# Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor κB

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**Cells of the monocyte–macrophage lineage play a central role in the orchestration and resolution of inflammation. Plasticity is a hallmark of mononuclear phagocytes, and in response to environmental signals these cells undergo different forms of polarized activation, the extremes of which are called classic or M1 and alternative or M2. NF-κB is a key regulator of inflammation and resolution, and its activation is subject to multiple levels of regulation, including inhibitory, which finely tune macrophage functions. Here we identify the p50 subunit of NF-κB as a key regulator of M2-driven inflammatory reactions in vitro and in vivo. p50 NF-κB inhibits NF-κB–driven, M1-polarizing, IFN-β production. Accordingly, p50-deficient mice show exacerbated M1-driven inflammation and defective capacity to mount allergy and helminth-driven M2-polarized inflammatory reactions. Thus, NF-κB p50 is a key component in the orchestration of M2-driven inflammatory reactions.**

Macrophages play an essential role in homeostasis and defense and are characterized by high functional heterogeneity (1–5). Analogous to the Th1 and Th2 dichotomy of T cell polarization, macrophages can be polarized by the microenvironment to mount specific M1 or M2 functional programs (1, 2, 6). Classic or M1 macrophage activation in response to microbial products or IFN-γ is characterized by high capacity to present antigen; high IL-12 and IL-23 production and consequent activation of a polarized type I response; and high production of nitric oxide (NO) and reactive oxygen intermediates. Thus, M1 macrophages are generally considered potent effector cells that kill intracellular micro-organisms and tumor cells and produce copious amounts of proinflammatory cytokines. In contrast, alternative activation of macrophages is promoted by various signals [e.g., IL-4, IL-13, glucocorticoids, IL-10, Ig complexes/Toll-like receptor (TLR) ligands] that elicit different M2 forms, able to tune inflammatory responses and adaptive Th2 immunity, scavenge debris, and promote angiogenesis, tissue remodeling, and repair (2).

The molecular basis of macrophage polarization has not been fully elucidated. Members of the NF-κB/Rel family regulate many genes involved in immunity and inflammation (7). Two major signaling pathways control the activation of the NF-κB (7). The classic pathway is stimulated by proinflammatory cytokines, such as TNF-α and IL-1, as well as by recognition of pathogen-associated molecular patterns, and is mostly involved in innate immunity (7). In addition, an alternative pathway of NF-κB activation, mainly involved in adaptive immunity, is activated by certain members of the TNF cytokine family but not by TNF-α itself (7). The NF-κB family consists of 5 members: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel (7, 8), which may form different homo- and heterodimers associated with differential regulation of target genes. Because of its central role in inflammation and immunity, the NF-κB system is equipped with several negative regulators (9). For instance, p50 and p52 homodimers act as repressors because these proteins lack a transcription activation domain, present in RelA, RelB, v-Rel, and c-Rel (7). Accumulation of p50 homodimers has been observed in endotoxin-tolerant macrophages (10), as well as in tumor-associated macrophages (11, 12), suggesting an important role in the modulation of immune and inflammatory functions in infection and cancer. Other negative regulators of NF-κB activation have been identified in disease and include the LPS-inducible splice variant of myeloid differentiation 88 (MyD88), termed MyD88s, the single Ig IL-1 receptor-related molecule (SIGIRR)/TIR8, ST2, IRAK-M, SOCS1 (9, 13), and the Src homology 2-containing inositol-5′-phosphatase, SHIP (14). Exposure to microbial components, such as bacterial LPS, has long been known to induce tolerance to the same agonist in terms of in vivo toxicity and macrophage production of inflammatory mediators, such as TNF (15). This phenomenon has been sometimes referred to as *immunoparalysis* (16), which might play a role in sepsis mortality (17), and it was recently pointed out that in septic patients a reprogramming of the macrophage, rather than an "immunoparalysis," might occur (16, 18). Here we report that LPS-tolerant macrophages indeed have the phenotype of M2-polarized cells. Our findings indicate that p50 NF-κB plays an essential role in the orientation of macrophage polarization both in vitro and in vivo, suggesting a crucial role of this regulatory subunit in the control of M1- vs. M2-driven inflammation.

