interesting to note that there have been no reports of increased susceptibility to infection in DAP12-deficient humans (a disease named Nasu-Hakola syndrome)[49]. Rather than displaying an immune deficiency, Nasu-Hakola patients have bone defects and brain pathologies, similar to DAP12-deficient mice[50,51]. In fact, our data predict that these patients may have enhanced resistance to certain pathogens.

METHODS

Mice. C57Bl/6 mice were purchased from NCI Frederick or Charles River. DAP12-deficient mice[15] were backcrossed to C57Bl/6 mice for 9 generations. Syk-deficient mice[23] were backcrossed to C57Bl/6 mice for 6 generations. Fetal liver chimeras were generated from syk-deficient embryos as described[24]. All experiments were in accordance with protocols approved by the University of California San Francisco Institutional Animal Care and Use Committee.

Macrophages and DCs. Bone marrow-derived macrophages were grown as described[52]. After 6 days, non-adherent cells were removed and macrophages were re-plated for use in experiments. Bone marrow-derived DCs were grown as described in media containing 10 ng/ml recombinant GM-CSF (Peprotech)[53]. At day 8, non-adherent cells containing DCs were removed and purity and maturation was assessed by flow cytometry. Cells were stained with antibodies to CD11c, CD86 and I-A^b (BD Pharmingen). The non-adherent cells were typically 60-75% $CD11c^+$ DC which were 80-90% immature (CD86/I-A^b low/intermediate).

TLR stimulation and cytokine measurement. For all experiments, day 6 bone marrow-derived macrophages were plated and adhered overnight. Titrations of TLR stimuli were added at 10x concentration. To exclude LPS contamination, all stimuli used, with the exception of LPS and *E. coli*, were treated with polymixin B (10 μg/ml) (Sigma-Aldrich) prior to addition to cells. TLR stimuli were as follows: *Salmonella minnesota* R595 LPS (List Biological Laboratories); CpG DNA (ODN 1826) and peptidoglycan (Invivogen);

zymosan, heat-killed *E. coli* and *S. aureus* (Molecular Probes); poly (I:C) (Amersham Pharmacia); synthetic bacterial lipopeptide Pam_3CSK_4 (Roche). For cytokine secretion, the amounts of TNF, IL-6, IL-12 p40 and IL-10 in duplicate supernatants were measured by ELISA (TNF, eBioscience; IL-6, IL-12 p40, and IL-10, BD Pharmingen). For TNF measurement by intracellular cytokine staining, cells were stimulated for 4 h (zymosan and CpG DNA) or 14 h (LPS) in the presence of Brefeldin A (10 μg/ml) for the final 4 h. Macrophages were blocked with anti-FcR mAb 2.4G2, and stained after fixation and permeabilization (CalTag) with a PE-labeled anti-TNF (BD PharMingen). Day 8 DCs were stimulated with LPS or CpG DNA for 4 h in the presence of Brefeldin A. DCs were surface stained with FITC-labeled anti-CD11c prior to fixation, permeabilization and staining with PE-labeled antibody to TNF or IL-12 p40 (BD Pharmingen). Cells were analyzed by flow cytometry using a FACScan (BD) running CellQuest software (BD) and analyzed with FlowJo software (TreeStar). In some experiments, wild-type and DAP12-deficient macrophages were cultured in Transwell dishes (Costar) separated by a 0.2 μM filter. In these experiments, TLR stimuli were added to both the top and bottom chambers.

Activation through Fc receptors. 96 well plates were coated with a 1 mg/ml solution of DOTAP (Sigma) for 10 minutes at room temperature, washed with PBS and incubated overnight with monoclonal antibodies against FcγRII/III (hybridoma 2.4G2) in 0.1 M bicarbonate buffer (pH 9.0). Macrophage supernatants were collected at 16 hrs and tested for cytokines by ELISA.

Retroviral transduction of macrophages. VSVg pseudotyped retroviruses were generated by using the following constructs in the pMX-pie vector, in which the cDNA is followed by an IRES-GFP to identify infected cells: control empty vector, N-terminus FLAG-tagged DAP12, and N-terminus FLAG-tagged mutant DAP12, in which both tyrosines in the ITAM have been changed to phenylalanine[52]. After 72 h, the cells were activated and TNF was measured by using intracellular cytokine staining. Infected cells were gated upon as GFP+, and the percentage staining for TNF was determined.

Kinetics of MAPK phosphorylation. Macrophages were activated with 0.5 ng/ml LPS, lysed at the indicated times in lysis buffer containing 1% Triton X-100, protease inhibitors, and sodium orthovanadate (1mM) (Sigma-Aldrich). Cytoplasmic extracts were analyzed by SDS-PAGE and Western blot by using antibodies specific for phosphorylated and non-phosphorylated p38 MAPK, p42/44 ERK and JNK and HRP-conjugated anti-rabbit IgG (Cell Signaling Technologies). Western blots were developed with ECL Plus (Amersham Pharmacia).

Endotoxic shock. Mice were injected with 10 μg of *S. minnesota* LPS and 20 μg of D-galactosamine (Sigma-Aldrich) and were monitored for 12 h for survival or for failure to have a righting response, at which point they were euthanized. To determine plasma TNF levels, mice were injected with 100 μg of *E. coli* 055:B5 LPS (Sigma-Aldrich). At the indicated times, a sample of blood was taken and plasma TNF was determined by ELISA.

***Listeria monocytogenes* infection.** Mice were injected i.v. with the indicated amount of *Listeria monocytogenes* 10403S (a gift of E. Pamer) grown to mid-log phase (OD_{600}=0.1) in tryptic soy broth (Fisher Scientific). At day 3 after infection, the livers and spleens were homogenized, serial dilutions were plated on tryptic soy broth plates and incubated overnight at 37^{o}C, and the cfu per organ was determined by colony count.

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Figure Legends

Figure 1 DAP12-deficient macrophages secrete increased amounts of cytokines after TLR stimulation. Bone marrow-derived macrophages from wild-type or DAP12-deficient mice were incubated with the indicated concentrations of LPS, CpG DNA, synthetic bacterial lipopeptide or zymosan for 16 h. Supernatants were collected and the amounts of TNF (**a**), IL-6 (**b**) and IL-12 p40 (**c**) were measured by using ELISA. No IL-6 was detected after incubation of macrophages with zymosan. Data are representative of 10 (LPS, zymosan) or 5 (lipopeptide, CpG DNA) (**a**) or 4 independent experiments (**b, c**).

