TCAIM Decreases T Cell Priming Capacity of Dendritic Cells by Inhibiting TLR-Induced Ca^{2+} Influx and IL-2 Production

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We previously showed that the T cell activation inhibitor, mitochondrial (*Tcaim*) is highly expressed in grafts of tolerance-developing transplant recipients and that the encoded protein is localized within mitochondria. In this study, we show that $CD11c^+$ dendritic cells (DCs), as main producers of TCAIM, downregulate *Tcaim* expression after LPS stimulation or in vivo alloantigen challenge. LPS-stimulated TCAIM-overexpressing bone marrow–derived DC (BMDCs) have a reduced capacity to induce proliferation of and cytokine expression by cocultured allogeneic T cells; this is not due to diminished upregulation of MHC or costimulatory molecules. Transcriptional profiling also revealed normal LPS-mediated upregulation of the majority of genes involved in TLR signaling. However, TCAIM BMDCs did not induce *Il2* mRNA expression upon LPS stimulation in comparison with Control-BMDCs. In addition, TCAIM overexpression abolished LPS-mediated Ca^{2+} influx and mitochondrial reactive oxygen species formation. Addition of IL-2 to BMDC–T cell cocultures restored the priming capacity of TCAIM BMDCs for cocultured allogeneic $CD8^+$ T cells. Furthermore, BMDCs of IL-2–deficient mice showed similarly abolished LPS-induced T cell priming as TCAIM-overexpressing wild type BMDCs. Thus, TCAIM interferes with TLR4 signaling in BMDCs and subsequently impairs their T cell priming capacity, which supports its role for tolerance induction. *The Journal of Immunology*, **2015, 194: 3136–3146.**

T cell activation inhibitor, mitochondrial (*Tcaim*; also known as *Toag-1*) is highly expressed in graft and blood of tolerance-developing allogeneic kidney and heart graft recipients. In contrast, 3–5 days prior to graft rejection *Tcaim* mRNA is downregulated up to 10-fold in both compartments (1, 2). TCAIM is highly conserved among different species, but no homologies to protein families of known functions were detectable. The protein contains a mitochondrial leader sequence and a J-domain within the N- and C terminus, respectively. So far little is known about the specific function of TCAIM. It is highly expressed in $CD11c^+$ dendritic cells (DCs) and $CD4^+$ T cells (1). Keeren et al. (3) showed that TCAIM is exclusively localized within mitochondria. Increasing data identified mitochondria to be crucial for innate immune signaling. West et al. (4) demonstrated that TNF receptor associated factor (TRAF6) translocates to mitochondria in macrophages after TLR1/2/4 stimulation. Subsequently, TRAF6 ubiquitinates the mitochondrial protein evolutionarily conserved signaling intermediate in Toll pathway (ECSIT) and thereby induces mitochondrial reactive oxygen species (mROS) production (4). ECSIT is involved in complex I assembly (5), which is the main producer of ROS (6). mROS potentiate MAPK signaling by inactivating MAPK phosphatases (7). Consequently, protein-modification by ROS can result in a variety of functional changes, such as activation, inactivation, or multimerization of proteins (8).

As professional Ag-presenting cells, DCs communicate between innate and adaptive immunity. Upon activation within the tissue, they migrate to draining lymph nodes. Within the lymph nodes, DCs activate Ag-specific T cells because of their high expression levels of costimulatory proteins (e.g., CD86, CD80), MHC class II (MHC-II) molecules and increased secretion of proinflammatory cytokines (9). It has also been reported that DCs are able to produce IL-2 early upon TLR-mediated activation (10, 11). This might explain the high T cell priming capacity of DCs, because they are supposed to be the only IL-2–producing cells in the myeloid compartment (12, 13). *Il2* expression is initiated upon activation of NFAT, which in turn is controlled via dephosphorylation by calcineurin. In DCs, calcineurin is activated by an increased intracellular $Ca2^+$ concentration, initiated by CD14 signaling or Dectin-1 ligand binding (14–16).

We studied the effect of *Tcaim* overexpression on the priming capacity of LPS-matured murine bone marrow–derived DCs

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Abbreviations used in this article: AP1, activating protein-1; BMDC, bone marrow–derived DC; CPD, cell proliferation dye; CRAC, calcium release activated channel; DC, dendritic cell; ER, endoplasmic reticulum; ESCIT, evolutionarily conserved signaling intermediate in Toll pathway; MHC-II, MHC class II; *mHprt*, mouse hypoxanthine phosphoribosyltransferase; moDC, monocyte-derived DC; mROS, mitochondrial reactive oxygen species; QRT-PCR, quantitative real-time PCR; rmIL-2, recombinant mouse IL-2; TCAIM, T cell activation inhibitor, mitochondrial; TMRM, tetramethylrhodamine-methyl ester.

(TCAIM-BMDCs) as compared with control transduced BMDCs (Control-BMDCs). We showed that LPS- but not polyinosinic-polycytidylic acid (poly-IC)–stimulated TCAIM-BMDCs have a reduced priming capacity for both $CD4^+$ and $CD8^+$ T cells. Interestingly, this was not due to reduced LPS-mediated upregulation of MHC-II and CD86 expression or IL-6 production. In contrast, TCAIM abolished LPS-mediated Ca^{2+} influx, mROS formation and diminished *Il2* expression. LPS-mediated *Il2* expression was instrumental for the priming capacity of BMDCs. Addition of IL-2 restored the LPS-induced T cell priming capacity of TCAIM-BMDCs for $CD8^+$ T cells. Thus, TCAIM interferes with crucial LPS-signaling steps at the mitochondria and may thereby inhibit inflammation.

Materials and Methods

Mice

Male BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice were purchased from Charles River Laboratories. Mice were 6–8 wk of age and were allowed free access to food and water. The mice were kept according to German Guidelines on the use of animals in research (from the Berliner Sentsverwaltung).

Skin transplantation and DC subset sorting

C57BL/6 mice received a transplant from tail skin of BALB/c mice as previously reported (17). After 2 and 7 d, spleens and lymph nodes were collected and DC subset sorted on a FACSAria II cell sorter (BD Biosciences, Heidelberg, Germany) according to the gating strategy depicted in Supplemental Fig. 1.

Generation of BMDCs

BMDCs were produced using a standard protocol as described by Inaba et al. (18). Briefly, C57BL/6 bone marrow cells were cultured in six-well non–tissue-treated culture plates (Falcon) in complete medium (RPMI 1640 with stable glutamine supplemented with 10% superior FBS and 1% penicillin–streptomycin; Biochrome). Recombinant mouse GM-CSF (6 ng/ml; Miltenyi Biotec) was added, and medium was changed every other day.

Adenoviral transduction

Immature DCs were transduced on day 6 with a TCAIM encoding adenovirus (TCAIM-BMDCs) or a control virus encoding *Egfp* (Control-BMDCs). To increase the transduction-rate 8 μg/ml Polybrene (Sigma-Aldrich) was added to the mixture. The mixture was centrifuged for 77 min, 3500 rpm, and 14°C. Cells were placed carefully for an additional 120 min in the incubator (37°C, 5% CO_2, and 90% humidity). The maturation was induced on day 7 by adding 1 μg/ml LPS or 50 μg/ml poly-IC for 24 h (both from Sigma-Aldrich).