## Results

**Endotoxin Tolerance Promotes M2 Skewed Inflammation.** We characterized the gene expression profile expressed by LPS-tolerant monocytes/macrophages (Fig. 1). To induce LPS tolerance (L/L), monocytes were incubated in the presence of LPS (L) (100 ng/mL) for 20 h (step 1). Cells were then washed and maintained in standard medium (M) for 2 h (step 2) and subsequently rechallenged with LPS for an additional 4 h (step 3). This time schedule was also adopted for the other experimental groups. In particular, untreated monocytes (M/M) were maintained in standard medium throughout the entire experimental period, whereas LPS-activated monocytes (M/L) were maintained in standard medium during steps 1 and 2 and activated with LPS in

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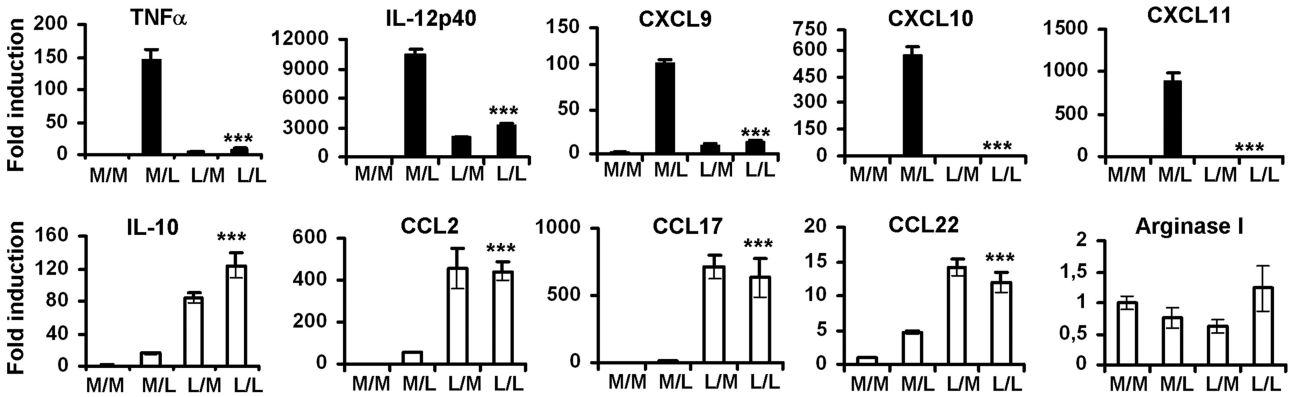


**Fig. 1.** LPS-tolerant monocytes express an M2 cytokine/chemokine profile. Total RNA from control (M/M), activated (M/L), tolerant (L/M), and tolerant monocytes rechallenged with LPS (L/L) were analyzed by RT-PCR for the expression of representative M1 genes (filled bars) and M2 genes (open bars). Results are given as fold increase over the mRNA level expressed by untreated cells (M/M) and are representative of at least 3 different experiments; shown are mean ± SD from triplicate values. For ELISA, results are the average of 3 independent experiments ± SD. ***$P < 0.001$, *t* test.

step 3. A group of "tolerized" monocytes (L/M), which did not receive a second challenge with LPS in step 3, was included to be compared with LPS-rechallenged monocytes (L/L) and to ascertain their effective status of unresponsiveness to LPS. Total RNA was then extracted, and the expression of representative M1 and M2 genes (2) was evaluated by RT-PCR (Fig. 1). In agreement with previous reports (18), expression of the prototypic M1 gene TNF-α was highly induced in LPS-activated monocytes (M/L), whereas its expression was drastically reduced in tolerance (L/L). Similarly, M1-associated genes (*IL-12p40*, *CXCL9*, *CXCL10*, and *CXCL11*) (6) were highly expressed in LPS-activated monocytes (M/L) and remained extremely low in tolerant cells (L/L). In contrast, mRNA level of the M2 cytokine IL-10 and the Th2-recruiting chemokines *CCL2*, *CCL17*, and *CCL22* (19) was consistently higher in tolerant (L/L) as compared with LPS-activated monocytes (M/L). These results suggested that tolerant monocytes acquire an alternative-activation program, with high expression of M2 immunomodulatory genes. Accordingly, we found high levels of the M2 chemokines CCL2, CCL17, and CCL22 in the supernatants of tolerant monocytes, collected 24 h after step 3 (supporting information Fig. S1), whereas IL-10 was secreted at similar levels in M/L and L/L monocytes. In addition, as reported in both IL-4– and IL-13–treated monocytes (20), Arginase I gene expression was impaired in both activated (M/L) and tolerant (L/L) monocytes (Fig. 1 and Fig. S1). We also evaluated whether the M2 profile in tolerant monocytes was stable, by extending the interval before the LPS rechallenge (step 2) up to 48 h. As a result, tolerant monocytes stably displayed defective expression of M1-associated genes (*TNFα*, *IL-12p40*, *CXCL9*, *CXCL10*, and *CXCL11*), along with high expression of M2-associated genes (*IL-10*, *CCL2*, *CCL17*, and *CCL22*) (Fig. S1). Because monocytes are macrophage precursors, a similar analysis was performed on both human monocyte-derived macrophages (M-DM) and mouse peritoneal macrophages (peritoneal exudate cells, PEC). Similarly to monocytes, LPS-tolerant M-DM (Fig. S2*A*) and PEC (Fig. S2*B*) displayed defective expression of M1-associated genes (*TNFα*, *IL-12p40*, *CXCL9*, *CXCL10*, and *CXCL11*) and enhanced expression of M2-associated genes (*CCL2* and *CCL17*). Surprisingly, as compared with their LPS-activated counterparts (M/L), whereas tolerant PEC (L/L) displayed increased expression of *IL-10* mRNA, LPS-tolerant M-DM expressed lower levels of *IL-10* mRNA. This unexpected dissociation in IL-10 expression between mouse and human macrophages was also confirmed for *CCL22* and *Msr1* and indicates selective interspecies differences. In addition, this discrepancy is also likely due to the M-CSF–dependent "in vitro generation" of M-DM, which does not faithfully recapitulate the in vivo conditions of macrophage differentiation. In support of this, it was reported that M-CSF–driven monocyte-to-macrophage differentiation strongly affects their transcriptional profile (21). However, high expression of the prototypic murine M2 genes *Ym1*, *Fizz1*, and *Arginase I* (22) further confirmed the M2-skewing of tolerant PEC (Fig. S2*B*). Cross-tolerance between TLRs and cytokine receptors (e.g., IL-1 and TNF receptors) has been previously reported (18). On the basis of this, we investigated the responsiveness of LPS-tolerant monocytes to both the M1-polarizing signal IFN-γ and the M2-polarizing signal IL-4 (21). As shown, LPS-tolerant monocytes expressed lower levels of M1 genes (*TNFα*, *CXCL9*, *CXCL10*, and *CXCL11*) in response to IFN-γ (Fig. S3*A*). In contrast, LPS-tolerant monocytes retained full responsiveness to IL-4, in terms of M2-associated chemokines expression (*CCL17* and *CCL22*) (Fig. S3*B*). These data suggest that LPS-tolerant monocytes maintain full capacity to respond selectively to prototypic alternative or M2 activation signals.