Figure 2 DAP12-deficient macrophages produce more TNF after TLR stimulation. **(a, b)** Bone marrow-derived macrophages were incubated with 0.4 ng/ml LPS, 0.04 μM CpG DNA or 10 particles zymosan per macrophage for 4 h (CpG DNA and zymosan) or 14 h (LPS) in the presence of Brefeldin A for the final 4 h. TNF levels were then assessed by using intracellular immunofluorescent staining and flow cytometry and the data are represented by one-parameter histograms (**a**) or by the mean fluorescent intensity (MFI) of the TNF-producing population (**b**). Data are representative of 4 independent experiments. (**c**) Bone marrow-derived macrophages (day 7) were stimulated with 0.5 ng/ml of LPS or 10 zymosan particles per macrophage. Supernatants were removed at the indicated time points, and TNF concentrations were measured by using ELISA. Data are representative of 3 independent experiments.

Figure 3 Re-introduction of DAP12 reduces the TNF production by DAP12-deficient macrophages in an ITAM-dependent manner. Bone marrow-derived macrophages from wild-type or DAP12-deficient mice were transduced with an empty vector (control) retrovirus or retroviruses encoding wild-type DAP12 (DAP12) or a DAP12 ITAM mutant (DAP12 Y→F). The macrophages were activated with zymosan (10 per macrophage) or CpG DNA (0.01 μM) in the presence of Brefeldin A for 4 hrs and TNF production was assessed by flow cytometry. Transduced cells were gated on (based on GFP fluorescence) and the percentage of TNF-producing cells was determined. (**a**) Histograms of TNF expression in wild-type and DAP12-deficient macrophages transduced with control or DAP12-expressing retrovirus from one representative experiment. (**b, c**) The percentage of transduced cells producing TNF represented as the mean +/- SEM of 3 independent experiments (zymosan) or 2 independent experiments (CpG DNA).

Figure 4 Syk-deficient macrophages secrete increased levels of pro-inflammatory cytokines in response to TLR stimulation. Bone marrow-derived macrophages from wild-type mice, DAP12-deficient mice or syk-deficient fetal liver chimeras were incubated with the indicated concentrations of LPS, CpG DNA, or synthetic bacterial lipopeptide for 16 h. Supernatants were collected and the amounts of TNF (**a**), IL-6 (**b**) and IL-12 p40 (**c**) were measured by using ELISA. Data are representative of 3 independent experiments.

Figure 5 Soluble inhibitory factor production does not explain differences between wild-type and DAP12-deficient macrophages. (**a)** Bone marrow-derived macrophages from wild-type and DAP12-deficient mice were stimulated with the indicated concentrations of LPS, zymosan, CpG DNA and lipopeptide for 16 h. Supernatants were then assayed for TNF or IL-10 by using ELISA. (**b**) Wild-type and DAP12-deficient macrophages were cultured in the upper and lower chambers of Transwell culture plates separated by a porous membrane. Cells in both the top and bottom chambers were treated with CpG DNA or zymosan for 4 h, and TNF-producing cells in the bottom chambers were determined by using flow cytometry as described in **Fig. 2a**. In the histograms shown, the type of cells in the upper and lower chambers of the Transwell plates are indicated as cells in bottom chamber:cells in top chamber. The percentage of TNF positive cells is indicated in the upper right corner of each histogram. Data shown are representative of 2 independent experiments.

Figure 6 LPS-induced ERK phosphorylation is increased in DAP12-deficient macrophages. Bone marrow-derived macrophages from wild-type or DAP12-deficient mice were stimulated with 0.5 ng/ml LPS for the indicated time (in min) at which cells were lysed. Cytoplasmic extracts were analyzed by SDS-PAGE and Western blot by using antibodies specific for p38 MAPK, p42/44 ERK and JNK or for phosphorylated versions of these proteins. Data are representative of 4 independent experiments.

Figure 7 DAP12-deficient mice are more susceptible to endotoxic shock than wild-type mice. (**a**) Wild-type (n=10) or DAP12-deficient (n=9) mice were injected i.p. with

10 μg LPS and 20 μg D-galactosamine and then monitored for survival for 12 h. Data are representative of 4 independent experiments. (**b**) Wild-type (n=5) and DAP12-deficient (n=5) mice were injected with 100 μg of LPS. At the indicated times, a sample of blood was drawn and plasma was analyzed for TNF by using ELISA. The data are reported as mean +/- SEM and are representative of 3 independent experiments. (**c**) Mice were treated as described in (**b**) and plasma TNF concentrations at one hour post-injection are represented as the mean of 25 mice assayed in 5 independent experiments +/- SEM. The difference between wild-type and DAP12-deficient mice is significant using the Students t-test, $p<0.03$ (two-tailed distribution).

Figure 8 DAP12-deficient mice have an enhanced innate immune response to infection with *Listeria monocytogenes*. Wild-type and DAP12-/- mice were infected i.v. with 5×10^4 (**a**) or 1×10^4 (**b**) cfu of *Listeria monocytogenes* strain 10403S. 3 days after infection, the number of cfu in the spleens and livers of the mice were determined. The data are represented as the cfu in each individual mouse (circles) and the mean (horizontal line). In (**a**), there were 5 mice per group; in (**b**) there were 6 wild-type mice and 4 DAP12-deficient mice per group. These data are representative of 4 independent experiments.