Coculture

Lymph node cells of BALB/c mice were harvested. The organs were homogenized through a 100-μm cell strainer. The cell suspension was filtered using a 40-μm cell strainer after centrifugation. Cells were used as alloreactive responder cells in cocultures with LPS-stimulated transduced C57BL/6 BMDCs. To quantify the proliferation of $CD4^+$ or $CD8^+$ T cells a maximum of 2×10^7 cells was stained with 10 μM Cell Proliferation Dye eFluor 450 (CPD450; eBioscience) for 20 min at room temperature in the dark. Cells were washed once. After mixing BMDCs (4×10^4) and CPD450-labeled responder cells (5×10^5) in a 96-well plate, cells were incubated for 5 d. In some cocultures, recombinant mouse IL-2 (Peprotech) was added in increasing concentrations (20 and 200 ng/ml).

Flow cytometry

Coculture: intracellular cytokine staining. Cells were washed once with medium and restimulated for 4 h at 37°C with 1 μg/ml ionomycin and 10 ng/ml PMA (Biotrend Chemikalien). After 2 h, 2 μg/ml Brefeldin A (Sigma-Aldrich) was added. Cells were stained for live dead using Fixable Viability Dye eFluor 506 (eBioscience) and subsequently for surface expression of CD3e, CD4, and CD8a at 4°C for 20 min. Cells were fixed and permeabilized with the BD Biosciences Cytofix/Cytoperm Fixation/Permeabilization Kit. For intracellular staining following Abs were used: CD3e-FITC, CD4-Pacific Blue, CD8a-allophycocyanin-Cy7, IFNγ-allophycocyanin (all from BioLegend), and the staining was performed for 30 min at 4°C. Samples were measured on an LSR II (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

Coculture: surface staining and proliferation determination. On day 5 of coculture CPD450-labeled lymph node cells were harvested carefully. They were stained for live dead and then for surface expression of CD3e, CD4, CD8a, and CD44 as described above (CD3e-FITC, CD4-Alexa Fluor 700, CD8a-allophycocyanin-Cy7, and CD44-PeCy7; all from BioLegend). Responder cell proliferation was measured by the dilution of CPD450 intensity. Surface expression and proliferation was measured on an LSR Fortessa (BD Biosciences) and analyzed using FlowJo software.

Surface expression

Splenocytes and lymph node cells collected from naive C57BL/6 mice or transplant recipient mice for sorting of DC subsets were stained for surface expression of a lineage mixture (CD3/CD19/CD49b/F4-80-PB), CD11c-PerCP, B220-AF700, CD199-PE-Cy7, CD64-allophycocyanin, Ly6C-FITC, CD4-BV785, CD8a-BV650 (all from BioLegend), and CD103-PE (eBioscience).

BMDCs were harvested with ice-cold PBS and stained for surface expression of CD11c, IA(b) and CD86 (CD11c-allophycocyanin, IA(b)-PE and CD86-Biotin, Streptavidin-PerCP) (all from eBioscience). The cells were measured on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software.

Cytokine production

The cytokine concentrations in the supernatants taken 6, 12, and 24 h after 1 μg/ml LPS or 50 μg/ml poly-IC stimulation were determined using a Cytometric Bead Assay from Bender MedSystem according to the FlowCytomix Mouse/Rat Basic Kit instructions. We measured TNF-α and IL-6 on a FACSCalibur (BD Biosciences) and quantified the concentrations using the FlowCytomix Pro software (Bender Medsystem). As the sensitivity for mouse IL-2 with the CBA technology is low, we used the Luminex xMAP Technology. Supernatants were treated exactly according to the Milliplex MAP Kit protocol (Millipore) and were analyzed with the Bio-Plex200 System (Bio-Rad).

Measurement of mitochondrial membrane potential $\Delta\Psi$

BMDCs were stimulated for 24 h with 1 μg/ml LPS or not. After a washing step with prewarmed PBS, BMDCs were incubated with 150 nM tetramethylrhodamine-methyl ester (TMRM) (Invitrogen) in PBS containing 2% BSA for 15 min at room temperature in the dark. After a washing step with a 150-pM TMRM solution, cells were stained with CD11c-allophycocyanin and measured within 30 min on a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software.

Measurement of mROS

BMDCs were stimulated for 1 and 3 h with 1 μg/ml LPS or not. After a washing step with prewarmed PBS, BMDCs were incubated with 5 μM final concentration MitoSox (Molecular Probes) in HBSS containing 1 nM calcium, 1 mM magnesium. After a washing step with PBS/10% FCS cells were additionally stained with CD11c-allophycocyanin and measured within 30 min on a FACSCalibur (BD Biosciences). Data were analyzed with FlowJo software.

Ca^{2+} influx

BMDCs were resuspended in prewarmed cell loading medium (HBSS containing 1 nM calcium, 1 mM magnesium, 1% FBS, and 0.5% BSA) and 4 mM probenecid (Santa Cruz). Cells were incubated for 30 min at 37°C with 2 μg/ml Indo-1 AM (Life Technologies). After two washing steps with cell loading medium at room temperature, cells were stained at 4°C with CD11c-allophycocyanin. Cells were rested and warmed to room temperature before measuring. The LSRFortessa (BD Biosciences) was used to quantify first the calcium flux without stimulus. Afterwards, the same cells were stimulated with 10 μg/ml LPS or 1 μg/ml ionomycin and immediately recorded.

Detection of apoptotic cells

After a surface staining step (CD11c-allophycocyanin), BMDCs were washed twice with cold PBS. Cell pellets were resuspended in 1× Annexin-V binding Buffer. Cells were stained with Annexin-V-PE and 7-amino-actinomycin D according to the protocol (Annexin-V-PE Apoptosis Detection Kit I; BD Biosciences) and measured within 1 h using an LSRFortessa (BD Biosciences).

RT² profiler PCR array: mouse TLR signaling pathway microarray

BMDCs were stimulated 2 d after transduction with 1 μg/ml LPS for 1 and 12 h. RNA was extracted using NucleoSpin RNA II (Macherey-Nagel) following manufacturer's instructions. In total, 3 μg RNA was used for cDNA synthesis according to the QuantiTect Reverse Transcription (Qiagen) manual. The Mouse Toll-Like Receptor Signaling Pathway Microarray (Qiagen) was performed following the instructions with some modifications. cDNA synthesis reaction mix (60 μl) was diluted with 60 μl RNase-free H_2O. Diluted cDNA was mixed with additional H_2O and SYBR Green PCR Master Mix (Life Technologies). The experimental mixture (25 μl) was pipetted to each well of the 96-well PCR array. A two-step cycling program was started using an ABI 7500 Sequence Detection System. Raw data were analyzed with 7500 System Software Version 1.3.1.2.1 (ABI). Mouse hypoxanthine phosphoribosyltransferase (*mHprt*) was used as a housekeeping gene.

PCA and clustering

All steps of the analysis were performed in R 2.15.0. The quantitative real-time PCR (QRT-PCR) data were normalized by subtracting the corresponding Ct-values of *mHprt* as housekeeping gene. The resulting ΔCt values of all target genes present on the arrays were used in a principle component analysis. The calculations were performed by a single value decomposition of the centered, not-scaled data matrix. The two first principle components are shown in Fig. 6B. Variances over all samples were calculated for each target gene. Highly variable genes with a variance ≥4 were selected and used in subsequent clustering; therefore, data were scaled and a hierarchical clustering was performed using euclidean distances and a complete linkage.

