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NFATc1 releases BCL6-dependent repression of CCR2 agonist expression in peritoneal macrophages from *Saccharomyces cerevisiae* infected mice

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The link between the extensive usage of calcineurin (CN) inhibitors cyclosporin A and tacrolimus (FK506) in transplantation medicine and the increasing rate of opportunistic infections within this segment of patients is alarming. Currently, how peritoneal infections are favored by these drugs, which impair the activity of several signaling pathways including the Ca^{++}/CN/NFAT, Ca^{++}/CN/cofilin, Ca^{++}/CN/BAD, and NF-κB networks, is unknown. Here, we show that *Saccharomyces cerevisiae* infection of peritoneal resident macrophages triggers the transient nuclear translocation of NFATc1β isoforms, resulting in a coordinated, CN-dependent induction of the *Ccl2*, *Ccl7*, and *Ccl12* genes, all encoding CCR2 agonists. CN inhibitors block the CCR2-dependent recruitment of inflammatory monocytes (IM) to the peritoneal cavities of *S. cerevisiae* infected mice. In myeloid cells, NFATc1/β proteins represent the most prominent NFATc1 isoforms. NFATc1/β ablation leads to a decrease of CCR2 chemokines, impaired mobilization of IMs, and delayed clearance of infection. We show that, upon binding to a composite NFAT/BCL6 regulatory element within the *Ccl2* promoter, NFATc1/β proteins release the BCL6-dependent repression of *Ccl2* gene in macrophages. These findings suggest a novel CN-dependent cross-talk between NFAT and BCL6 transcription factors, which may affect the outcome of opportunistic fungal infections in immunocompromised patients.

Keywords: BCL6 · CCL2 · CCR2 · Inflammatory monocytes · NFATc1 · Opportunistic infection

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Introduction

Current success in solid organ transplantation depends on the use of triple-drug immunosuppression regimens [1], based on the calcineurin (CN) inhibitors cyclosporin A (CsA) and tacrolimus (FK506). In complexes with immunophilins both drugs bind to the large catalytic subunit A of CN and suppress its Ser/Thr-specific protein phosphatase activity [2]. By inhibiting CN, CsA and FK506 suppress the immunoreceptor-mediated activation of NFATc factors in lymphocytes and, thereby, immune responses [3] leading to graft rejection. However, severe side-effects including a high susceptibility to opportunistic infections, hamper the clinical applications of CsA- and FK506-based regimens. CN is a ubiquitously expressed phosphatase with multiple downstream targets, such as the Ca^{++}/CN/NFAT, Ca^{++}/CN/cofilin, Ca^{++}/CN/BAD and NF-κB networks [4, 5]. Although ablation of CN-B compromises neutrophil-dependent killing of fungal pathogens ex vivo [6], it is still not completely clear how CsA and FK506 facilitate fungal and other opportunistic infections. We will show here that in peritoneal macrophages CsA and FK506 inhibit the synthesis of chemokines that attract neutrophils and inflammatory monocytes (IMs) to the site of infection.

Tissue-specific resident macrophages [7] are key components of the heterogeneous mononuclear phagocyte system, which is defined on the basis of common ontogeny and phagocytic activity [8]. The mononuclear phagocyte system provides the first line of innate immune responses against invading pathogens. In the peritoneal cavity, resident macrophages constitute the largest population of phagocytotic cells. However, the successful clearance of peritoneal infections critically depends on the rapid recruitment of neutrophils and IMs [9] from a specific short-lived $CX_3CR1^{lo}CCR2^{+}$ subset of peripheral blood monocytes [10]. Peritoneal infections trigger the release of several CCR2-specific chemokines from peritoneal resident macrophages (prMΦ), such as CCL2, CCL7, and CCL13. However, significantly reduced numbers of recruited IMs and the inability to control toxoplasmosis in mice deficient for CCL2 or CCR2 suggest a critical nonredundant role for the CCR2/CCL2 axis in the clearance of peritoneal infections [11, 12]. Aberrant CCL2 expression is associated with autoinflammatory disorders and leads to an increased monocyte infiltration in several organ-specific diseases [13] whereas Tg overexpression of CCL2 resulted in myocarditis [14].

Constitutive secretion of the inflammatory chemokines CCL2, CCL3, CCL6, and CCL7 as well as IL-1α from macrophages is a major reason for severe T_H2-type inflammation in BCL6-deficient mice [15, 16]. This indicates that the *Ccl2* gene located within the orthologous cluster of chemokine genes on chromosome 17 (chr 17, q11.2) in humans and on chromosome 11 in mice (Supporting information Fig. 1A) is under stringent negative control of the transcriptional repressor BCL6. BCL6 is a member of the BTB/POZ factor family, which represses transcription by binding to several DNA sequence motifs [17]. BCL6 binds to DNA through C2H2-type zinc fingers (ZF) and recruits transcriptional corepressors through the BTB/POZ and RDII domains. The expression and activity of BCL6 are regulated by a transcriptional autoregulatory loop [18], through the p300-dependent acetylation [19] and by phosphorylation-dependent degradation via the ubiquitin-proteasome pathway [20–22]. We will show here that BCL6 and NFATc1 directly interact and compete for the binding to a composite BCL6/NFAT sequence motif within the *Ccl2* promoter. Thereby, they control the expression of CCL2 in macrophages.

The family of NFAT transcription factors consists of the four closely related NFATc1, c2, c3, c4 and the distantly related NFAT5 protein [3]. In bone marrow-derived murine macrophages all four NFATc proteins are expressed. While both NFATc3 and c4 are permanently localized in the nuclei, an increase in intracellular Ca^{++} level results in the nuclear translocation of both NFATc1 and c2 and specifies them as putative targets of activation signals in macrophages [23]. Due to the two alternative promoters P1 and P2, two poly A addition sites and alternative splicing events the *Nfatc1* gene is expressed in numerous cell types as six alternative protein isoforms, which differ in their proliferative and apoptotic activities [24, 25]. Similar to NFATc2 which supports anergy and suppresses proliferation [3], the P2-directed NFATc1β isoforms exert antiproliferative activities while the P1-directed α-isoforms support the proliferation and survival of lymphocytes. In peripheral T- and B-lymphocytes immune receptor signals induce the switch from the synthesis of NFATc1β-isoforms directed by the constitutively active *Nfatc1* P2 promoter to the NFATc1α-isoforms directed by P1 [26].

The yeast *Candida albicans* is a frequent causal agent of opportunistic infections in immune compromised patients. However, *C. albicans* is able to escape murine macrophages upon infection through the growing of hyphae. Therefore we used *S. cerevisiae*, a rare pathogen in human [27], as a model organism to study opportunistic peritoneal infection in mice. Here, we show that in peritoneal macrophages NFATc1/β proteins are the most prominent NFATc1 factors, which are induced upon *S. cerevisiae* infection. Although NFATc1/β ablation did not affect the differentiation of ES cells and HSCs to monocytes/macrophages, in peritoneal resident macrophages NFATc1/β ablation inhibited the expression of CCL2 and related chemokines, impaired the recruitment of IMs, and delayed the clearance of peritoneal yeast infection.