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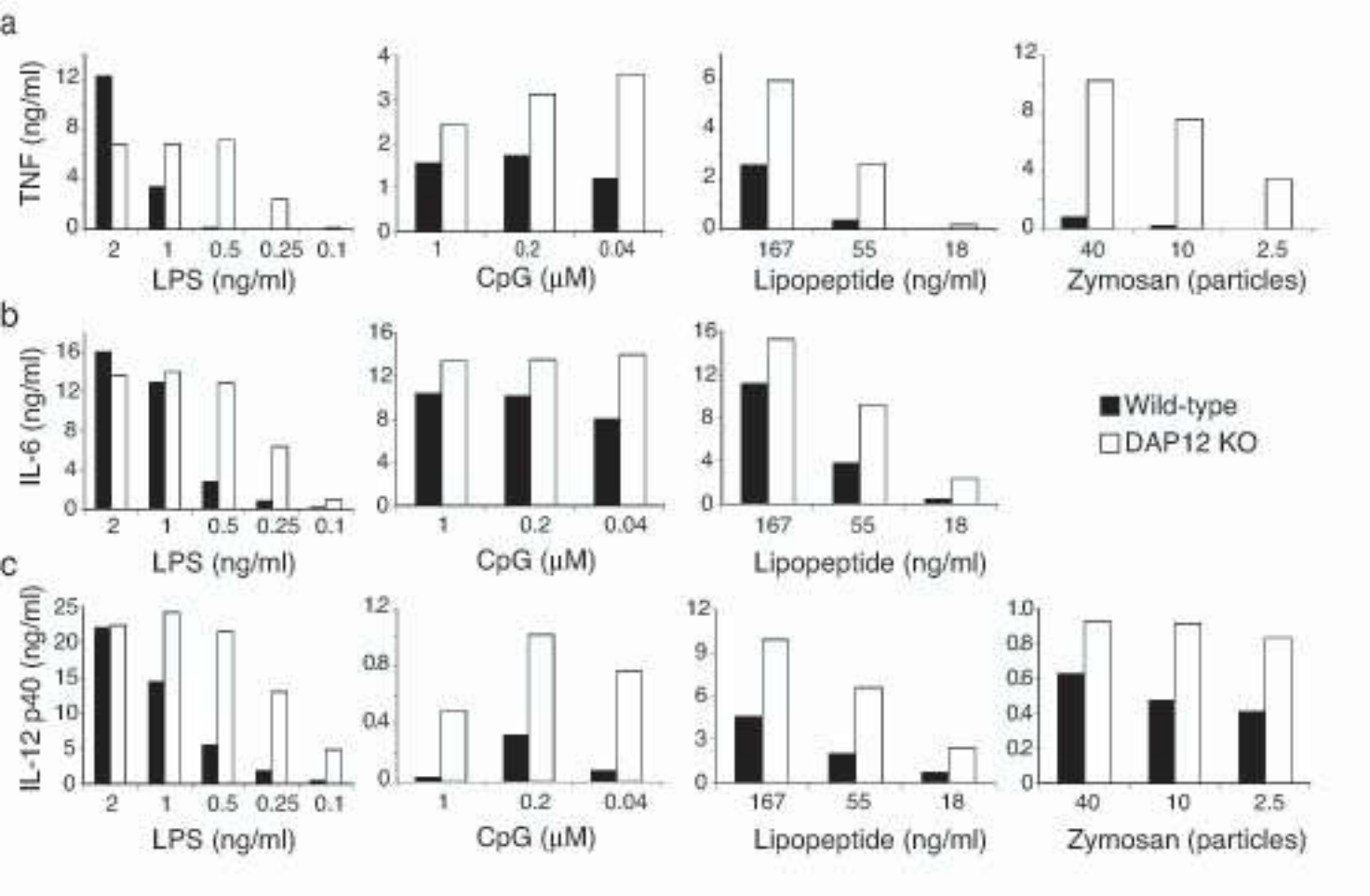
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0.5
0.25
0.1
LPS (ng/ml)
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2
1
0
1
0.2
0.04
CpG (μM)
6
4
2
0
167
55
18
Lipopeptide (ng/ml)
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8
4
0
40
10
2.5
Zymosan (particles)
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LPS (ng/ml)
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0.04
CpG (μM)
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8
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55
18
Lipopeptide (ng/ml)
Wild-type
DAP12 KO
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0.5
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0.1
LPS (ng/ml)
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0.4
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1
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CpG (μM)
12
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0
167
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18
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2.5
Zymosan (particles)

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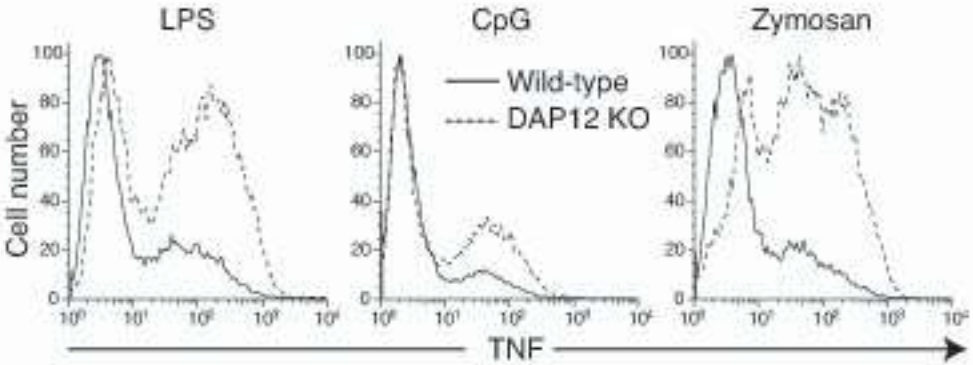
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Wild-type
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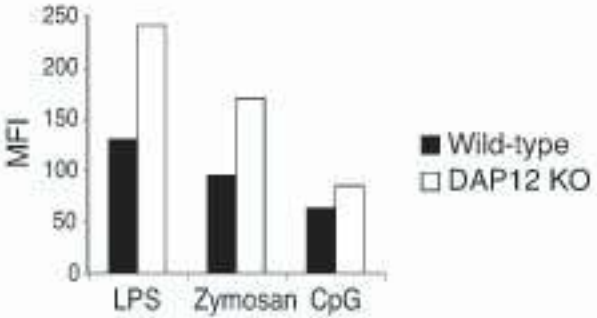
Cell number
TNF