**NF-κB p50 Drives the M2 Polarization of "Tolerant" Macrophages.** We analyzed in LPS-tolerant monocyte/macrophages the activation of both the TLR4/MyD88/Mal- and TLR4/TRIF/TRAM-dependent activation of NF-κB (7, 13) and STAT1 (13), respectively. NF-κB activation is mainly associated with nuclear translocation of the p50/p65 heterodimer (7, 8). In addition, the p50 NF-κB protein may form inhibitory homodimers that are able to repress transcription of NF-κB target genes (7, 8, 18). As expected (10), Western blot analysis of the NF-κB subunits showed an increase of p50 NF-κB nuclear localization in LPS-tolerant monocytes (L/L) (Fig. S4*A*), whereas a partial inhibition of p65 nuclear translocation was observed. We also evaluated the activation of STAT1. Whereas a significant level of phospho-STAT1 was present in both M/L and IFN-γ–stimulated monocytes, used as positive control (12), L/L monocytes displayed impaired STAT1 phosphorylation. Consistent with the gene expression profile, these results suggest that LPS tolerance in monocytes inhibits both the MyD88- and TRIF-dependent pathways. Peritoneal macrophages from p50−/− mice have been shown to be unable to undergo endotoxin tolerance-mediated suppression of TNF-α production (23). To explore the role of p50 in the expression of an M2 phenotype by tolerant macrophages, representative M1 and M2 cytokine and chemokine genes were analyzed by RT-PCR in WT and p50−/− macrophages. L/L p50−/− macrophages failed to develop LPS tolerance (Fig. 2*A*), assessed by the inducible expression of *TNF-α* and inducible NO synthase (*iNOS*). In addition, the absence of p50 significantly restored LPS induction of *CXCL9* in L/L macrophages. Most