Results

S. cerevisiae infection induces CN-dependent monocyte recruitment and chemokine expression

We used *S. cerevisiae* as a model organism to study opportunistic yeast infections in mice. The clearance of peritoneal *S. cerevisiae* infections was strongly affected by i.p. or s.c. administration of the CN inhibitors CsA and FK506 (Fig. 1A). This resulted in a decrease of accumulation of IMs ($Gr1^{hi}CD11b^{+}F4/80^{+}$), and CCL2 and CCL7 chemokines in peritoneal fluid (Fig. 1B), accompanied by a decrease in yeast-dependent induction of *Ccl2*, *Ccl7*, *Ccl12* mRNAs in $F4/80^{+}$ peritoneal cells (PECs, Fig. 1C and Supporting Information Fig. 1B and C). In contrast, the synthesis of *Ccl3*, *Ccl4*,

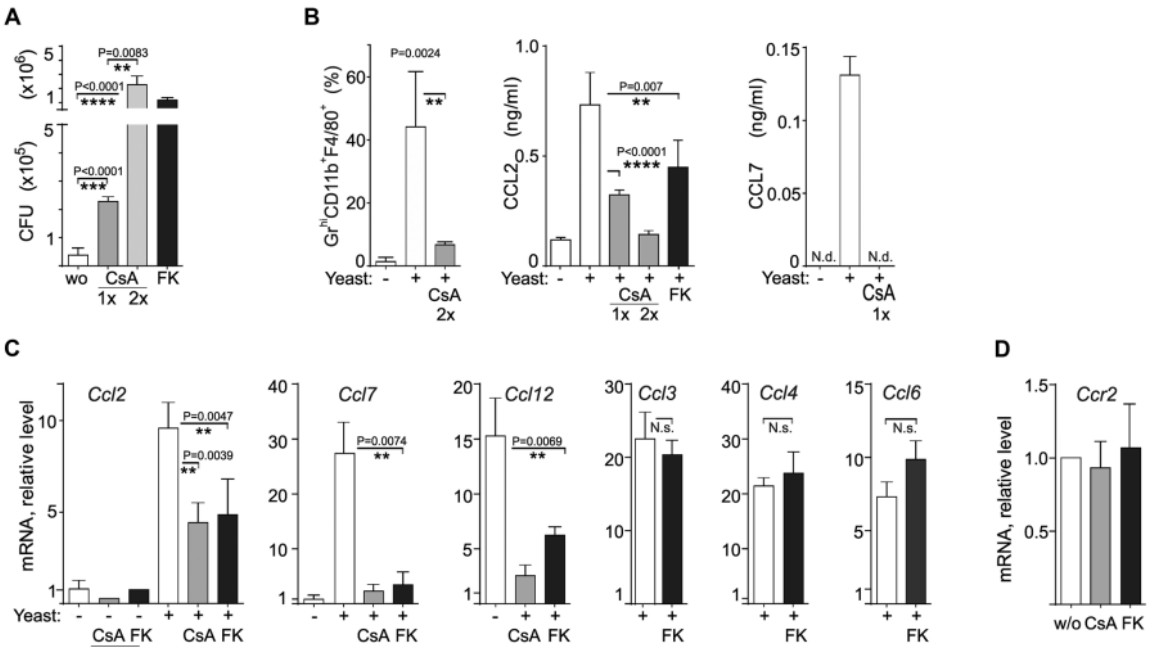

Figure 1. Peritoneal infection with *S. cerevisiae* triggers rapid CN-dependent recruitment of IMs and induction of chemokines in peritoneal macrophages. WT mice received CsA or FK506 treatment as a single (1×, 24 h before) or double (2×, 24 and 1 h) i.p. (FK506) or s.c. (CsA) injection before challenging i.p. with *S. cerevisiae*. (A) The CFU in peritoneal fluid 3 h after infection is shown. (B) The recruitment of IMs to the peritoneal cavity ($Gr1^{hi}CD11b^+F4/80^+$, left) was determined by flow cytometry, and the levels of unbound CCL2 (middle) and CCL7 (right) protein in peritoneal fluid 3 h after infection was determined by ELISA. (C) *Ccl2*, *Ccl7*, *Ccl12*, *Ccl3*, *Ccl4*, and *Ccl6* mRNAs in $F4/80^+$ PECs 3 h after infection was measured by real-time PCR analyses. (D) Peptone-elucidated $CD11b^+$ PECs were cultured for an additional 16 h in the presence or absence of CsA or FK506. *Ccr2* mRNA levels were measured by real-time PCR. (A–D) Data are shown as the mean + SD of three samples pooled from three (A–C) or two (D) independent experiments. Statistical significance was determined using unpaired Student's *t*-tests.

and *Ccl6* mRNAs remained unaffected by FK506. The expression of *Ccl2* transcripts in peritoneal $F4/80^+$ cells from unchallenged mice (Supporting Information Fig. 1B) and of CN-independent expression of *Ccr2* mRNA (Fig. 1D) indicated that peritoneal resident macrophages remain the major producers of chemokines responsible for CCR2/CCL2-dependent recruitment of IMs during yeast infection.

NFATc1 is a CN-sensitive factor induced by yeast infection in mouse peritoneal macrophages

To identify the CN-dependent factor(s) in peritoneal resident macrophages, we analyzed the activation of NFAT and NF-κB factors in these cells ex vivo. Stimulation of peritoneal resident macrophages with *S. cerevisiae* cells resulted in a rapid nuclear translocation of NFATc1 and RELA proteins, whereas NFATc3 and NFKB2 proteins remained cytosolic (Fig. 2A and Supporting Information Fig. 3). NFATc2 expression/translocation was detected only in a minor fraction of $F4/80^+$ cells. The time course of NFATc1 and RELA translocation indicated a transient translocation of NFATc1 followed by a persistent nuclear residence of RELA (Fig. 2A). While the nuclear translocation of NFATc1 was inhibited by CsA and FK506 (Fig. 2B and Supporting Information Fig. 3B), the translocation of RELA remained unaffected by CsA, and was even induced by FK506 alone, as described before [28]. Correspondingly, neither the expression of CCL2, nor the clearance of infection was affected in NFKB1-deficient mice (Supporting Information Fig. 2). From these data we concluded that NFATc1 proteins, which could be expressed as six alternative isoforms [26], are candidates for CN-dependent factors that control the yeast-mediated induction of CCR2 agonists in peritoneal resident macrophages.

Predominant expression of NFATc1/β isoforms in the myeloid lineage and in peritoneal macrophages

To elucidate the stage-specific expression of *Nfatc1* gene during myeloid cell development we studied the appearance of individual NFATc1 isoforms in hematopoietic progenitor cells. Both in hematopoietic stem cells, during hemopoietic differentiation of ES cells and in $F4/80^+$ PECs the expression *Nfatc1* gene was driven by the P2-promoter (Fig. 3A–C), and yeast infection resulted in an increase of P2-, but not P1-directed transcripts in $F4/80^+$ cells (Fig. 3D). This is in marked contrast to the *Nfatc1* gene

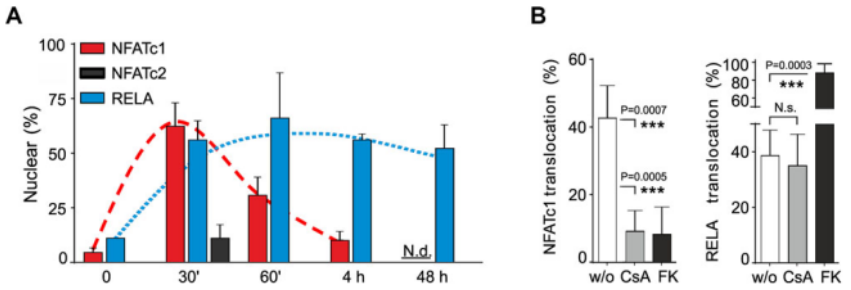

Figure 2. NFATc1 is a CN-sensitive factor induced by yeast infection in mouse peritoneal macrophages. (A) Mice were infected with *S. cerevisiae* and peritoneal F4/80$^+$ macrophages were isolated for ex vivo quantification of nuclear NFATc1, NFATc2, and RELA by confocal microscopy. N. d.: not detected. (B) Quantification of nuclear translocation of NFATc1 and RELA in peritoneal resident macrophages after 30 min of yeast infection in the presence of CsA or FK506. Data represent mean + SD of two experiments. More than 100 cells were analyzed per staining (see Supporting Information Fig. 2 for staining examples). Statistical significance was determined by using unpaired Student's *t*-tests.

expression in naïve splenic CD4$^+$ T cells in which P1- and P2-directed transcripts are generated at the same levels (Fig. 3E). To consolidate these findings at the protein level, we investigated NFATc1 expression in cells from *Nfatc1-Ex3-dsRed* knock-in (KI) mice.