QRT-PCR

RNA from BMDCs stimulated with LPS or poly-IC or from sorted DC subsets was isolated as described before. Up to 1 μg RNA was used for cDNA synthesis. QRT-PCR was done with TaqMan Universal PCR Master Mix (ABI) using FAM-TAMRA as reporter fluorescence and *mHprt* (MWG Biotech AG) as a normalizing control. The 7500 Real-Time PCR system was used to detect gene expression for mouse *tcaim* or IL-2 (*mIl2*). The primer sequences used to quantify mouse *tcaim* expression have been published previously (19), whereas the primer sequences used to quantify mouse *mIl2* are as follows: *Il2* sense 5′-TCGCCAGTCAAGAGCTTCAGACAAGCA-3′, anti-sense 5′-CATGCCGCAGAGGTCCAA-3′ (MWG Biotech AG). Primers applied for the detection of *mHprt* were reported previously (1). Each reaction was carried out in duplicate. For evaluation we used 7500 System SDS Software.

Rotenone treatment of murine BMDCs

Transduced BMDCs were treated on day 7 overnight (14 h) with 1 μM Rotenone (Enzo Life Sciences). In the morning, complete media was exchanged and the BMDCs were stimulated with 1 μg/ml LPS for 24 h or left unstimulated for coculture experiments. For *Il2* mRNA expression studies, Rotenone-treated cells were left untreated or stimulated with 1 μg/ml LPS for 1 h.

TF activation reporter array I

One day after transduction cells were seeded in a 96-well white-wall plate (Greiner Bio-One) at 70% confluency and incubated overnight. Recombinant baculovirus (30 μl) encoding a construct of a consensus sequence for one of the following transcription factors NF κ-light-chain-enhancer of activated B cells (NF-κB), NFAT, activating protein-1 (AP1) or CCAAT/enhancer binding proteins (C/EBP) followed by a luciferase reporter gene (Signosis) were added directly to the corresponding wells. Twenty-four hours later, cells were left untreated or were stimulated for 30 min and 3 h with 1 μg/ml LPS. After treatment, the media was carefully removed and cells were washed with 100 μl PBS. Cells were incubated with 40 μl passive lysis buffer (Promega) for 15 min at room temperature. Luciferase Assay Substrate (100 μl; Promega) was added and mixed gently. Luminescence was measured using Mithras LB 940 (Berthold Technologies) immediately after substrate addition.

Statistics

Flow cytometry data were analyzed with FlowJo software version 9.4.3. The analyses were performed using R 2.15.0. We have used two-way ANOVA with multiple comparisons to analyze the data. Confidence intervals and adjusted p values were calculated using the Studentized range statistic (Tukey honest significant difference method). A p value < 0.05 was considered to be statistically significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). Data are expressed as mean ± SEM if not mentioned in the figure legends.

Results

DCs downregulate Tcaim *expression following in vitro LPS stimulation or in vivo allogeneic skin transplant challenge*

First, we determined whether inflammatory stimuli influence *Tcaim* expression in DCs. Therefore, BMDCs were stimulated with LPS for 1, 3, 6, 12 ,and 24 h, and their TCAIM mRNA expression was analyzed. As shown in Fig. 1A, LPS stimulation resulted in a transient significant reduction of *Tcaim* mRNA expression, reaching its maximum at 3 h after stimulation.

Next, we investigated whether this also happens in vivo. To this end, we sorted different DC populations ($CD4^+$, $CD8^+$, $CD4^-$ $CD8^-$, $CD103^+$, monocyte-derived DCs [moDCs], and plasmacytoid DCs; gating strategy shown in Supplemental Fig. 1) from spleens and lymph nodes of naive C57BL/6 mice and mice 2 and 7 d after receiving a BALB/c skin transplant. $CD64^+$ moDCs were included because they are supposed to most closely resemble BMDCs. As can be seen in Fig. 1B, allogeneic skin transplantation resulted in an early but transient increase in frequencies of plasmacytoid DCs, whereas we detected a continuous increase in $CD4^+$, $CD103^+$, and moDCs. Especially those three DC subsets showed also a downregulation in TCAIM transcription following transplantation (Fig. 1C). For $CD103^+$ and moDCs this effect was transient, whereas in $CD4^+$ DCs this effect was even more pronounced 7 d posttransplant.

TCAIM-BMDCs show a reduced T cell priming capacity

To investigate whether the mitochondrial protein TCAIM affects BMDC function, we overexpressed *Tcaim* using adenoviral gene delivery, reaching a significant increase in TCAIM expression especially upon LPS stimulation (Supplemental Fig. 2).

Enhanced expression of *Tcaim* in BMDCs resulted in increased baseline proliferation of cocultured $CD8^+$ T cells (Fig. 2). LPS and poly-IC stimulation of Control-BMDCs resulted in an increase of proliferating cocultured allogeneic $CD4^+$ and especially $CD8^+$ T cells (Fig. 2). TCAIM overexpression led to a reduced priming capacity of LPS-stimulated BMDCs (Fig. 2). We observed a substantially diminished proliferation and CD44 expression of cocultured $CD4^+$ and $CD8^+$ T cells (Fig. 2). Interestingly, this reduced priming capacity of TCAIM-BMDCs was specific for LPS-stimulation and not observed upon poly-IC stimulation.

In addition, we analyzed cocultured T cells for their cytokine production after restimulation with PMA–ionomycin. Again LPS and to a reduced extent also poly-IC stimulation of Control-BMDCs increased the frequencies of IFN-γ expressing cocultured allogeneic $CD4^+$ and, even more pronounced, $CD8^+$ T cells (Fig. 3A). We observed reduced IFN-γ production of cocultured $CD4^+$ and $CD8^+$ T cells with LPS-stimulated TCAIM-BMDCs compared with Control-BMDCs. This reached significance when applying the Wilcoxon test ($p < 0.05$), but did not reach significance when correcting for multiple comparisons because of low numbers (n = 4–5). In contrast, poly-IC stimulation of TCAIM-BMDCs induced a similar increase in IFN-γ expressing cocultured allogeneic $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells cocultured with LPS- or poly-IC–stimulated TCAIM- and Control-BMDCs showed no altered IL-17 and TNF-α production compared with cocultures with unstimulated BMDCs (Fig. 3B, 3C).

Tcaim *overexpression does not affect LPS-mediated MHC-II and CD86 upregulation*

Next, we tested whether an altered surface expression and cytokine secretion of TCAIM-BMDCs explains the diminished priming