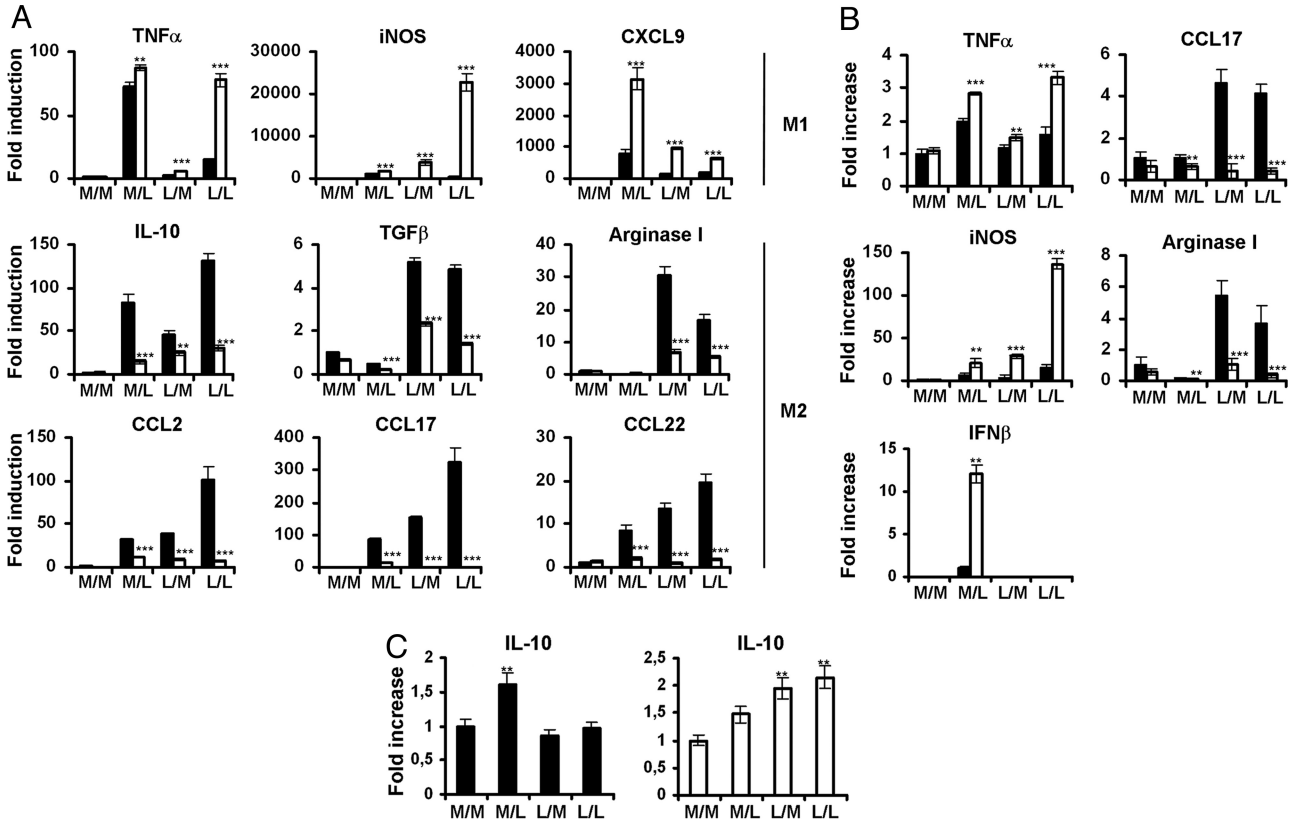


**Fig. 2.** Role of p50 NF-κB in macrophage polarization. (*A*) peritoneal macrophages from WT (filled bars) and p50−/− (open bars) mice were either untreated (M/M), activated 4 h with LPS (M/L), or tolerized without (L/M) or with LPS rechallenge (L/L). Representative M1 and M2 cytokine and chemokine genes were analyzed by RT-PCR (*A*) and chromatin immunoprecipitation (ChIP) of RNA Pol II (*B*) on representative M1 (TNFα, iNOS, and IFNβ) and M2 (CCL17 and Arginase I) genes, as well as of the NF-κB subunits p50 (open bars) and p65 (filled bars) on the IL-10 gene promoter (*C*). Results were normalized to β-actin level and expressed as fold induction with respect to the control cell population. Data are representative of 3 independent experiments; shown are mean ± SD from triplicate values. $^{**}P < 0.01$; $^{***}P < 0.001$, *t* test.

strikingly, in all conditions p50−/− macrophages displayed impaired expression of M2-related genes *IL-10*, *TGF-β*, *CCL2*, *CCL17*, *CCL22*, and *Arginase I*. Protein levels and enzymatic activities of iNOS (nitrite) and Arginase (arginase activity) fully confirmed these results (Fig. S4*B*). We next asked whether p50 NF-κB could play a differential role in the transcription of M1 and M2 genes. To address this point, we performed ChIP assay for the RNA polymerase II (Pol II). We observed that, in the absence of p50 NF-κB, Pol II recruitment by M1 gene promoters (*TNF-α*, *iNOS*, and *IFN-β*) was increased, whereas its recruitment on M2 gene promoters (*CCL17* and *Arginase I*) was inhibited (Fig. 2*B*). This scenario was even more evident in LPS tolerance, supporting the idea that p50 NF-κB is an essential orchestrator of M2-type gene transcription. In agreement, the recruitment of p50 NF-κB by the M2 gene promoter IL-10 was increased in LPS tolerance (Fig. 2*C*). Thus, p50 is a negative regulator of M1-associated gene expression, whereas it plays a nonredundant positive role in induction of M2-associated genes.