In the Supporting Information Figure 4, the construction of *Nfatc1-Ex3-dsRed* knock-in (KI) mice is described. In these mice, the exon3 of *Nfatc1* gene is fused with a DNA cassette encoding monomeric dsRed protein. This leads to the generation of chimeric NFATc1-dsRed proteins, which enables a direct quantification of NFATc1/α and β protein expression. Using these mice, we observed a more than 40-fold higher expression of NFATc1/β-dsRed protein in F4/80$^+$ cells (Fig. 3F) indicating that in peritoneal resident macrophages NFATc1 protein is expressed as a nearly equimolar blend of NFATc1/βA, βB, and βC isoforms.

Ablation of NFATc1/β proteins results in delayed clearance of *S. cerevisiae* peritoneal infection

To ablate NFATc1/β expression, we crossed *Nfatc1-P2*$^{flx/flx}$ mice (Supporting Information Fig. 4A) with *CMV-cre* mice for the deletion of P2 promoter and of exon2 sequences in all cells. *Nfatc1P2*$^{\Delta/\Delta}$ mice (called here as *Nfatc1P2*Δ mice) were born at the expected Mendelian ratios. They were viable and indistinguishable from their *Nfatc1P2*$^{\Delta/flx}$ or *Nfatc1P2*$^{flx/flx}$ littermates, or from mice with the *LysM-cre*-mediated deletion of P2+*exon2* sequences in myeloid cells only (*Nfatc1P2*$^{flx/flx}$*LysM-cre*). Immunoblots confirmed the expected decrease of the NFATc1 expression in peritoneal resident macrophages isolated from *Nfatc1P2*Δ mice (Fig. 3G). The phenotypes of HSCs, multipotent progenitors, common myeloid progenitors, osteoclast progenitors, and monocytes in BM did not reveal any significant difference between *Nfatc1P2*Δ mice and their WT littermates (Supporting Information Fig. 5). The numbers of *Nfatc1P2*Δ peritoneal resident macrophages and their phagocytic and antigen-presenting properties remained unaffected. However, after i.p. yeast infection, *Nfatc1P2*Δ mice showed a significantly delayed clearance of pathogens (Fig. 4A), reduced recruitment of IMs (Fig. 4B), and decreased secretion of CCL2 in the peritoneal cavity (Fig. 4C). While the basal *Ccl2* mRNA levels in peritoneal resident macrophages were similar in WT and *Nfatc1P2*Δ mice, yeast infection resulted in a significantly reduced induction of *Ccl2* mRNA in *Nfatc1P2*Δ mice (Fig. 4D). However, it did not affect the transcription of CN-independent *Ccl3*, *Ccl4*, *Ccl6*, and *Ccr2* genes (Fig. 4E). Expression of a constitutively active version of NFATc1 in Tg *Rosa26-caNfatc1xCMV-cre* mice was sufficient to rescue *Ccl2* expression and the clearance of infection in NFATc1/β-deficient mice (Fig. 4F). This indicated that upon yeast infection the induction of CCL2 in peritoneal resident macrophages is controlled by NFATc1. And indeed, forced expression of NFATc1 proteins was sufficient to activate transcription of endogenous *CCL2* and *CCL8* (but not *CCL1*, *CCL7*, or *CCL11*) genes and to induce secretion of CCL2 protein (Fig. 5A and B) in human HEK293T cells. Tg expression of constitutively active version of NFATc1/A in *Rosa26-caNfatc1xCMV-cre* mice (Fig. 5C) was sufficient to activate *Ccl2* gene transcription and CCL2 protein production in bone marrow-derived murine macrophages cells. ChIP assays demonstrated the binding of NFATc1 to the *Ccl2* locus in J774 macrophages in which a large part of NFATc1 is located in the nucleus (Fig. 5D).

The *Ccl2* gene is a direct target of NFATc1/β proteins in macrophages

Inspection of the 5′ sequences of human and mouse *Ccl2* genes and EMSA analyses revealed multiple conserved NFAT-binding sites (Supporting Information Fig. 6A and B), which overlap with well-characterized NF-κB regulatory elements. In transient transfections, coexpression of NFATc1 (and of RELA/NFKB1 but not of RELB/NFKB2) strongly transactivated a luciferase reporter construct spanning the upstream region of the *Ccl2* gene (+77/−3009). While progressive truncation of 5′ region resulted in a decrease of RELA/NFKB1-mediated transactivation, the proximal *Ccl2* promoter region (−313/+77), but not a *Ccr2* promoter,