b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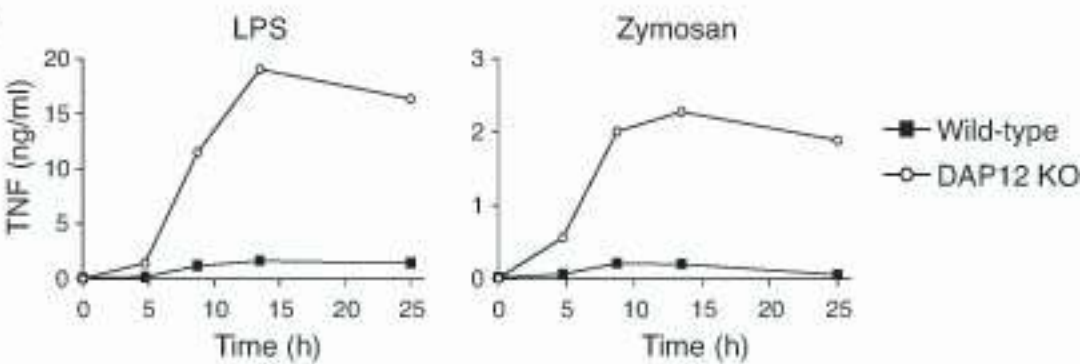
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Zymosan
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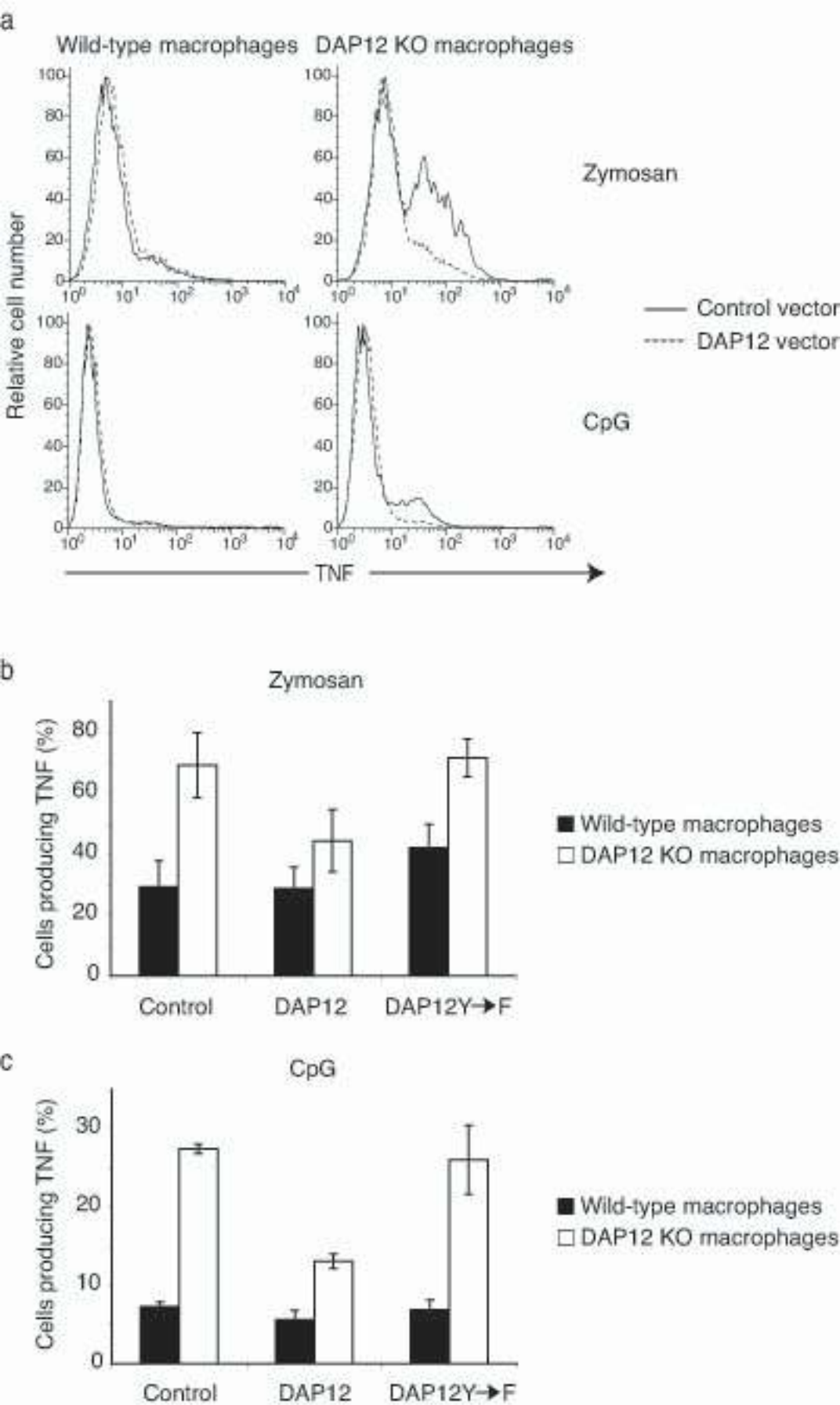
Time (h)
Wild-type
DAP12 KO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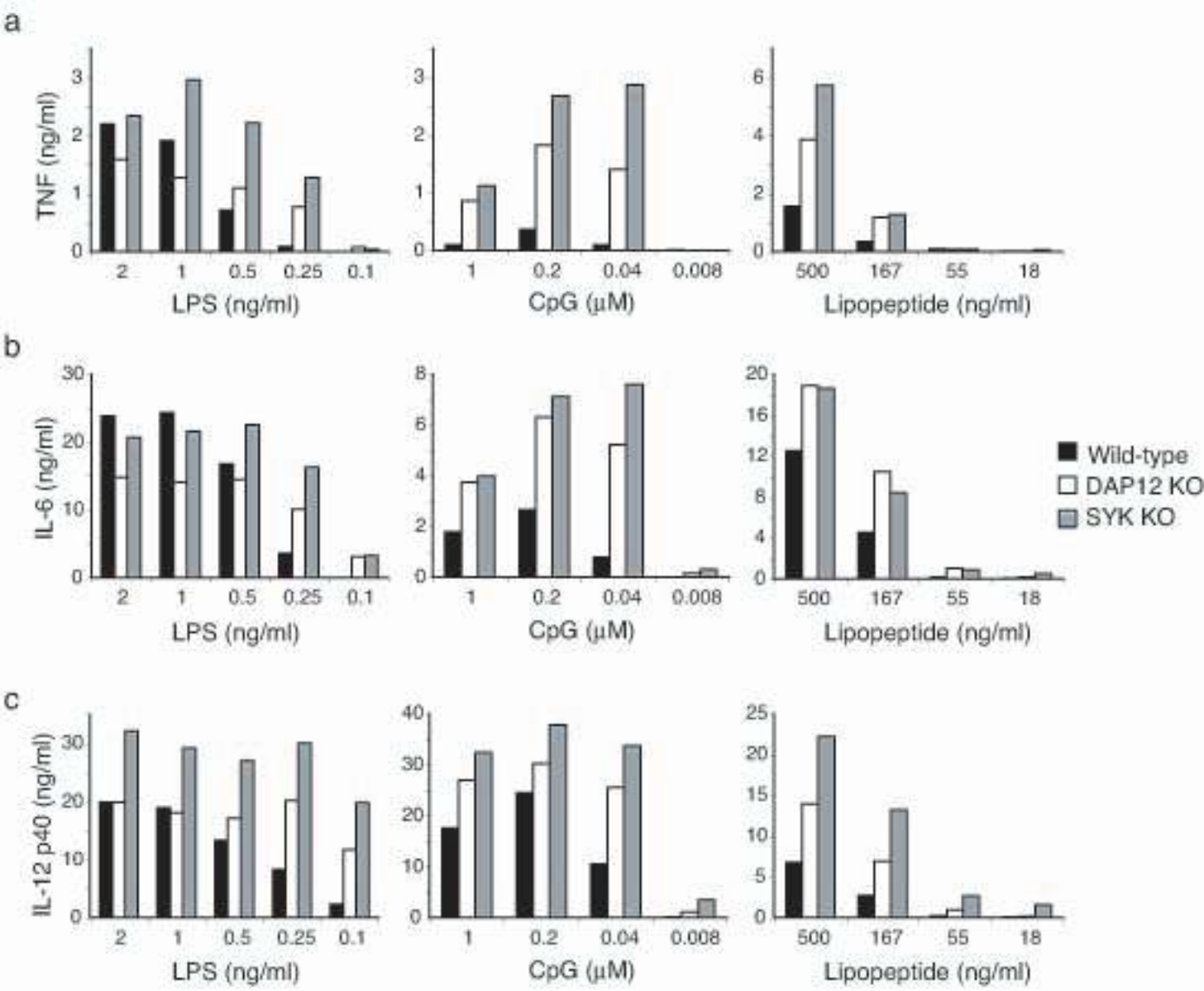
Wild-type macrophages
DAP12 KO macrophages
Zymosan
CpG
Relative cell number
TNF
Control vector
DAP12 vector
b
Zymosan
Cells producing TNF (%)
Wild-type macrophages
DAP12 KO macrophages
Control
DAP12
DAP12Y→F
c
CpG
Cells producing TNF (%)
Wild-type macrophages
DAP12 KO macrophages
Control
DAP12