**p50 NF-κB Inhibits IFN-β Production and STAT1 Activity.** TLR4 activation promotes the IRF-3–dependent *IFN-α/β* gene transcription that, in autocrine manner, sustains activation of STAT1, leading to the transcription of M1-inflammatory and IFN-dependent genes, such as *CXCL10* and *CXCL9* (24). To investigate whether p50 NF-κB could play a role in the modulation of IFN-α/β–dependent M1 gene transcription, we analyzed its expression in both WT and p50−/− peritoneal macrophages. Lack of p50 NF-κB resulted in enhanced mRNA expression and secretion of IFN-β (Fig. 3*A*), paralleled by enhanced expression of *CXCL9* and *CXCL10* (Fig. 3*B*). To ascertain the actual role of IFN-β in the increased expression of *CXCL9* and *CXCL10*, we used IRF-3–deficient macrophages, because this factor was shown to play an essential role in the transcription of the *IFN-β* gene (25). In addition to the impaired expression of *IFN-β*, LPS-activated IRF-3–deficient macrophages displayed defective expression of both *CXCL10* and *CXCL9*, as well as lower *TNF-α* and *iNOS* mRNAs (Fig. 3*B*). Similar results were obtained when the biologic activity of IFN-β was blocked by using a specific anti–IFN-β antibody (Fig. S5). These results suggest that p50 NF-κB inhibits the NF-κB–driven and M1-polarizing IFN-β production. To test this hypothesis, the murine macrophage cell line RAW 267.4 was transfected with a luciferase reporter plasmid containing the murine IFN-β promoter region −125 to +55 (26), along with increasing amounts of a murine p50 expression plasmid (11). Luciferase activity was measured after 6 h of LPS stimulation. In agreement with previous studies (27), increasing amounts of p50 correlates with dose-dependent inhibition of LPS-induced *IFN-β* promoter activity (Fig. 3*C*). To ascertain that inhibition of *IFN-β* gene transcription correlates with increased DNA binding of the p50 NF-κB subunit, supershift analysis was performed by using PEC nuclear extracts in the presence of an $^{32}$P-dCTP-labeled IFN-β NF-11B double-strand oligonucleotide, spanning the *IFN-β* promoter region −64 to −55 (28). As shown, in tolerant PEC (L/L) the anti-p50 NF-κB antiserum supershifted a significant portion of the DNA/protein complex, whereas the p65 antiserum poorly affected DNA/protein complex formation (Fig. 3*D*). These result are in agreement with the nuclear levels of both p50 and p65 NF-κB