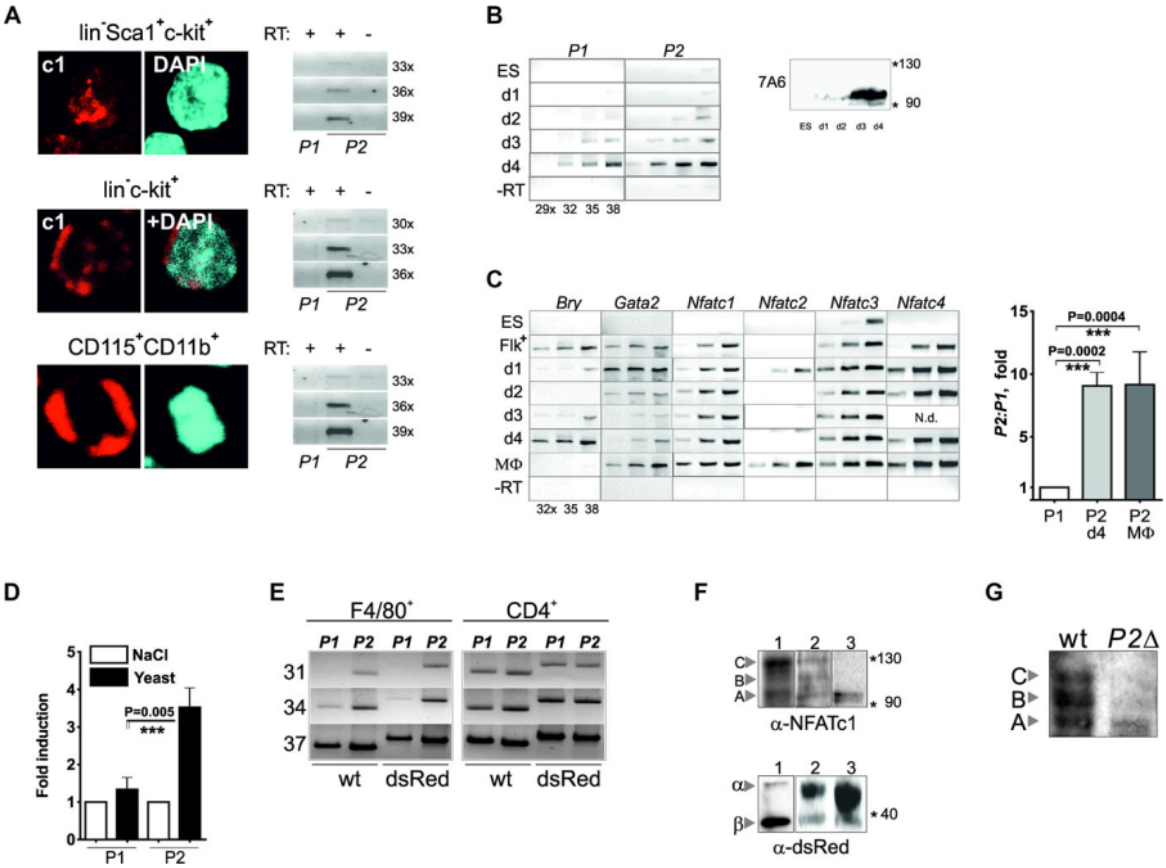

Figure 3. Predominant expression of NFATc1/β isoforms in the myeloid lineage and in peritoneal resident macrophages. BM cells were isolated from WT mice and sorted by flow cytometry into HSC (lin−Sca1+c-kit+), BM progenitor (lin−c-kit+), and monocyte (CD115+CD11b+) populations. (A) Representative immunofluorescence stainings for NFATc1 expression (left) and RT-PCR analyses of *Nfatc1* P1- and P2-directed transcripts (right) in HSC, BM progenitors, and monocytes. (B) Induction of *Nfatc1* transcripts (left) and immunoblot analyses (right) during formation of embryoid bodies from WW6 ES cells. Equal loading of immunoblots was shown by Ponceau staining. (C) Induction of *Nfatc1* mRNA during hemopoietic differentiation of *GFP-Bry* ES cells (left) and quantification of P1- and P2-transcripts on d4 of blast culture (right). (D) Induction of P2-directed transcripts in peritoneal resident macrophages 1 h after yeast infection. (E) RT-PCR analyses of *Nfatc1* P1- and P2-directed transcripts in peritoneal resident macrophages and in naïve splenic CD4+ T cells from WT and *Nfatc1-Ex3-dsRed* KI mice. (F) Above, immunoblot analyses of NFATc1 expression in freshly isolated peritoneal resident macrophages (lane 1), after in vitro cultivation for 7 days (2) and in naïve splenic CD4+ T cells (3). The positions of NFATc1/A, NFATc1/B, and NFATc1/C isoforms are indicated. Below, expression of NFATc1/α and NFATc1/β isoforms in peritoneal resident macrophages (1) and in naïve (2) or stimulated (anti-CD3+CD28, 24 h) splenic CD4+ T cells (3) from *Nfatc1-Ex3-dsRed KI* mice. Positions of NFATc1/α- and -β-dsRed fusion proteins are indicated. (G) Immunoblots of NFATc1 expression in peritoneal resident macrophages isolated from WT and *Nfatc1-P2Δ* littermate mice. NS: nonspecific protein band. (A–G) Data shown are representative of three independent quantification experiments and (C, D) are shown as mean + SD of three samples. Statistical significance was determined by using unpaired Student's *t*-tests.

was still transactivated fivefold by NFATc1/β, i.e. more than the *IL2* promoter under the same conditions (Supporting Information Figs. 6A and 7). Together these data indicated that *Ccl2* is a direct NFATc1 target gene in macrophages.

NFATc1/β proteins release the BCL6-dependent repression of the *Ccl2* gene in macrophages

The induction of *Ccl2* transcription is under the tight control of BCL6-dependent repression. The proximal promoter region of the *Ccl2* gene contains three potential NFAT motifs (Fig. 6A) that overlap with a single, evolutionary conserved BCL6-binding site (at position −157/−148) [16]. Correspondingly, the basal NFAT- and REL/NFKB1-dependent luciferase activities of the proximal *Ccl2* promoter (−331/+77) reporter were strongly suppressed by BCL6 (Fig. 6B). The NFATc1/αC- and NFATc2-induced activities of a reporter controlled by three NFAT sites, and the RELA/NFKB1-induced activity of a reporter controlled by seven NF-κB sites were also repressed by BCL6. Colocalization of NFATc1 and BCL6 in J774 macrophages (Fig. 5D) suggested an interaction between both proteins. In addition, the prototypical "M0"-type