DAP12Y→F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TNF (ng/ml)
3
2
1
0
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LPS (ng/ml)
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0.2
0.04
0.008
CpG (μM)
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Lipopeptide (ng/ml)
b
IL-6 (ng/ml)
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LPS (ng/ml)
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CpG (μM)
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55
18
Lipopeptide (ng/ml)
Wild-type
DAP12 KO
SYK KO
c
IL-12 p40 (ng/ml)
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LPS (ng/ml)
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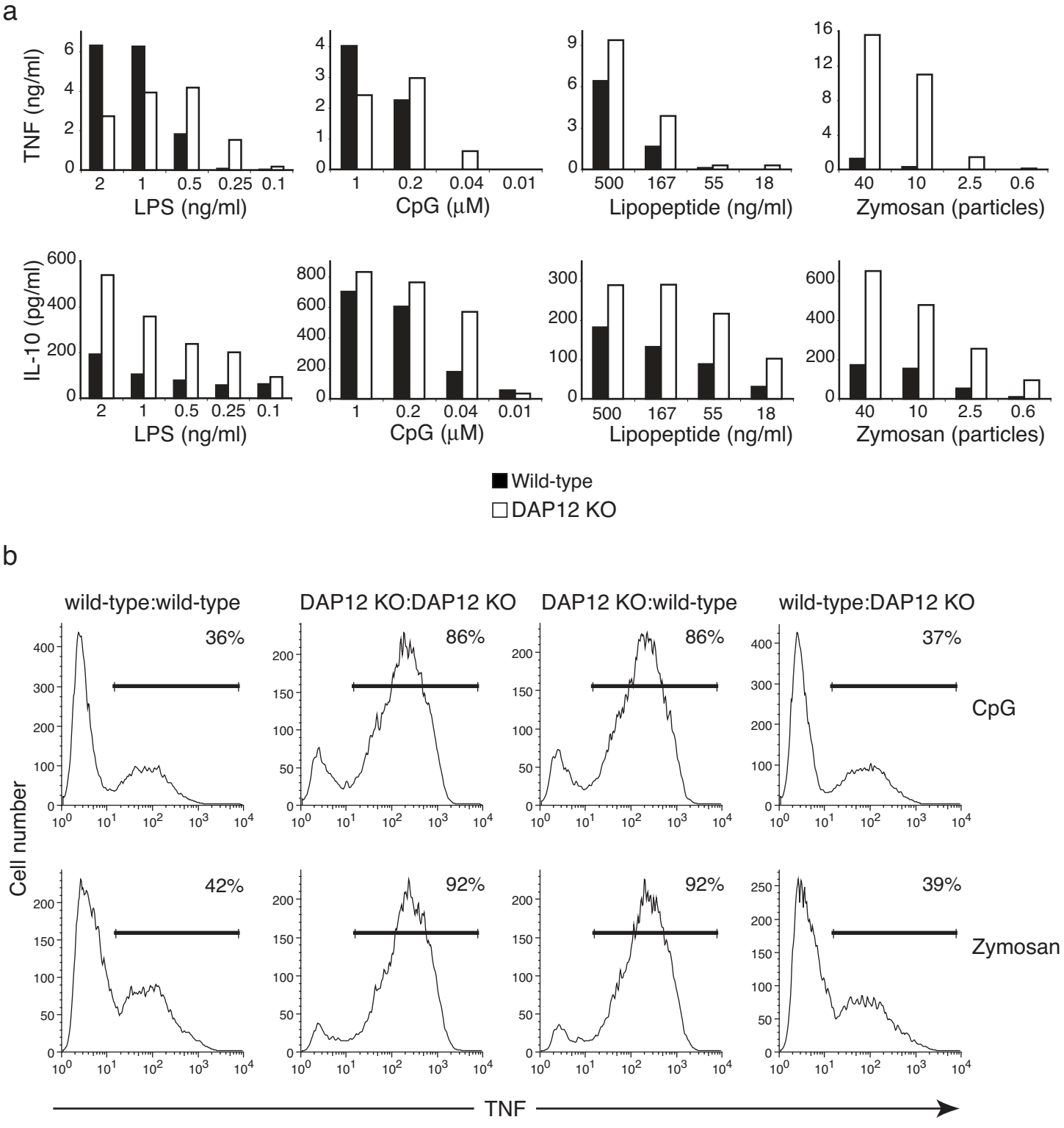