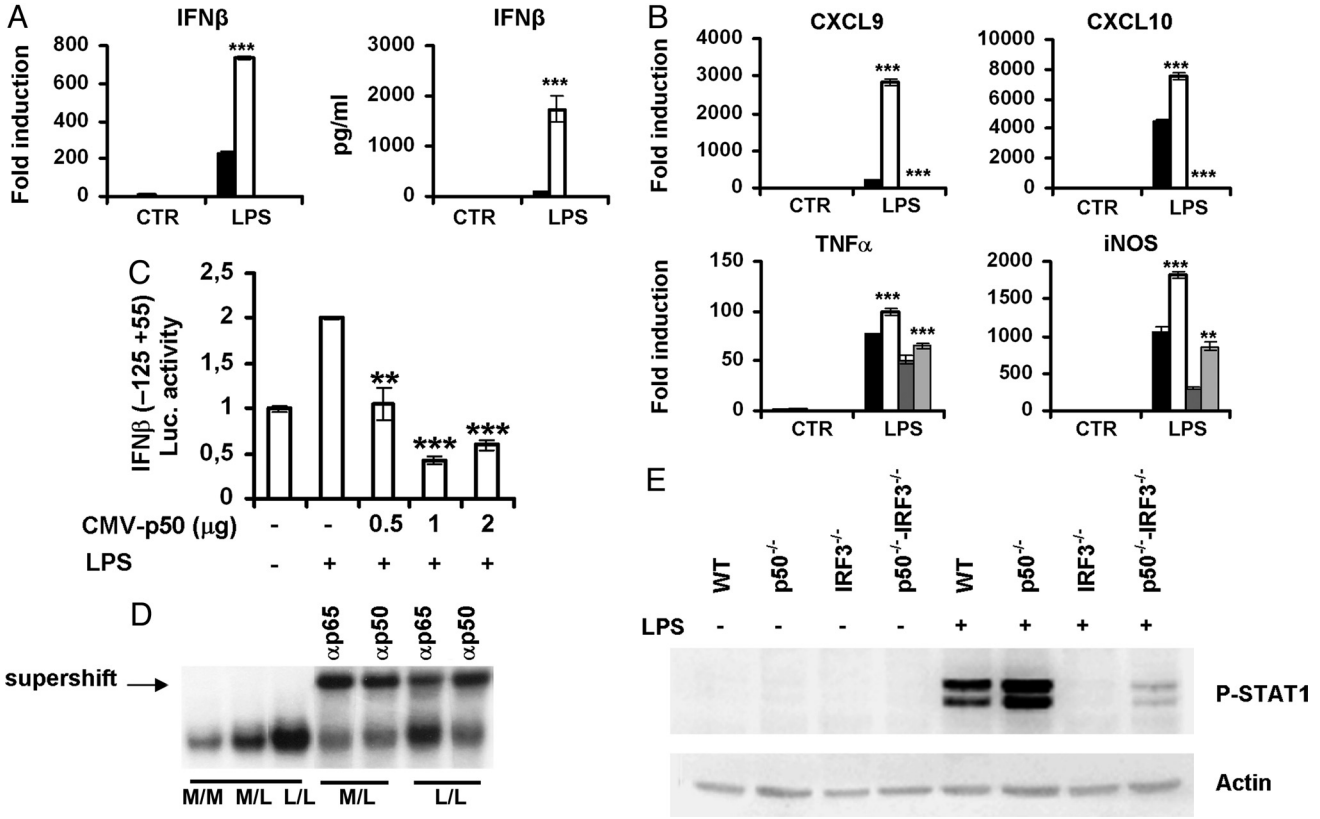


**Fig. 3.** p50 NF-κB acts as negative regulator of IFN-β production and STAT1 activity. PEC were isolated from WT (black bars), p50−/− (white bars), IRF-3−/− (dark gray bars) and double p50/IRF-3−/− (light gray bars) mice, as indicated. Next, cells were activated with LPS (100 ng/mL) and analyzed for IFN-β expression (*A* and *B*). Total RNA was extracted 4 h after LPS treatment (100 ng/mL) and analyzed by RT-PCR. Supernatants were collected 24 h after LPS and tested by ELISA for IFN-β. (*C*) RAW 267.4 cells were cotransfected with 0.5 μg of the luciferase reporter plasmid containing the murine IFN-β promoter region −125 to +55 and increasing amounts of a CMV-driven p50 NF-κB expression vector, as indicated. Luciferase activity is expressed as fold induction with respect to untreated cells. Results shown are the average ± SD of 3 independent experiments. $^{**}P < 0.01$, *t* test. ***, $P < 0.001$. (*D*) EMSA analysis of the NF-κB complexes binding the IFN-β promoter region −64 to −55 (28) in PEC untreated (M/M), M1-activated (M/L), and LPS-tolerant (L/L). Antisera against the p50 and p65 NF-κB subunits were used for supershift analysis, as indicated. (*E*) Peritoneal macrophages were isolated from WT, p50−/−, IRF-3−/−, and double p50/IRF-3−/− mice and stimulated with LPS for 90 min. Next STAT1 phosphorylation was analyzed by Western blot. Equal loading is visualized by actin expression.

observed by Western blot (Fig. S4*A*). Finally, because the expression of *TNF-α* and *iNOS* mRNAs was partially restored in the double p50/IRF-3–deficient macrophages as compared with IRF3−/−, we hypothesized that p50 NF-κB could act as a negative regulator of STAT1 activity, which is a recognized activator of *CXCL9*, *CXCL10* (24), *iNOS* (29), and *TNF-α* gene transcription (30). We confirmed this hypothesis in Western blot analysis (Fig. 3*E*), whereby lack of p50 resulted in higher STAT1 phosphorylation in response to LPS. Collectively these results suggest that in the absence of the p50 regulatory subunit, increased production of IFN-β occurs, which favors M1 polarization. *IFNα* gene expression was not measured because it was shown to be poorly induced in both WT and p50−/− peritoneal macrophages (31).

**In Vivo Role of p50 in M1 vs. M2 Polarized Inflammation.** The role of p50 was then investigated in pathologic conditions associated with polarized immune responses. First, we evaluated the role of p50 in diseases associated with M1 polarized inflammation, such as the Shwartzman reaction (32). The Schwartzman reaction is a type I and IFN-γ–dependent inflammatory response that leads to a lethal shock syndrome that can be induced by 2 consecutive injections of LPS (32). WT and p50−/− mice were primed with a low i.p. dose of LPS (30 μg), and after 16 h mice were i.v. rechallenged with a high dose of LPS (200 μg). We observed a dramatic difference in the survival rate (Fig. S6). In particular, 24 h after LPS rechallenge only WT mice were still alive, indicating that in response to bacterially derived LPS, p50 NF-κB controls the extent of M1 polarized inflammation in vivo. M2 macrophages and their products play a central role in both allergic inflammation and response to parasites. We therefore investigated the role of p50 NF-κB in models representative of polarized type II inflammation. To investigate the role of p50 in macrophage polarization during allergy, mice were sensitized with ovalbumin (OVA) plus alum at day 0 and day 10; then from day 19 to day 24 mice were aerosol challenged each day for 20 min with OVA and killed 24 h after the last treatment. Control mice were sham-sensitized with alum only and next OVA-challenged as for the other groups. As expected, circulating IgE levels increased in OVA-sensitized WT mice. In contrast, no IgE increase was observed in OVA-sensitized p50−/− mice (Fig. S7*A*). Furthermore, we observed a drastic decrease of CCL17 and CCL22 in the bronchoalveolar lavage (BAL) fluid recovered from p50−/− OVA-sensitized mice, as compared with their WT counterparts (Fig. S7*A*). Chitin and Arginase-dependent pathways contribute to airway hyperresponsiveness (33–35), as well as to the pathogenesis of asthma. On the basis of this, we performed confocal microscopy on F4/80$^+$ BAL cells from both WT add p50−/− mice and observed defective expression of Arginase I in p50−/− F4/80$^+$ BAL cells, in contrast to its strong expression by the WT counterpart (Fig. S7*B*). Finally, the lungs were examined histologically. In agreement with a previous report (36) and with the defective expression of M2 chemokines observed in p50−/− mice, poor or