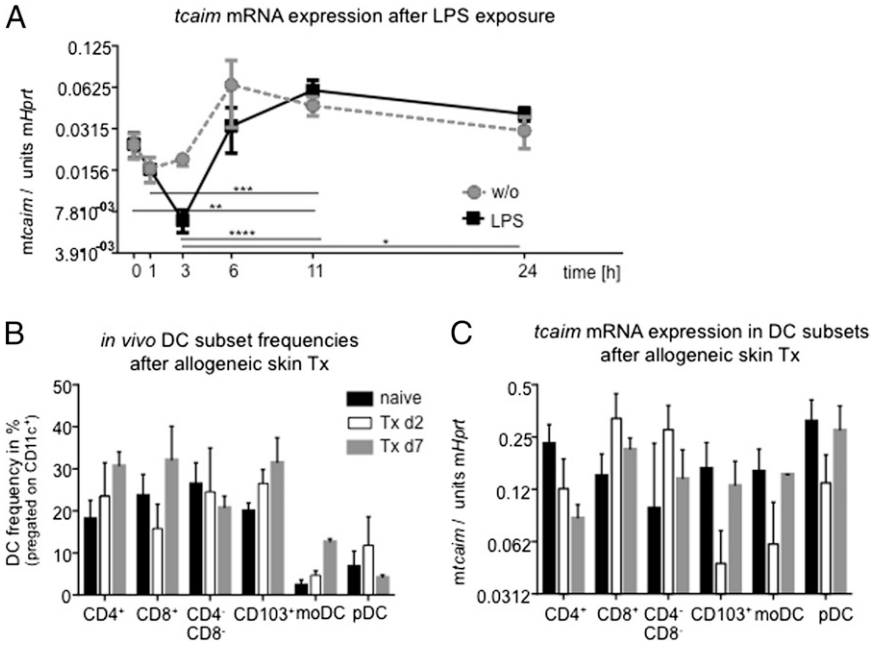


FIGURE 1. DCs downregulate *Tcaim* expression following in vitro LPS stimulation or in vivo allogeneic skin transplant challenge. (**A**) *Tcaim* mRNA expression after LPS stimulation. BMDCs were stimulated with 1 μg/ml LPS for the indicated time points (0, 1, 3, 6, 12, and 24 h). Shown are the *Tcaim* mRNA expression results of $n = 4$–5 independent experiments, as mean ± SEM. (**B**) Changes in DC subset frequencies in spleen and lymph nodes following allogeneic skin transplantation (skin Tx). Indicated DC subsets were sorted from spleen and lymph nodes of naive C57BL/6 mice or mice, which received an allogeneic BALB/c skin transplant 2 or 7 d prior to sample collection. (**C**) *Tcaim* mRNA expression of sorted DC subsets. Shown are the frequencies and *Tcaim* mRNA expression results of $n = 2$–3 independent experiments, as mean ± SEM. Cells of three mice were pooled per experiment. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

capacity. No differences in upregulation of MHC-II or CD86 upon LPS and poly-IC stimulation between TCAIM-BMDCs and Control-BMDCs were detectable. In contrast, baseline CD86 expression was even slightly increased in unstimulated TCAIM-BMDCs (Fig. 4A, 4B). In addition, LPS-induced IL-6 secretion was not affected by TCAIM overexpression (Fig. 4C). TCAIM-BMDCs did also release TNF-α upon LPS stimulation; however, we observed reduced TNF-α secretion by 42% in comparison with Control-BMDCs. Poly-IC–induced TNF-α and IL-6 production was only slightly lower in TCAIM-BMDC cultures as compared with Control-BMDCs (Fig. 4C).

TCAIM-BMDCs display abolished release of LPS-induced mROS

As a mitochondrial protein TCAIM might also interfere with cell survival and thereby affect the T cell priming capacity of DCs. Therefore, we analyzed the percentage of apoptotic or necrotic cells in unstimulated and LPS-stimulated TCAIM- and Control-BMDCs.

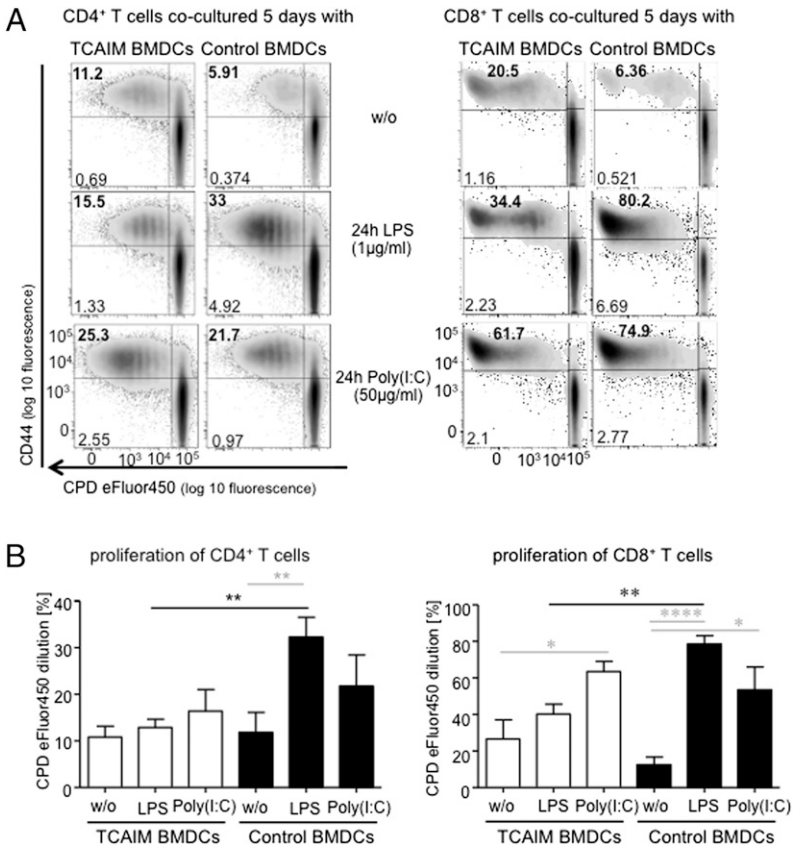


FIGURE 2. TCAIM-BMDCs fail to prime alloreactive T cells. (**A**) Representative dot plots showing CD44 expression and proliferation of $CD4^+$ (*left panel*) and $CD8^+$ T cells (*right panel*) cocultured for 5 d with LPS- or poly-IC-matured or immature TCAIM-BMDCs or Control-BMDCs. (**B**) The *left panel* summarizes the proliferation results of $n = 4$–8 independent experiments. Depicted are the mean ± SD of the proliferated cells (CPD negative/low) pregated on living $CD3^+CD4^+$ T cells. The right graph summarizes the proliferation of living $CD3^+CD8^+$ T cells. The mean ± SEM calculated from $n = 3$–6 independent experiments are shown. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