Lipopeptide (ng/ml)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LPS (ng/ml)
CpG (μM)
Lipopeptide (ng/ml)
Zymosan (particles)
IL-10 (pg/ml)
Wild-type
DAP12 KO
b
wild-type:wild-type
DAP12 KO:DAP12 KO
DAP12 KO:wild-type
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86%
86%
37%
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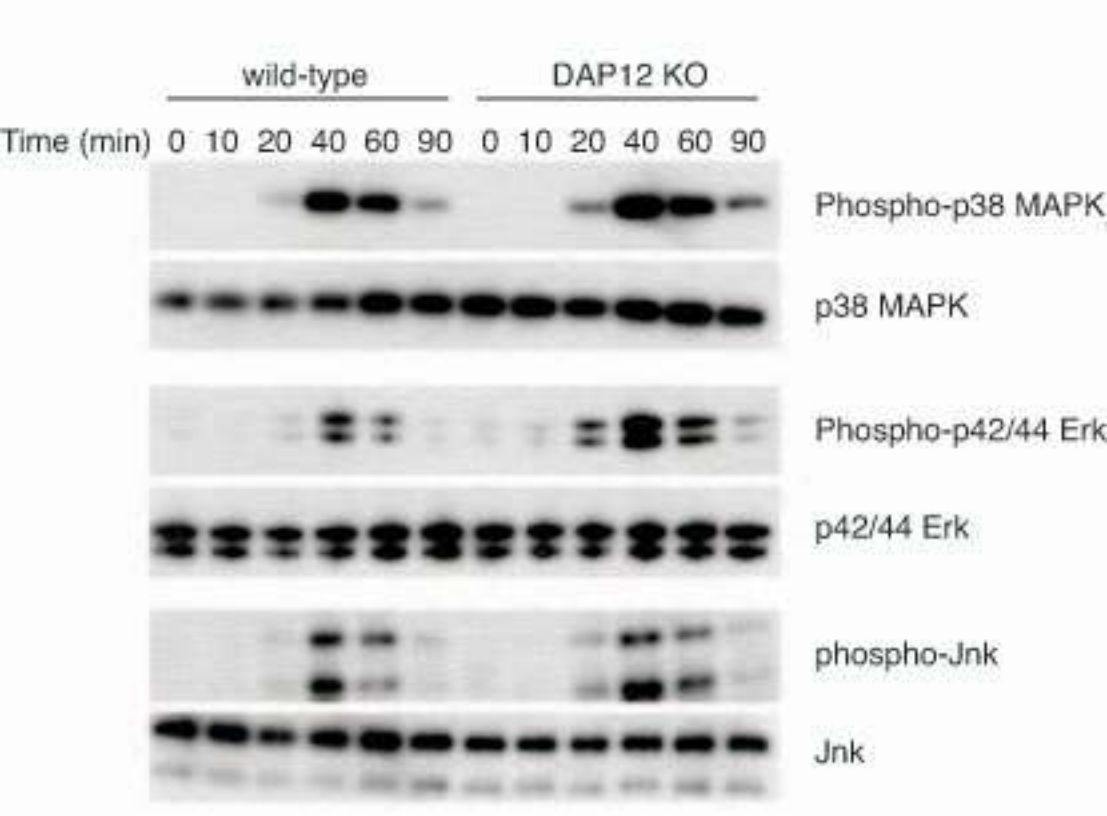
42%
92%
92%
39%
Zymosan
Cell number
TNF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DAP12 KO
Time (min) 0 10 20 40 60 90 0 10 20 40 60 90
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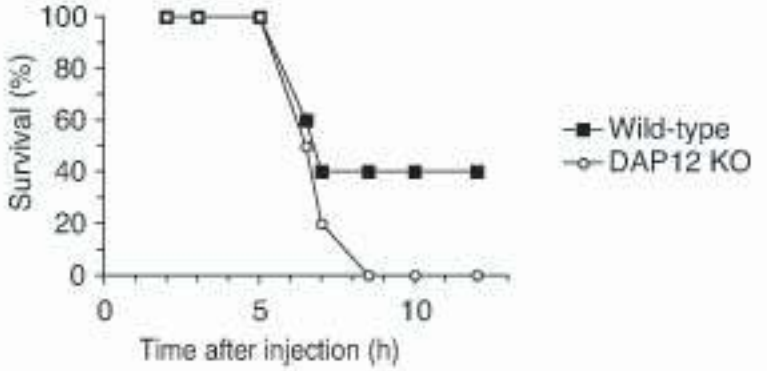
p38 MAPK
Phospho-p42/44 Erk
p42/44 Erk
phospho-Jnk
Jnk