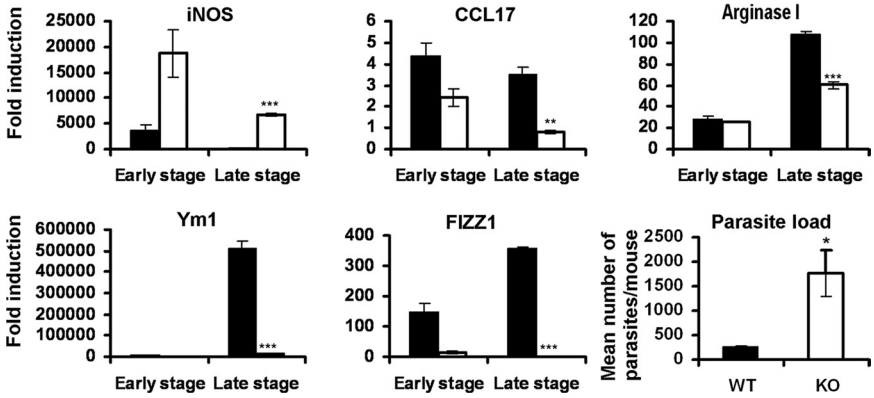


**Fig. 4.** Essential role of p50 NF-κB in the development of M2-polarized inflammation associated with chronic *T. crassiceps* infection. WT and p50−/− mice were i.p. inoculated with 25 nonbudding *T. crassiceps* metacestodes. Expression of M1 and M2 genes by WT (filled bars) and p50−/− (open bars) peritoneal macrophages was analyzed in naïve, early-stage infected (<4 weeks), and late-stage infected (>6 weeks) mice. Data are normalized to actin gene expression and shown as fold increase in mRNA expression with respect to WT naïve macrophages. At each time point, the results shown are the mean ± SEM of 4 naïve and infected mice. Parasite loads were evaluated at 8 weeks after infection. $^{**}P < 0.01$; $^{***}P < 0.001$, *t* test, mean ± SEM, $n = 4$.

absent eosinophil and neutrophil inflammation was observed in p50−/− mice upon antigen challenge, as compared with WT mice (Fig. S7*C*).

Alternatively activated macrophages play a central role in helminth infections (37). We then infected WT and p50−/− mice by i.p. inoculation of *Taenia crassiceps* metacestode and analyzed peritoneal cell recruitment and phenotype at early (<4 weeks) and late (>6 weeks) stages of infection. In accordance with previous data (38), in the course of infection the percentage of mature macrophages ($CD11b^{high}$ $F4/80^{high}$) and T and B cells gradually decreased in both WT and p50−/− animals, whereas neutrophils ($CD11b^{+}$ $Ly6G^{+}$) and eosinophils ($CD11b^{+}$ $CCR3^{+}$) increased (Fig. S8*A*). Strikingly, a higher recruitment of neutrophils and a lower percentage of eosinophils, along with lower levels of circulating IgE, were observed in p50−/− than in WT infected mice (Fig. S8*A*). In the absence of p50, either untreated or LPS-activated peritoneal cells secreted higher levels of NO and expressed lower arginase activity at all stages of infection (Fig. S8*B*), consistent with an M1-skewing of the inflammatory response. In accordance, gene expression analysis confirmed a consistent inhibition of the M2-associated genes *Arginase I*, *Ym1*, *Fizz1*, and *CCL17* in p50−/− vs. WT macrophages (Fig. 4). Finally, in agreement with the protective role of type 2 immune response during helminth infection reported in C57BL/6J mice (39), we observed a drastic increase of parasite load in p50−/− mice (Fig. 4).