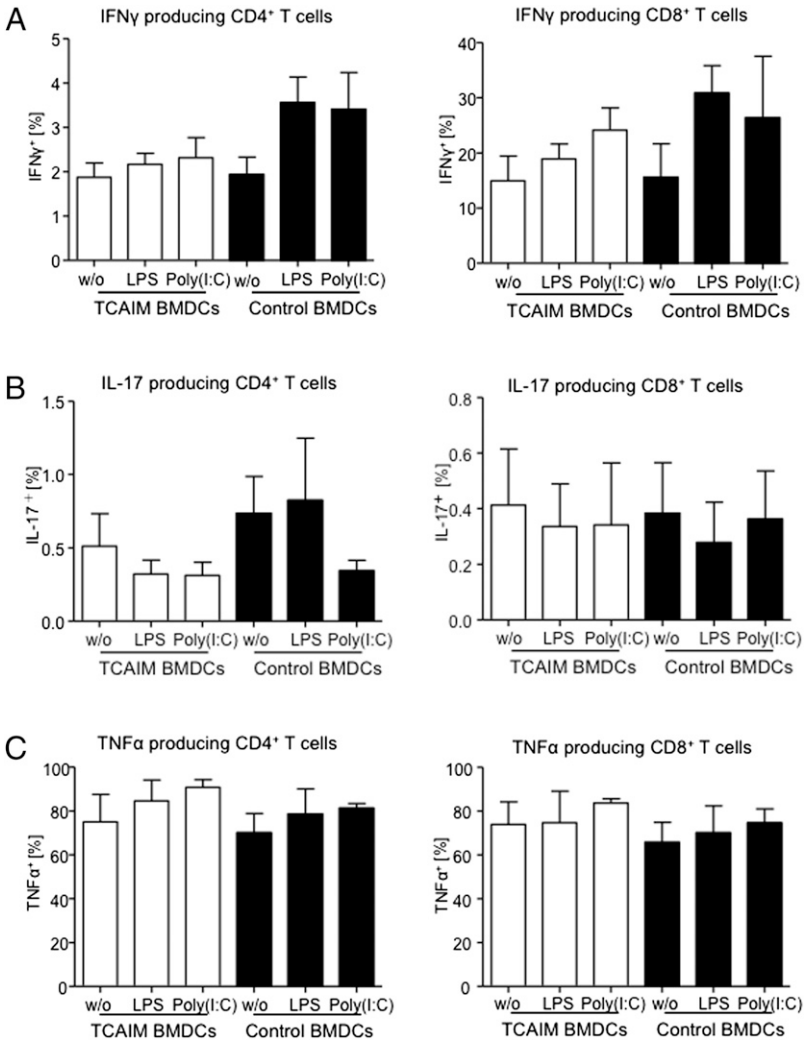


FIGURE 3. TCAIM-BMDCs induce fewer cytokine producing alloreactive T cells. (**A**) The *left panel* depicts the results of $n = 3–5$ independent experiments for $CD4^+$ T cells and $n = 3–4$ independent tests for $CD8^+$ T cells cocultured with allogeneic BMDCs. Depicted is the percentage of IFN-γ–producing living $CD3^+CD4^+$ or $CD8^+$ T cells. The results are shown as mean ± SEM; $p < 0.05$ (Wilcoxon-test) for $CD4^+$ and $CD8^+$ cocultured with LPS-stimulated TCAIM-BMDCs versus LPS-stimulated Control-BMDCs. In addition, the percentages of (**B**) IL-17– and (**C**) TNF-α–producing living $CD3^+CD4^+$ (*left panels*) or $CD8^+$ (*right panels*) T cells are shown. The mean ± SEM calculated from $n = 3–5$ independent experiments is shown.

As shown in Fig. 5A, TCAIM overexpression did not affect cell viability of BMDCs. In addition, recovery of BMDCs in absolute cell numbers was not different between TCAIM-BMDCs and Control-BMDCs (TCAIM-BMDCs: 55% ± 5.7; Control-BMDCs: 55.5% ± 3.4).

ATP and mROS production are dependent on mitochondrial membrane potential ΔΨ. To determine whether LPS-stimulated TCAIM-BMDCs show an altered ΔΨ, we used TMRM. TMRM is a lipophilic cation that accumulates ΔΨ-dependent in negatively charged mitochondria (20, 21). Fig. 5B shows the percentages of TMRM accumulation in unstimulated and LPS-stimulated TCAIM- and Control-BMDCs. *Tcaim* overexpression showed no effect on the mitochondrial membrane potential.

In addition, we investigated whether *Tcaim* overexpression alters mROS release (Fig. 5C). We measured mROS in TCAIM-BMDCs and Control-BMDCs, which were left unstimulated or stimulated with LPS for 1 h and 3 h. TCAIM-BMDCs showed a significantly diminished mROS production compared with Control-BMDCs. As described by West et al. (4, 22), we could not detect mROS induction upon poly-IC stimulation (data not shown).

Gene array analysis reveals abolished LPS-induced Il2 expression in TCAIM-BMDCs

To reveal the underlying mechanism for the diminished T cell priming capacity of TCAIM-BMDCs, we studied their LPS-induced transcription using a TLR signaling pathway PCR Array. TCAIM-BMDCs and Control-BMDCs were stimulated for 1 or 12 h with LPS or were left unstimulated. We visualized the results of three independent experiments using an unsupervised heat map analysis, meaning that the sample arrangement occurred respectively to expression similarities and not to groups. A clustering of all unstimulated, 1-h LPS-stimulated, or 12-h LPS-stimulated samples implied that the stimulatory effect of LPS exceeds generally the effect of *Tcaim* overexpression (Fig. 6A). A principal component analysis, in which triplicates of TCAIM- and Control-BMDCs of three stimulation time points are depicted, also showed a uniform distribution of TCAIM- and Control-BMDCs according to their stimulation (Fig. 6B). Surprisingly, of the 84 genes analyzed, only expression of *Il2* was significantly downregulated in TCAIM-BMDCs compared with Control-BMDCs. To verify the array results, we performed QRT-PCR on additional samples. LPS stimulation of Control-BMDCs resulted in an up to 20-fold upregulation of *Il2* mRNA expression (Fig. 6C). Already in unstimulated BMDCs, TCAIM overexpression reduced the basal *Il2* transcription. LPS-induced *Il2* mRNA expression was significantly decreased in TCAIM-BMDCs compared with Control-BMDCs. Il2 mRNA expression was not increased upon poly-IC stimulation in TCAIM- and Control-BMDCs compared with unstimulated cells. Furthermore, we detected a time-dependent increase in IL-2 protein secretion in supernatants of LPS-stimulated Control-BMDCs, which we did not observe in supernatants of LPS-stimulated TCAIM-BMDCs (Fig. 6D).

Il2 transcription in T cells and in DCs is regulated by intracellular Ca^{2+} increase with subsequent NFAT/NF-κB/AP1 acti-