a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20
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Survival (%)
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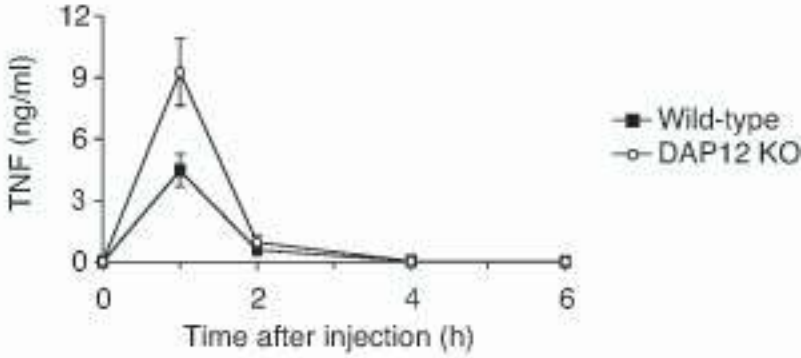
5
10
Time after injection (h)
Wild-type
DAP12 KO

b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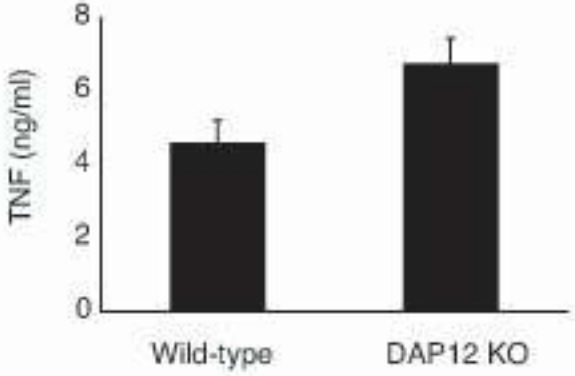
TNF (ng/ml)
0
2
4
6
Time after injection (h)
Wild-type
DAP12 KO