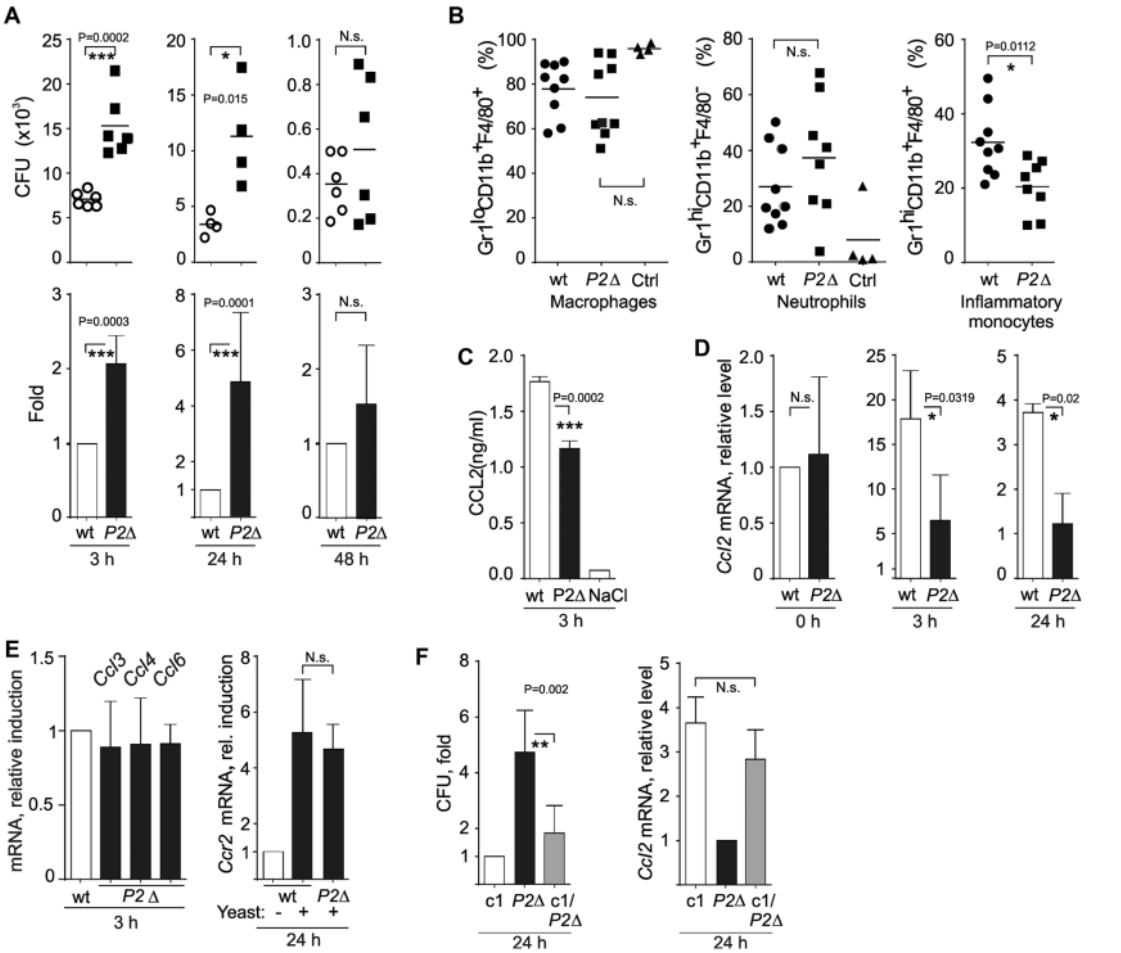

Figure 4. NFATc1/β deficiency results in the delayed clearance of peritoneal yeast infection, impaired recruitment of IMs, and decreased expression of CCL2. WT and *Nfatc1*P2Δ littermates were challenged with i.p. yeast infection and peritoneal fluid was analyzed at the time indicated. (A) Numbers of yeast CFU in peritoneal fluid 3, 24, and 48 h after infection, single experiment (above) and compilation (below). (B) Composition of peritoneal myeloid cells 24 h after infection, in comparison with noninfected WT and *Nfatc1*P2Δ littermates (Ctrl) was determined by flow cytometry. (C) Unbound CCL2 protein in peritoneal fluid 3 h after infection was measured by ELISA. (D) *Ccl2* mRNA in F4/80$^+$ cells from unchallenged mice (left), 3 and 24 h after infection (middle and right, respectively) was determined by RT-PCR. (E) *Ccl3*, *Ccl4*, *Ccl6*, and *Ccr2* mRNAs in F4/80$^+$ cells 3 or 24 h after infection was measured by RT-PCR. (F) Relative amount of yeast CFU in peritoneal fluid (left) and *Ccl2* mRNA levels in F4/80$^+$ cells isolated from *Rosa26-caNfatc1*$^+$*CMV-cre*$^+$*Nfatc1P2*$^{\Delta/wt}$ (c1), *Rosa26-caNfatc1*$^-$*CMV-cre*$^+$*Nfatc1P2*Δ (P2Δ), and *Rosa26-caNFATc1*$^+$*CMV-cre*$^+$ *Nfatc1P2*Δ(c1/P2Δ) littermates 24 h after yeast infection. (A, B) Each symbol represents an individual mouse and (A–F) data are shown as mean + SD of three samples pooled from (A–D) three or (E, F) two independent experiments with two to eight mice per group. Statistical significance was determined by using unpaired Student's *t*-tests.

BCL6-binding motif [29] shows a striking homology to the NFAT consensus motif (Supporting Information Fig. 8). In EMSA experiments both NFATc1/βC and BCL6 bound to a probe from the *Ccl2* promoter spanning the nucleotides 157/−148 (Fig. 6C). While mutations within the BCL6 motif [16] abolished not only BCL6 but also NFATc1/βC binding, mutations within the putative NFAT motifs 1 and 2 only slightly affected NFATc1/βC, but abolished any BCL6 binding. This indicated that both proteins compete for the binding to the same critical BCL6 motif [16]. Oligonucleotide pull down assays indicated that BCL6 can recruit endogenous NFATc1 protein, which is expressed in HEK293T cells as a single NFATc1/A isoform (Fig. 6D and E). Finally, in ChIP assays we observed that upon peritoneal yeast infection the binding of BCL6 to the *Ccl2* promoter in peritoneal resident macrophages is gradually replaced by NFATc1 and RELA (Fig. 6F). This indicates that yeast infection triggers NFATc1-dependent release of BCL6-mediated repression of *Ccl2* gene expression in peritoneal resident macrophages.

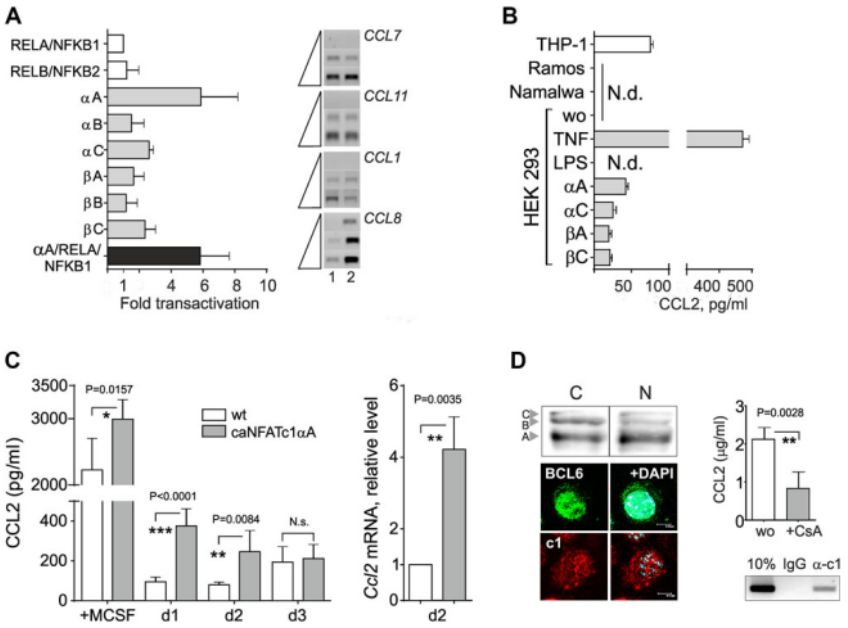

Figure 5. The *Ccl2* gene is a direct transcriptional target of NFATc1 in macrophages. Transactivation of endogenous *CCL2* gene in HEK293T cells. (A) On left, HEK293T cells were transfected with the indicated expression vectors, and mRNA was isolated to measure expression of *CCL2* by RT-PCR. Levels have been normalized to the expression of *GAPDH* mRNA. On right, HEK293T cells were transfected with the empty vector (lane 1) or with NFATc1/αA expression vector (lane 2), and mRNA was isolated to measure expression of *CCL7*, *CCL11*, *CCL1*, and *CCL8* mRNAs by RT-PCR.). (B) Human monocytic THP-1 cells, Ramos and Namalwa Burkitt lymphoma cells and HEK293T cells were induced with TNFα or LPS for 24 h or cultivated without the treatment. In addition, HEK293T cells were transfected with indicated expression vectors and further cultivated for 40 h without stimulation. CCL2 secretion was analyzed by ELISA and normalized in respect to transfection efficiency (60–85%, according flow cytometry of eGFP expression). N.d.: not detectable. (C) BM macrophages were derived from unchallenged *Rosa26-caNfatc1xCMV-cre* and WT littermate mice. *Ccl2* expression was analyzed after 3 days of culture in the presence of M-CSF and at days 1–3 after M-CSF withdrawal. On the right, expression of *Ccl2* RNA was determined in bone marrow-derived murine macrophages cells at day 2 after M-CSF withdrawal. (D) Left top, cytosolic (C) and nuclear (N) protein extracts were prepared from J774 macrophage cells and analyzed by immunoblotting using antibody directed against NFATc1. Positions of NFATc1/A, NFATc1/B, and NFATc1/C isoforms are indicated. Left bottom, representative immunofluorescence stainings of J774 cells using antibodies directed against BCL6 and NFATc1. Right above, J774 cells were cultivated for 24 h in the presence or absence of CsA. CCL2 secretion was determined by ELISA. Right below, ChIP assay. Chromatin of J774 cells was cross-linked with formaldehyde and after purification immunoprecipitated with control antibody (IgG) or antibody directed against NFATc1 (α-c1). NFATc1 binding to the distal regulatory region of *Ccl2* gene was detected using PCR. (A–E) Data are shown as the mean + SD of three samples pooled from three (A, B) or two (C–E) independent experiments. Statistical significance was determined by using unpaired Student's *t*-tests.