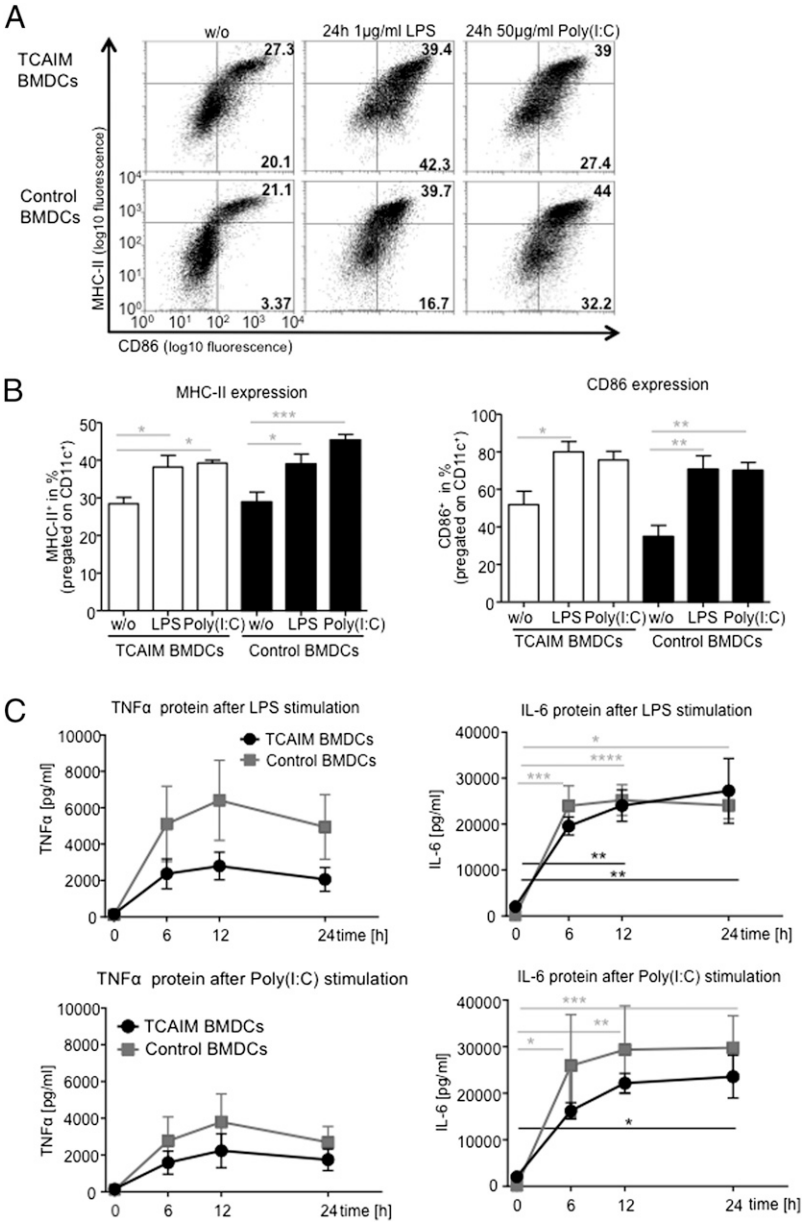


FIGURE 4. *Tcaim* overexpression does not affect LPS-mediated MHC-II and CD86 upregulation. (**A**) Representative dot plots showing the expression of MHC-II and CD86 of CD11c$^+$ immature and 24 h LPS- or poly-IC–matured TCAIM-BMDCs or Control-BMDCs analyzed by flow cytometry. (**B**) MHC-II (*left panel*) and CD86 (*right panel*) expression in the percentage of CD11c$^+$ cells stimulated with LPS (1 μg/ml) or poly-IC (50 μg/ml) for 24 h or left unstimulated TCAIM-BMDCs and Control-BMDCs of $n = 5–6$ independent experiments are shown, depicted as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (**C**) Effect on cytokine release upon *Tcaim* overexpression. The graphs show the cytokine production of LPS-stimulated (1 μg/ml) or poly-IC-stimulated (50 μg/ml) stimulated BMDCs analyzed using a cytometric bead array. Supernatants were taken 0, 6, 12, and 24 h after stimulation. The left figure displays the TNF-α secretion of $n = 2–7$ independent experiments, whereas the right graph shows the IL-6 release of $n = 1–9$ independent experiments. Results are depicted as mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

vation; therefore, we analyzed whether TCAIM overexpression affects LPS-induced Ca^{2+} signaling. Fig. 6E shows the intracellular Ca^{2+} influx after LPS and ionomycin stimulation of TCAIM-BMDCs and Control-BMDCs. In Control-BMDCs and TCAIM-BMDCs, we observed a rapid increase of intracellular Ca^{2+} after ionomycin stimulation, which was lower in TCAIM-BMDCs. TCAIM-BMDCs were completely unable to increase free cytosolic Ca^{2+} upon LPS stimulation. This effect was highly reproducible (Fig. 6F).

Addition of IL-2 restores T cell priming capacity of TCAIM-BMDCs

Next we questioned whether the addition of recombinant mouse IL-2 (rmIL-2) would restore the diminished T cell priming capacity of TCAIM-BMDCs. We added 20 or 200 ng/ml rmIL-2 to the coculture of LPS-stimulated transduced BMDCs and allogeneic lymph node cells. We observed a dose-dependent induction of CD8$^+$ T cell proliferation in cocultures with LPS-matured TCAIM-BMDCs (Fig. 7A), whereas the addition of rmIL-2 increased but did not normalize proliferation of cocultured CD4$^+$ T cells. The proliferation in cocultures with LPS-stimulated Control-BMDCs was not affected by the addition of rmIL-2.

Last, we tested whether IL-2 production by BMDCs determines their T cell priming capacity. Therefore, BMDCs generated from bone marrow of IL-2–deficient and IL-2$^{+/-}$ heterozygous control mice were cocultured with allogeneic lymph node cells. Indeed, IL-2–deficient BMDCs failed to increase their T cell priming capacity upon LPS stimulation (Fig. 7B). This failure was more pronounced for priming of CD8$^+$ T cells as compared with CD4$^+$ T cells.

Inhibition of mROS generating complex I reduces Il2 expression and T cell priming capacity of LPS-stimulated BMDCs

Rotenone, a systemic complex I inhibitor, was used to inhibit the mROS production in BMDCs (23), as complex I is one important source of mROS (6). To evaluate the effect of Rotenone on *Il2* mRNA expression, we treated the cells overnight with 1 μM Rotenone or control medium and then stimulated them for 1 h with LPS or left them unstimulated. Overnight incubation with

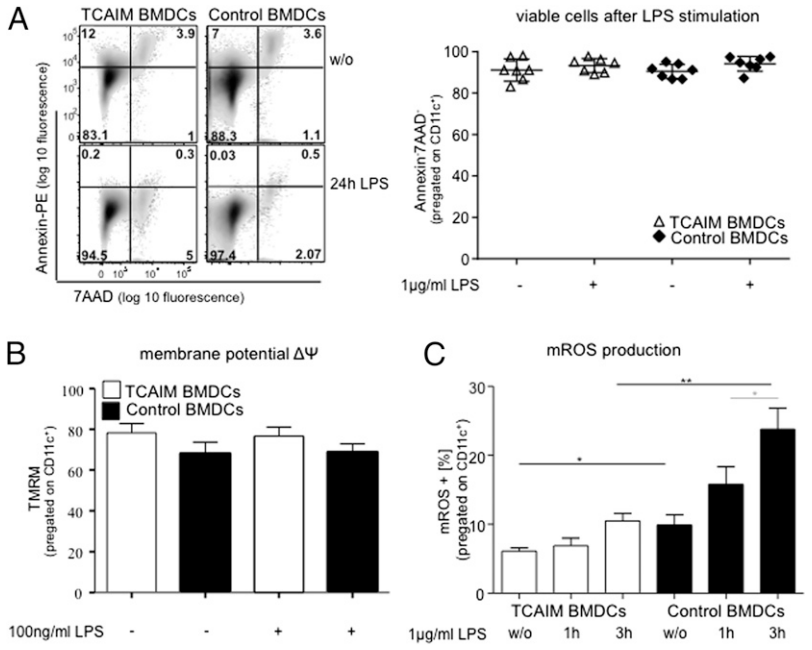


FIGURE 5. *Tcaim* overexpression abolishes release of mROS, which regulate T cell priming capacity of BMDCs. (**A**) Effect of *Tcaim* overexpression on apoptosis induction. Dot plots of unstimulated or 24 h LPS-stimulated (1 μg/ml) BMDCs stained for Annexin-V and 7-actinomycin D are depicted. Results of seven independent experiments are shown as mean ± SEM. The cells were pregated as $CD11c^+$. (**B**) Effect on $\Delta\Psi$ upon *Tcaim* overexpression. $\Delta\Psi$ was measured 24 h after LPS-stimulation in $CD11c^+$ TCAIM- and Control-BMDCs using TMRM. Results are depicted from five independent experiments as means ± SEM. (**C**) MROS produced by unstimulated or LPS (1 μg/ml) stimulated TCAIM- and Control-BMDCs after indicated time points (0, 1, and 3 h) are depicted as means ± SEM. Results of 10 independent experiments are shown. $*p < 0.05$, $**p < 0.01$.

Rotenone had no effect on *Il2* mRNA expression of LPS-stimulated TCAIM-BMDCs (Fig. 8A). In contrast, *Il2* mRNA expression is considerably attenuated in LPS-stimulated Rotenone-treated Control-BMDCS. Furthermore, Rotenone-treated LPS-matured Control-BMDCs had a diminished T cell priming capacity in comparison with untreated LPS-matured Control-BMDCs (Fig. 8B). This effect was more prominent for cocultured $CD8^+$ T cells than for $CD4^+$ T cells. In contrast, Rotenone-treatment of LPS-stimulated TCAIM-BMDCs did not reduce their residual low T cell priming capacity.