c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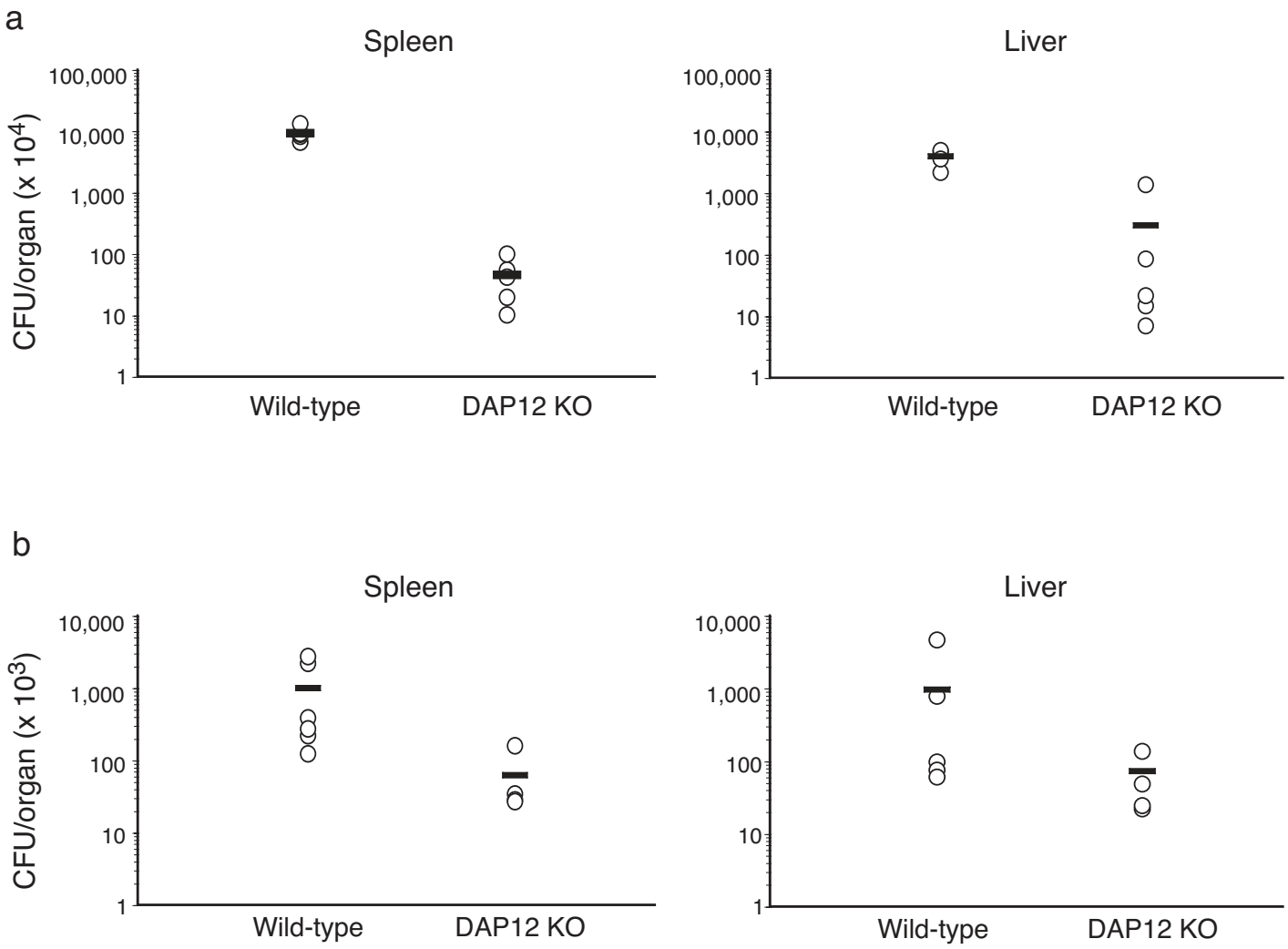
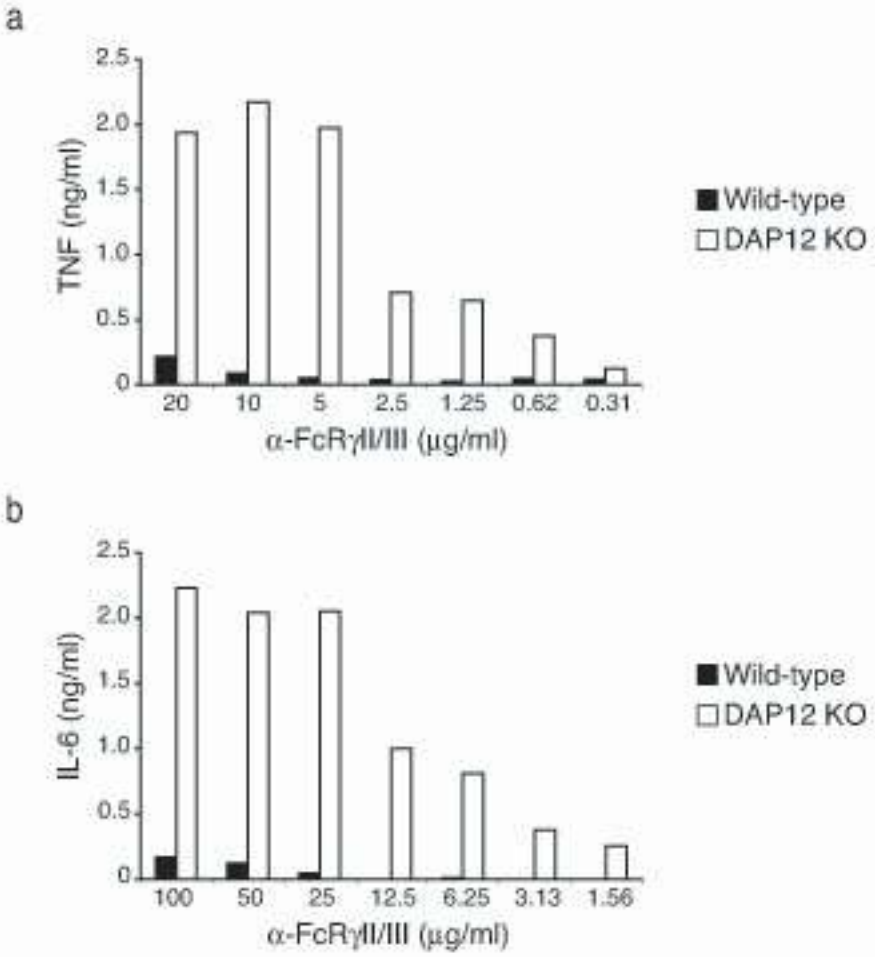
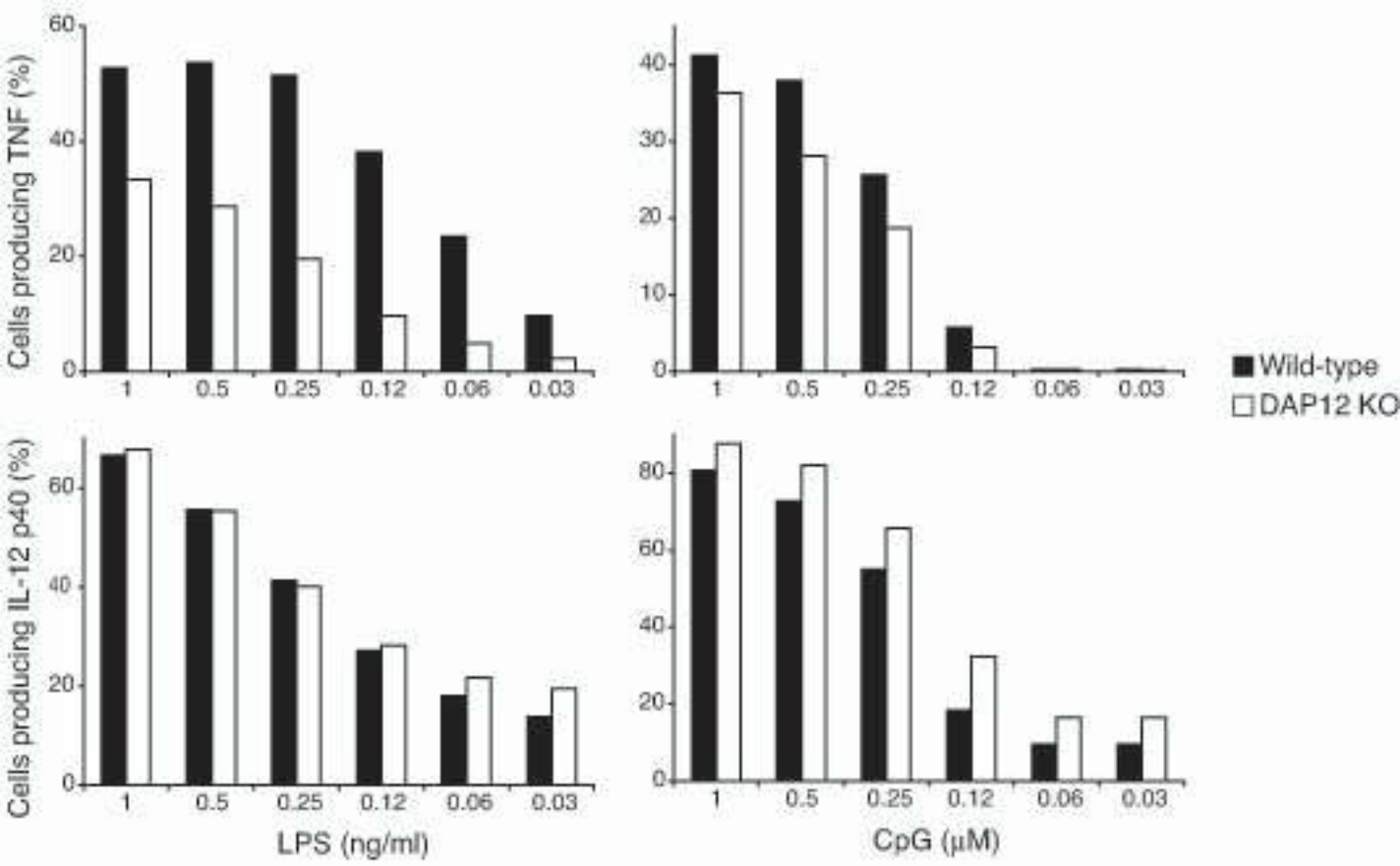
6
4
2
0
TNF (ng/ml)
Wild-type
DAP12 KO


a
Spleen
Liver
CFU/organ (x 10^4)
100,000
10,000
1,000
100
10
1
Wild-type
DAP12 KO
b
Spleen
Liver
CFU/organ (x 10^3)
10,000
1,000
100
10
1
Wild-type
DAP12 KO



Supplementary Figure 1. DAP12-deficient macrophages produce more TNF and IL-6 in response to FcγRII/III crosslinking. Wild-type and DAP12-deficient bone marrow-derived macrophages were plated onto wells coated with antibody to FcRγII/III (CD16/32) (hybridoma 2.4G2) and supernatants were harvested after 16 hours. The amount of TNF (a) and IL-6 (b) in the supernatants were quantitated using ELISA. The data shown are representative of 4 independent experiments.



Supplementary Figure 2. TLR stimulation of wild-type and DAP12-deficient dendritic cells. Bone-marrow derived dendritic cells (day 8) were cultured with LPS or CpG DNA for 4 hrs in the presence of Brefeldin A. Cells were stained for CD11c and then fixed, permeabilized and stained with PE-labeled antibodies to TNF or IL-12 p40. The percentage of CD11c-gated cells producing TNF or IL-12 p40 is shown. The data shown are representative of 2 independent experiments.