Discussion

In this study, we show that the CN inhibitors CsA and FK506 facilitate peritoneal fungal infections through the blockage of CCL2/CCR2-dependent recruitment of IMs. Monocytes and macrophages are a major source of CCL2 [30–32]. In peritoneal resident macrophages, yeast infections induce the coordinated activation of *Ccl2*, *Ccl7*, and *Ccl12* genes that encode CCR2 ligands and execute the regulation of CCL2/CCR2 axis [33, 34]. The transcription of these genes, the subsequent recruitment of IMs, and the clearance of infection are sensitive to CsA and FK506. Consequently, our study revealed a novel mechanism of proposed link [6] between the extensive usage of these immunosuppressants in transplantation medicine and the increase in opportunistic fungal infections in patients with a compromised immune system [35].

Our results show that NFATc1/β proteins are critical CsA/FK506-sensitive factors within the dectin-1 pathway. Albeit the effect of NFATc1/ß ablation is less dramatic than upon CsA/FK506 treatment, inactivation of NFATc1/ß expression resulted in a delayed clearance of yeast infection (Fig. 4). This might be due to the strong pleiotropic activity of these CN inhibitors. However, in contrast to the situation in T cells [4, 5], the translocation of RELA protein remained insensitive to CsA/FK506 in peritoneal resident macrophages, and the noncanonical NF-κB pathway was not activated by yeast infection. These data are consistent with the normal clearance of peritoneal yeast infection in mice deficient for RELA.

In the absence of NFATc1/β, the decreased expression of CCL2, CCL7, and CCL12 in peritoneal resident macrophages impeded the subsequent recruitment of IMs and led to a delayed clearance of fungal infection. We show that nuclear translocation of NFATc1 is

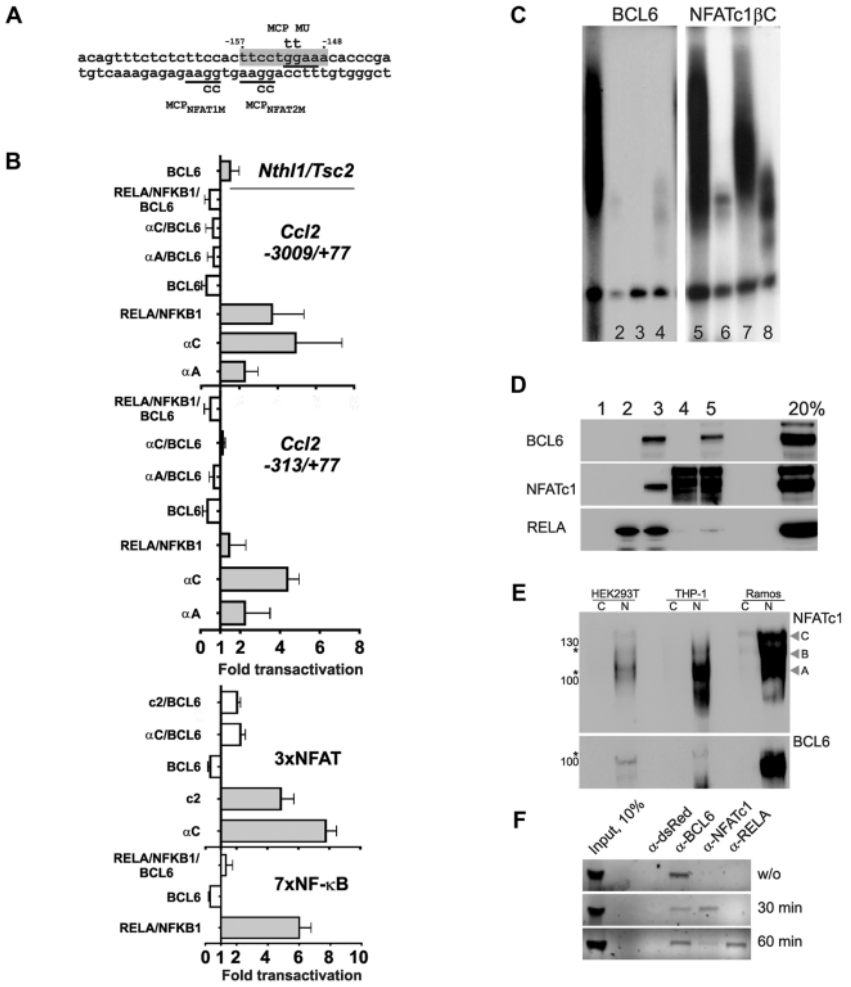

Figure 6. Yeast infection triggers NFATc1-dependent release of BCL6-mediated repression of *Ccl2* gene in peritoneal resident macrophages. (A) Sequence of the evolutionary conserved BCL6-binding site (shaded) within the *Ccl2* promoter. Three potential NFAT-binding motifs are underlined. Mutations abolishing BCL6 binding (MCP MU) [16] and mutations within the potential NFAT motifs (MCP_{NFAT1M} and MCP_{NFAT2M}) are indicated. (B) HEK293 cells were transfected with luciferase reporters driven by the complete or proximal *Ccl2* regulatory region, *Nfhl1/Tsc2* bidirectional promoter, three copies of NFAT-binding site or seven copies of the NF-κB binding site, alone or together with indicated combinations of expression vectors. Luciferase activities were determined after 36 h and presented relative to those of cells transfected to express the luciferase reporter alone, set as 1. (C) HEK293T cells were transfected with vectors expressing BCL6 (lanes 1–4) or NFATc1/αC (lanes 5–8) and then incubated with MCP WT probe (lanes 1, 5), MCP_MU (2, 6), $MCP_{NFAT1M+NFAT2M}$ (3, 7), or $MCP\_MU_{NFAT1m+NFAT2M}$ probes (4, 8). EMSA analyses were performed to assess NFATc1/αC and BCL6 binding. (D) HEK293T cells were transfected with empty vector (lane 2) or vectors encoding expression of BCL6 (lane 3), NFATc1/αC (lane 4), or BCL6+NFAT/αC (lanes 1 and 5). Whole cell protein extracts were prepared and incubated with a biotinylated MCP WT probe, immobilized on streptavidin beads (lane 1: empty beads). After extensive washing, the proteins were released and analyzed in immunoblots using indicated antibodies. (E) Cytosolic (C) and nuclear (N) protein extracts were prepared from HEK293T, THP-1, and Ramos cells. Immunoblot analyses were performed using antibodies directed against NFATc1 and BCL6. (F) ChIP assays. PECs were isolated from control mice (w/o) or 30 or 60 min after yeast injection. Chromatin was immunoprecipitated with the antibodies indicated and amplified with primers specific for the distal regulatory region of *Ccl2* gene. Input, PCR amplification of total chromatin before immune precipitation. (B–F) Data shown are representative of (B) five or (C–F) two independent experiments and shown as the mean + SD. Statistical significance was determined by using unpaired Student's *t*-tests.

sufficient to release the BCL6-mediated repression of *Ccl2* gene transcription in vitro and in vivo and, reciprocally, BCL6 can directly repress NFAT- and NFKB1/RELA-dependent transcription. Competition between NFATs and BCL6 for a binding to a subset of BCL6-controlled regulatory elements specifies a novel cross-talk between transcription factors with zinc-fingers and CN-dependent regulatory domains. In addition to macrophages, NFATs and BCL6 are coexpressed in nuclei of germinal center B and T_{FH} cells, and in

Burkitt lymphoma cells suggesting that this interaction is involved in normal and malignant B-cell development ([36, 37], and our unpublished data).