## Discussion

Polarized inflammation is a hallmark of several pathologic conditions, including infection and cancer (2, 38), and plays a central role in disease progression and/or resolution. In this study we report that "tolerant" macrophages are indeed a skewed population of M2 macrophages and that the p50 subunit of NF-κB is a key orchestrator of M2-driven inflammatory reactions. Accumulation of p50 NF-κB homodimer in monocyte/macrophages was described as mediating tolerance to LPS and was found in peripheral blood monocytes of septic patients (10). Likewise, massive accumulation of p50 NF-κB homodimer was also observed in the nuclei of tumor-associated macrophages from a murine fibrosarcoma (40), and analysis of their transcriptional profile characterized these cells as a unique M2-polarized macrophage population (40). Here we show that endotoxin-tolerant macrophages express a long-lasting M2 phenotype with impaired expression of M1 functions. Ablation of the p50 NF-κB subunit prevented development of tolerance, assessed by the selective restoration of M1 mediators (e.g., TNF-α and iNOS) and inhibition of M2 cytokines and chemokines, in response to LPS rechallenge. Foster et al. (41) have shown that differential TLR-induced chromatin modifications play a role in 2 distinct classes of LPS-tolerizeable and -nontolerizeable genes. We report now that tolerizeable cytokine and chemokine genes belong to a large extent to the M1 inflammation program, whereas nontolerizeable genes include prototypical M2 cytokines and chemokines. Accordingly, we demonstrated that p50 NF-κB plays a divergent transcriptional role, by promoting Pol II recruitment on M2 promoter genes (e.g., *CCL17* and *Arginase I*) and limiting its recruitment by M1 promoter genes (*iNOS*, *IFN-β*, and *TNF-α*). Thus, tolerance not only spares selected effector functions of macrophages (41) but actually represents an alternative form of macrophage polarization. We also demonstrated that the NF-κB p50 subunit, which in the form of homodimers has regulatory activity, is essential for an M2 "tolerant" phenotype in vitro and in vivo. Our results also suggest that p50 NF-κB acts by suppressing NF-κB–driven, M1-polarizing, IFN-α/β production. Accordingly, p50-deficient mice show exacerbated M1-driven inflammation during the Shwartzman reaction and defective capacity to mount allergy and helminth-driven M2-polarized inflammatory reactions. Recent evidence from Hagemann et al. (42) suggested that NF-κB induction suppresses STAT1 activity. Although we recognize the validity of this mechanism in LPS-activated macrophages, our data suggest that this is an unlikely event in LPS tolerance, during which macrophages are characterized by low NF-κB activity (43) and impaired expression of NF-κB–dependent genes (e.g., *TNF*, *IL-12*, and *IFN-β*). Although additional studies are required to fully characterize the molecular events driving polarized inflammation, previous studies suggest that NF-κB is a potential player (42). In this work we demonstrate that NF-κB p50 is a key component in the orchestration of M2-driven inflammatory reactions.

## Materials and Methods

**Cell Culture.** Human monocytes and M-DM were obtained as previously described (44, 45).

**Real-Time PCR.** Total RNA was reverse-transcribed by the cDNA Archive kit (Applied Biosystems), amplified using Power Syber Green PCR Master Mix (Applied Biosystems), and detected by the 7900HT Fast Real-Time System (Applied Biosystems).

**ELISA.** Supernatants and sera were tested in sandwich ELISA (R&D Systems).

**Enzyme Activity.** NO was measured by Griess reaction as the amount of $NO_3^-$ and $NO_2^-$ produced, using a nitrate/nitrite assay kit (Cayman Chemicals). Arginase activity was measured in cell lysates as previously described (46).

**Western Blot Analysis.** For the NF-κB subunits, nuclear and cytosolic extracts were analyzed by SDS-PAGE (11). For phospho-STAT1 analysis, protein extracts were processed as described previously (40).

**EMSA.** EMSA was performed as previously described (11) by using a double-strand oligonucleotide containing the IFN-β promoter NF-κB binding site (28). Antisera against p50 (no. 1263) and p65 (no. 1207) NF-κB were used for supershift analysis (11).

**ChIP.** ChIP was performed with a polyclonal anti-RNA Pol II, p65 NF-κB (C-20) sc-372, and p50 NF-κB (C-19) sc-1190 antibody (Santa Cruz Biotechnology) (44).

**Transfection and Luciferase Assay.** The RAW 267.4 macrophage cell line was transfected using lipofectamine LTX reagent (Invitrogen). Plasmid containing the region of IFN-β promoter from −125 to +25 linked to the luciferase reporter gene was obtained from Dr. Tadatsugu Taniguchi (26). The cytomegalovirus (CMV) construct expressing the murine p50 NF-κB subunit was a kind gift of Dr. Howard Young (National Cancer Institute). CMV-βGal reporter plasmid (Invitrogen) was used as control of transfection efficiency. The total amount of DNA transfected in the different groups was normalized with the empty CMV construct. Luciferase activity was tested by using the luciferase assay system (Promega) according to the manufacturer's instructions and measured by a Synergy 2 luminometer (Biotek).

**Animals.** The study was designed in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and European Union directives and guidelines. p50 NF-κB–deficient mice (11) were kindly donated by Drs. Michael Karin and Giuseppina Bonizzi (University of California at San Diego Medical School). IRF-3–deficient mice (26) were kindly donated by Dr. Kate Fitzgerald (University of Massachusetts Medical School). p50−/− and IRF-3−/− mice were crossed to obtained double p50/IRF-3–deficient mice.

For additional information see *SI Materials and Methods*.

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