TCAIM alters transcription factor binding activity of NF-κB and C/EBPβ

To obtain insight into the molecular mechanisms of TCAIM-mediated reduction in IL-2 production and T cell priming capacity of BMDCs, we have performed preliminary experiments studying LPS-induced transcription factors activation. TCAIM- and Control-BMDCs were incubated with baculoviruses encoding luciferase constructs with transcription factor binding sites for NFAT, NF-κB, AP1, and C/EBPβ. The following day, BMDCs were stimulated with LPS for 30 min and 3 h, and transcription factor binding activity was determined with luciferase assay (Fig. 8C). We detected a low NFAT binding activity in BMDCs, but we did not observe a difference between TCAIM-BMDCs and Control-BMDCs. In contrast, we detected a LPS-induced increase in NF-κB and AP1 binding activity, which was especially lower for NF-κB in TCAIM-BMDCs. Strikingly, TCAIM-BMDCs displayed a transient increase in C/EBPβ binding activity, which we did not observe for Control-BMDCs.

Discussion

In this study, we show that increased expression of TCAIM in murine BMDCs leads to an impaired LPS-induced priming capacity for allogeneic $CD4^+$, but even more $CD8^+$ T cells. This was not due to abolished LPS-mediated upregulation of MHC-II or CD86 expression. Release of inflammatory cytokines and mediators such as IL-6, IL-12, or IL-1 was not affected. However, LPS-stimulated TCAIM-BMDCs were characterized by a reduced Ca^{2+} influx, mROS formation, and *Il2* expression. Adding IL-2 normalized proliferation of cocultured $CD8^+$ T cells. Furthermore, IL-2 deficient BMDCs failed to increase priming of cocultured $CD8^+$ T cells upon LPS stimulation. Interestingly, inhibiting mROS producing mitochondrial complex I did mimic *Tcaim* overexpression as it resulted in reduced LPS-mediated *Il2* expression and T cell priming capacity.

Mitochondria play an important and decisive role in cell signaling. Especially their role in immune cell signaling has gained much attention in recent years (24, 25). Apart from regulating apoptosis, mitochondrial release of ROS is instrumental for redox-sensitive signaling pathways such as activation of MAP kinases, NF-κB, and AP1 as well as mitochondria are indispensable for Ca^{2+} signaling (22, 26–29).

Mitochondria are efficient Ca^{2+} buffers (30). Because of close proximity to the plasma membrane, mitochondria can take up large amounts of inflowing Ca^{2+}, which prevents the inactivation of calcium release activated Ca^{2+} (CRAC) channels (31).

Ca^{2+} is an important second messenger in DCs and its influx occurs upon PLC-γ activation transiently by depleting intracellular stores, such as the endoplasmic reticulum (ER) or through opening of Ca^{2+} channels in the plasma membrane. Ca^{2+} influx is induced by certain but not all microbial products, such as LPS, CpG, or peptidoglycan, resulting in a calcineurin-dependent NFAT activation, subsequent nuclear translocation, and NF-κB activation by degradation of the NF-κB inhibitor IκB (31). Applying CRAC channel inhibitors results in a less mature phenotype (32) and a reduced IL-2 secretion (33).

TCAIM as a mitochondrial protein containing a J-domain could interfere with mitochondrial Ca^{2+} uptake, as J-domain proteins are described to be involved in mitochondrial translocases (34, 35). Mitochondrial calcium uptake can be mediated by the mitochondrial calcium uniporter. This transport is supported by the mitochondrial membrane potential $\Delta\Psi$ and leads to the production of mROS (30). Inhibition of the prolonged Ca^{2+} uptake after LPS-stimulation in TCAIM-BMDCs by disturbing the Ca^{2+} transport into mitochondria could explain the reduced mROS production. Furthermore, mitochondria play an active part in regulating inositol triphosphate–mediated Ca^{2+} release from the ER (36, 37). Mitochondrial Ca^{2+} uptake exerts positive feedback effects on the inositol triphosphate receptor-mediated ER Ca^{2+} mobilization (38). Thus, mitochondria can affect both Ca^{2+} release from the ER and Ca^{2+} entry across the plasma membrane, thereby shaping the

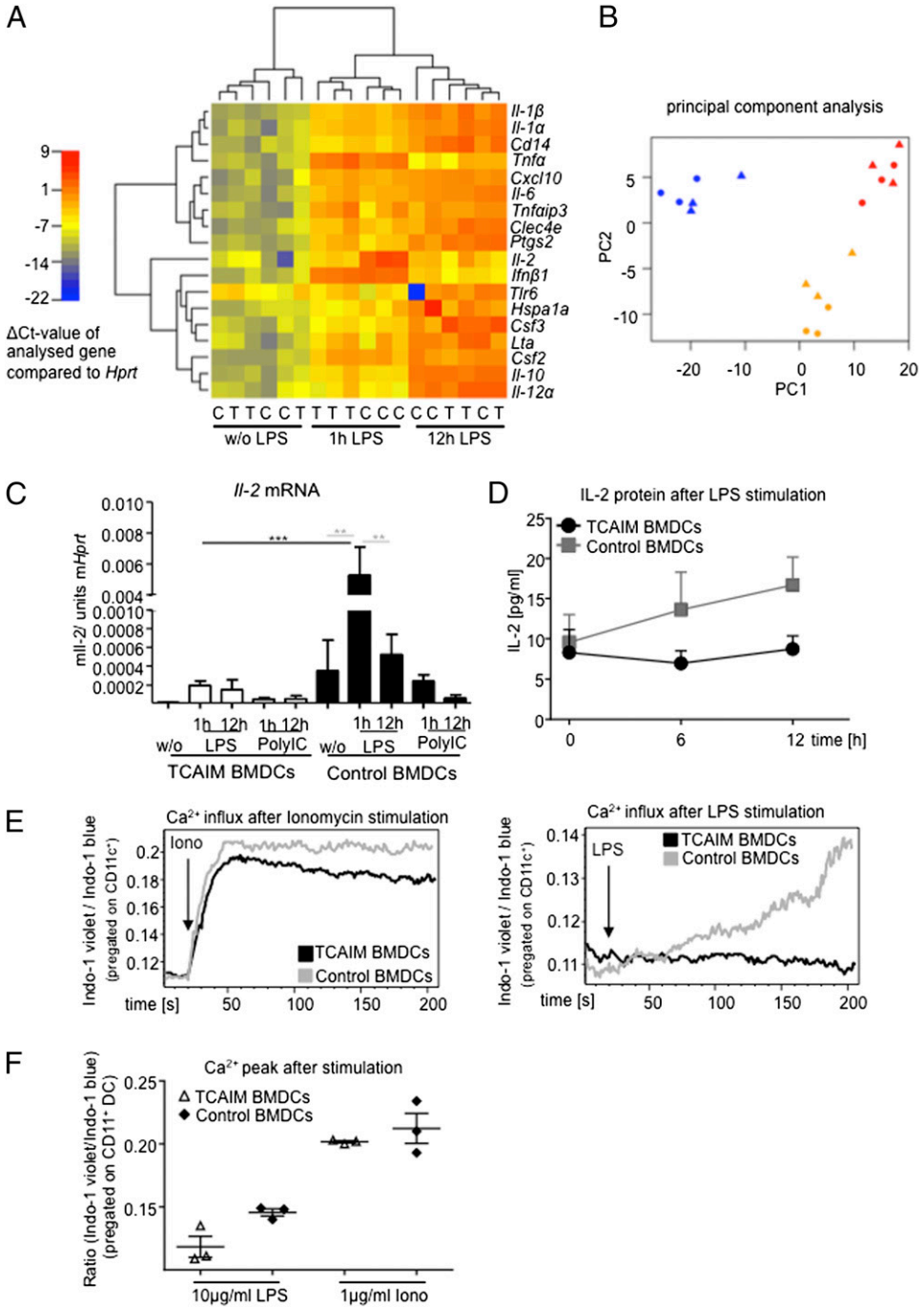
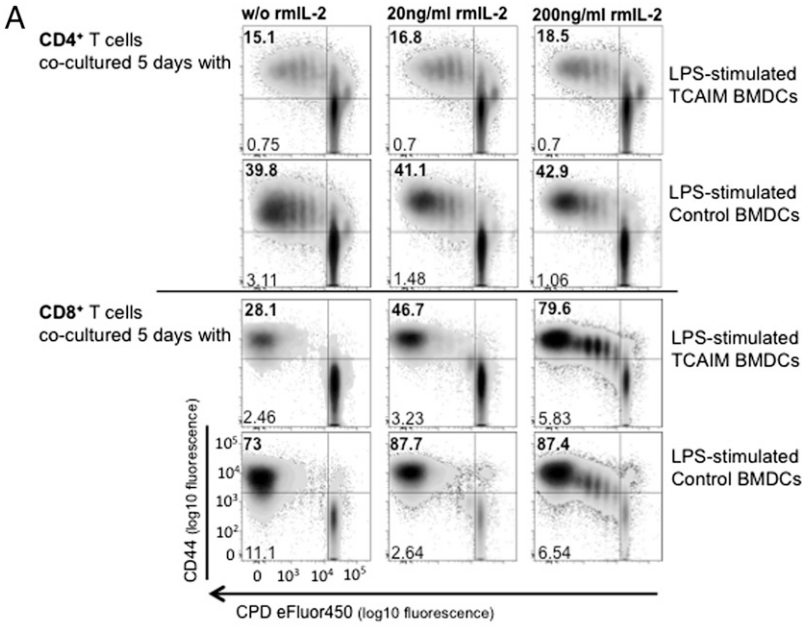