The role of CN/NFAT signaling pathway in innate immunity was mainly investigated using CN inhibitors and/or by overexpression of a constitutively active version of CN-B (PPP3CB) [6, 38, 39]. Certainly informative, this approach neglected the individual properties of four related but different CN-dependent NFATc factors that, in addition, are expressed as multiple protein isoforms in a cell-type specific manner. The phenotype of *Nfatc1 P2*Δ mice indicates that the NFATc1/β isoforms are dispensable for the critical role of NFATc1 during embryonic heart development [40], for the generation of peritoneal B1a cells [41], and for the homeostasis of adaptive immune system.

Throughout the embryonic development, *Nfatc1* transcripts remain undetectable at the preimplantation stages and in undifferentiated ES cells. Myeloid differentiation of ES cells predominantly activates the P2 promoter and induces expression of NFATc1/β proteins. In BM HSCs from adult mice mainly P2-directed *Nfatc1* transcripts are detectable. The nuclear localization of NFATc1 suggests involvement in maintenance and/or self-renewal of HSCs in their niche. In agreement with the phenotype of *Nfatc1 P2*Δ mice, cytosolic localization of NFATc1 in hematopoietic progenitors, in neutrophils [6], monocytes, and nonstimulated macrophages indicates that NFATc1 plays a minor, if any, role in a transcriptional control of myeloid development. The consequences of β-glucan/dectin-1/CN-triggered nuclear translocation of NFATc1 are cell-type specific. In neutrophils, conditional deletion of *CnB* resulted in a decreased sensitivity to disseminated *C. albicans* infection [6], likely because of defects in killing fungi. In macrophages, NFATc1/β deficiency did not affect phagocytotic capacity but, because of delayed and reduced level of CCL2 expression, compromised their ability to recruit IMs required for efficient clearance of infection.

CCL2 is produced by many cell types, including epithelial and endothelial cells, fibroblasts, astrocytes, microglia, and others [13], either constitutively or after induction by cytokines, oxidative stress, or other stimuli, which typically activate NF-κB signals [42]. Transcriptional profiling of macrophages from different tissue locations [30, 31] has confirmed a high heterogeneity of tissue-resident macrophages suggesting that identical physiological stimuli might activate alternative signaling pathways in different subpopulations of resident macrophages. Delayed induction of CCL2 expression during peritoneal yeast infection indicates that the NFATc1/β isoforms play a major role at early stages of derepression and/or activation of *Ccl2* transcription that remains largely controlled by canonical NF-κB signals. This mechanism is reminiscent of gradual replacement of rapidly activated NFKB1/RELA dimers with slowly activated NFKB2/RELB proteins [43] on the *Ccl22* promoter during stimulation of DCs by LPS.

Taken together, our study identified a novel mechanism that facilitates opportunistic fungal infections in immunocompromised patients. At the molecular level, we elucidated a novel cell-specific function of NFATc1/β isoforms as triggers for the release of BCL6-dependent transcriptional repression. The interplay between NFATc1 and BCL6 defines a novel molecular target for the design of cell-type specific antagonists [44] that control the CCL2/CCR2 axis in chronic inflammations. Inhibitors of interaction between both factors could provide alternatives to the current therapy regimens in transplantation medicine.

Materials and methods

Mice and cells

C57BL/6 mice were maintained in the Central Animal Facility of Medical Faculty, University of Wuerzburg, according to the institutional guidelines (acceptance AKZ 55.2-2531.01-80/10 from October 22, 2010) and used at 8–18 weeks of age. In in vivo experiments, mice were injected i.p. with 1×10^7 *S. cerevisiae* cells. For inhibitor studies, mice received s.c. injections of CsA (Sandimmune, 100 mg/kg) once (1 h before) or twice (12 and 1 h) before yeast injection, or a single i.p. injection of FK506 (10 mg/kg) 1 h before yeast injection. Mouse PECs were extracted by flushing the peritoneal cavity with ice-cold PBS/0.1% BSA. For the isolation of macrophages, 1×10^5 PECs were seeded in 1 mL of complete medium (DMEM/10% FCS/antibiotics) per well. Nonadherent cells were removed after cultivation for 4 h with complete medium. Adherent macrophages were incubated overnight. For RNA isolation, macrophages were purified from PECs using biotinylated F4/80 antibody and streptavidin-coated magnetic beads (Miltenyii Biotech). BM cells were isolated from the femurs and tibias of the hind legs.

Generation of *Nfatc1-Ex3-dsRed* knock-in mice

For the generation of targeting vector (Supporting Information Fig. 4A) the short and long arms of *Nfatc1* gene homology were isolated from BAC bMQ222j17 (Source Bioscience) as Bsu15I restriction fragments (spanning 6 and 9 kb) and cloned into the pBSKSII vector. The coding sequence of dsRed (*Discosoma species*) monomeric far-red fluorescent protein was amplified by PCR on the template of pLVX-DsRed-Monomer-C1 (Clontech). After addition of two SV40 termination signals, DNA was ligated as a fusion between Ser_{72} within the third exon of *Nfatc1* gene (common for all *Nfatc1* isoforms) and Ile_7 codon of *dsRed* coding sequence (further cloning details are available on request). All constructs were verified by sequencing. The final targeting vector was assembled in a pKSTKLOXP plasmid and after linearization with ScaII introduced by electroporation into JM8 ES cells followed by selection with G418 (300 μg/mL). ES cell culture was performed as described [45]. The DNA isolated from resistant ES cell clones was digested with BamHI or SdaI and tested for homologous recombination by Southern blot analysis with PCR-amplified and $\alpha-P^{32}$-dCTP-labeled 5′ and 3′ integration specific probes. Selected clones were reanalyzed by Southern blotting with a *neo*-specific

probe to exclude additional random integration events. Two independent ES clones were injected into E3.5 C57BL/6N blastocysts to generate chimeras. Germ-line transmission of the mutated allele was confirmed by the integration-specific long-distance PCR using primers specific for WT and knock-in alleles (Supporting Information Fig. 4B). Routine genotyping was performed with primers specific for the *exon3* of *Nfatc1* gene and *dsRed* sequences.

Cell culture, transient transfections, and reporter gene assays

J774, L929 cells (kindly provided by T. Rudel and M. Lutz, University of Wuerzburg, respectively) and HEK293T cells were maintained in DMEM (Gibco) supplemented with 10% FCS and 100 U penicillin/100 μg streptomycin (Gibco). Primary murine lymphoid cells were cultivated in RPMI-1640 supplemented with 10% FCS, antibiotics and, 50 μM β-ME (Gibco). HEK293T and L929 cells were transfected using polyethylenimine. Luciferase activities were measured in whole cell extracts 34–36 h after transfection (LUMIstar Omega), equalized for transfection efficiencies upon flow cytometry of eGFP expression or according to β-galactosidase activity of cotransfected RSV-β Gal expression plasmid.

Differentiation of ES cells in vitro

Generation of embryoid bodies from WW6 ES cells was performed as described [46]. For hemopoietic differentiation embryoid bodies were generated from *GFP-Bry* ES cells. For blast cultures, hemangioblast cells (GFP^+Flk1^+) were isolated and cultivated for 4 days as described [47].

DNA constructs

Vectors for the expression of NFATc2, BCL6, NFKB1, NFKB2, RELA, and RELB in eukaryotic cells, as well as *Nfat-*, *NF-κB-*, and *Il2* promoter-driven luciferase reporter constructs were published previously. For the construction of NFATc1 expression vectors cDNAs were synthesized from RNA purified from human peripheral blood monocytes. Individual *Nfatc1* isoforms were amplified with *Pwo* DNA polymerase (PeqLab) using isoform-specific primers (Supporting Information Table 2) and, after digestion with XbaI and BamHI, cloned into the pLVX-IRES-ZsGreen1 vector (Clontech). For the construction of *Ccl2* and *Nthl1/Tsc2* promoter-reporter constructs specific genomic regions were amplified with the long-distance PCR mix (Thermo) on murine genomic DNA template with specified primers. After digestion with MluI and BglII the DNA fragments were cloned into the pGL3-basic vector (Promega). For construction of bacterial GST-BCL6 expression vectors, corresponding domains of *Bcl6* cDNA were amplified on the template of pMSCV-BCL6-IRES-GFP [48] and after digestion with BamHI and EcoRV were cloned in-frame with *Gst* coding sequence into the pGEX-3X vector (GE Healthcare). All DNA constructs were verified by complete sequencing of inserted fragments.

Macrophage culture and stimulation

Peritoneal macrophages were maintained in DMEM supplemented with 10% FCS (Gibco) and 100 U penicillin/100 μg streptomycin (Gibco). For fungal stimulation heat-inactivated *S. cerevisiae* were added in a twofold excess to macrophages and incubated for the time periods indicated. For inhibitory studies macrophages were preincubated for 30 min with FK506 (10 μg/mL, Sigma-Aldrich), CsA (5 μg/mL Calbiochem), or DMSO as a solvent control. For the phagocytosis assays heat-inactivated, FITC-labeled *S. cerevisiae* cells were incubated with macrophages in a ratio of 10:1 yeast cells/cell in a 24-well plate. The plates were washed twice with medium to remove unbound yeast. Macrophages were harvested by scraping and analyzed by FACS. For the antigen-presentation assay, $CD4^+$ T cells were isolated from the spleens of OTII Tg mice using CD4 (L3T4) microbeads (Miltenyii Biotech) and labeled using the CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes). Macrophages (1×10^5 cells) were loaded with 1 μg/mL $OVA_{323–339}$ peptide (Sigma-Aldrich) for 1 h in RPMI-1640 supplemented with 10% FCS, 100 U penicillin/100 μg streptomycin and 50 μM β-ME (Gibco). A total of 1×10^5 CFSE-labeled T cells were added to the macrophage in a flat 96-well plate. After 3 days the T cells were harvested and analyzed by flow cytometry.

Yeast culture

Saccharomyces cerevisiae cells were cultivated in YPD medium supplemented with ampicillin (100 μg/mL). To determine the number of CFUs serial tenfold dilutions of peritoneal fluid from yeast-injected mice were plated on YPD plates and incubated at 30°C overnight. Yeast colonies were counted and the numbers of yeast cells per mouse were calculated. For ex vivo experiments heat-inactivated yeast cells were labeled with FITC using the EZ-Label™ protein Labeling Kit (Thermo Scientific).

Immunofluorescence staining

For immunofluorescence stainings, glass slides with attached macrophages were washed and fixed in 3% formaldehyde. After permeabilization in 0.1% Triton-X 100 the cells were incubated with primary and secondary antibodies. The cells were mounted in fluoroshield including DAPI (Sigma-Aldrich). The pictures were taken with a Leica Confocal microscope TCS SP5 II and analyzed with the Image Pro Plus software (Leica). For further demonstration purposes the digital images were processed with Photoshop (Adobe).

Antibodies, staining reagents, and flow cytometry

The antibodies used are compiled in Supporting Information Table 1. Flow cytometry data were acquired on a BD Canto II, and they were analyzed with FlowJo software package (Tri-Star).

Quantitative real-time PCR and RT-PCR

Total RNA was prepared with TRIzol reagent (Invitrogen) or the RNeasy Mini Kit (Qiagen). cDNA was synthesized with the "First Strand Synthesis Kit" (Thermo Scientific) using random primers. Quantitative real-time PCR was performed on the ABI 7000 real-time PCR machine with Power SYBR Green PCR master mix (Applied Biosystems) and appropriate primers (see Supporting Information Table 2). For semiquantitative RT-PCR amplification products were separated on agarose gels and quantified using gel analysis software (BioRad).

Protein extracts, pull-down assays and EMSAs

For the preparation of whole cell protein extracts, the cells were harvested and washed with ice-cold PBS buffer. Proteins were extracted with 4 packed cell volumes of RIPA buffer (50 mM Tris, pH 7.5, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 1 mM DTT) supplemented with protease inhibitor cocktail (HALT, Thermo Scientific). After three freeze-thaw cycles the lysates were clarified by centrifugation and protein quantity in supernatants was determined using Bradford's reagent (BioRad).

For DNA pull-down studies 5′-biotinylated double-stranded oligonucleotides were coupled to streptavidin-agarose beads (Thermo Scientific) in TEN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). Nonspecific binding was blocked by 30 min incubation in TEN buffer supplemented with 0.5 mg/mL of BSA and 200 μg/mL of denaturated salmon sperm DNA. The beads were washed for three times with TEN buffer. For binding reaction 1 mg of protein extract was preincubated for 30 min with 20 μg of polydI/dC at 4°C in 15 mM HEPES (pH 7.8), 50 mM NaCl, 75 μM $ZnSO_4$, 0.1 mM EDTA, 0.1 mM EGTA, 0.01% NP40, 0.1 mM DTT, 10% glycerol (final concentrations), and clarified by centrifugation (15 min at 13 000 rpm). Supernatant was combined with oligonucleotide-coated beads and incubated for 1 h with constant rotation. The beads were washed four times with buffer A/10% glycerol, boiled in Laemmli sample buffer and released proteins were subjected to immunoblotting.

For EMSAs γP^{32}-labeled double-stranded oligonucleotides were incubated with 5 μg of nuclear protein and 1 μg of polydI/dC for 30 min on ice in 20 mM HEPES (pH 7.9), 50 mM KCl, 1mM EDTA, 0.75 mM $ZnSO_4$, 1 mM DTT, 4% Ficoll [49]. DNA–protein complexes were resolved on 5% native PAA gels in 0.4× TBE buffer. The gels were fixed, dried, and exposed to X-ray films.

Immunoblotting

Western blots were performed by separation of indicated protein extracts on PAGE-SDS gels followed by immunodetection. Signals were developed by chemiluminescence detection using ECL Western Blotting Substrate (Thermo Scientific), captured and analyzed with the Fusion-SL gel imaging system (Vilber Lourmat). Equal loading of immunoblots was confirmed by Ponceau S staining.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described [50]. In brief, the chromatin from 5 × 10^7 J774 macrophages was cross-linked with 1% formaldehyde and the reaction was quenched with 125 mM glycine. Nuclei were isolated, sonicated, and after preclearing with protein G–Sepharose (Pierce) blocked with salmon sperm DNA (200 μg/mL) and BSA (0.5 mg/mL). Protein–DNA complexes were immunoprecipitated using anti-NFATc1 and an unrelated mouse IgG antibody as control. After washing, elution, and reversion of cross-links, DNA was isolated by phenol/chloroform and used in PCRs.

ELISA

Expression of CCL2 and CCL7 in the peritoneal fluid of yeast-injected and control mice and the secretion from human cell lines was measured with an enzyme-linked immune sorbent assay (mouse Ccl2 ELISA Ready SET Go, MCP-3 Instant ELISA, and human CCL2 ELISA Ready SET Go kits (eBioscience)).

Statistical analysis

Student's *t*-test or the Mann–Whitney U test was used for statistical analysis with the software GraphPad Prism 6. *p* values above 0.05 were considered as not significant.

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Abbreviations: **CN**: calcineurin · **CsA**: cyclosporin A · **IM**: inflammatory monocytes · **PEC**: peritoneal cell

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