FIGURE 6. Gene array analysis reveals an abolished *Il2* induction in TCAIM-BMDCs. (**A**) Analysis of gene expression using a mouse TLR signaling pathway PCR array in *Tcaim* overexpressing BMDCs upon LPS activation. Unsupervised heat map of three independent experiments, in which TCAIM (T) overexpressing and control-transduced BMDCs (**C**) were left unstimulated or stimulated for 1 and 12 h with 1 μg/ml LPS. From the 84 genes analyzed, the expression of the 16 genes shown was regulated upon LPS stimulation, normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT), when setting the variance threshold ($v > 4$) high. (**B**) Depicted is a principal component analysis of three independent experiments. Shown are the single experiment gene expression results normalized to HPRT of TCAIM-BMDCs (triangle) or Control-BMDCs (circle) at the following time points: 1 h (orange) and 12 h (red) LPS stimulated or unstimulated (blue). (C) *Il2* mRNA expression after LPS and poly-IC stimulation. TCAIM- and Control-BMDCs were stimulated with 1 μg/ml LPS or 50 μg/ml poly-IC for the indicated time points (0, 1, and 12 h). Shown are the *Il2* mRNA expression results of $n = 4$–8 independent experiments, as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. (**D**) IL-2 protein secretion into supernatants of LPS-stimulated TCAIM- and Control-BMDCs. Shown are the results of eight independent experiments as mean ± SEM. (**E**) Indo-1–labeled TCAIM- or Control-BMDCs were stimulated with 10 μg/ml LPS or 1 μg/ml ionomycin. Ca^{2+} influx is illustrated as the ratio of Indo-1 violet and Indo-1 blue over time. Depicted are exemplary histograms of pregated $CD11c^+$ BMDCs. (**F**) Summary of peak influx levels of three independent Ca^{2+} influx measurements.

size and duration of the intracellular Ca^{2+} signal (39). This could explain that TCAIM-BMDCs showed abolished LPS-mediated and reduced ionomycin-mediated increase in Ca^{2+}.

Interestingly, we could observe a stronger effect of TCAIM overexpression on the induction of proliferation and cytokine production by cocultured $CD8^+$ T cells than by $CD4^+$ T cells.



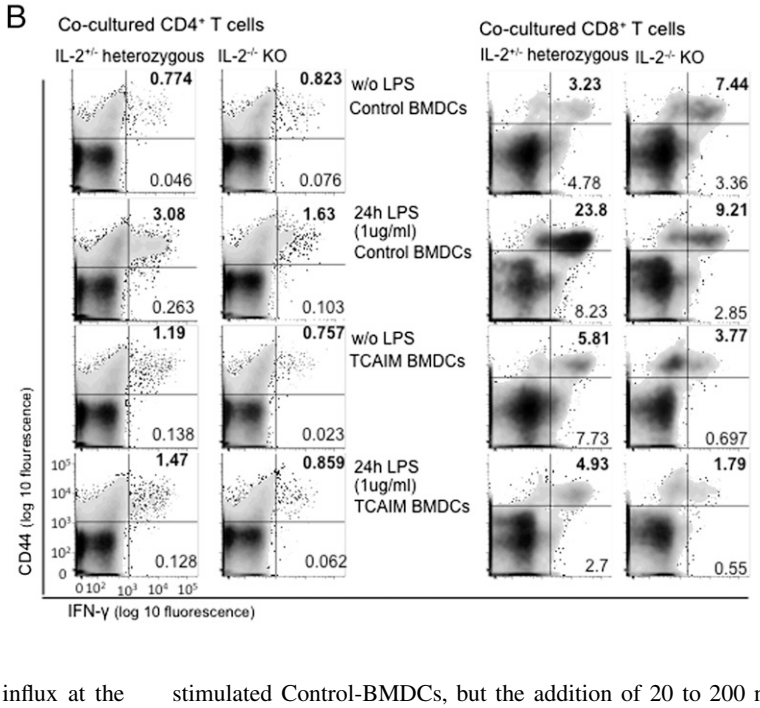


FIGURE 7. The addition of IL-2 can restore T cell priming capacity of TCAIM-BMDCs. (**A**) Representative dot plots showing CD44 expression and proliferation (CPD eFluor450), 5 d after cocultures of 5×10^5 BALB/c lymph node cells and 4×10^4 LPS-matured C57BL/6 TCAIM-BMDCs and Control-BMDCs upon the addition of 20 or 200 ng/ml recombinant mouse IL-2, are depicted. (**B**) Representative dot plots showing CD44 expression and IFN-γ production of living $CD3^+$ $CD4^+$ or $CD8^+$ T cells after PMA/ionomycin restimulation; 5×10^5 BALB/c lymph node cells were cocultured for 5 d with 4×10^4 unstimulated or LPS-matured $IL\text{-}2^{+/-}$ heterozygous or IL-2–deficient Control-BMDCs and TCAIM-BMDCs.

Shumilina et al. (31) described that the calcium influx at the immunologic synapse is dependent on the type of T cell contacting the DC. When the DC interacted with alloreactive $CD8^+$ T cells, the Ca^{2+} concentration rose within DCs. In contrast, when the DC formed a synapse with alloreactive $CD4^+$ T cells, only an increase of Ca^{2+} in the T cells was detectable (31, 40, 41). Thus, the more profound defect in proliferation and IFN-γ by cocultured $CD8^+$ T cells is perhaps due to the completely abolished calcium influx in TCAIM-BMDCs.

It is well known that during priming of naive $CD8^+$ T cells presence of $CD4^+$ T cells is critical (42, 43). Wolkers et al. showed that in the absence of $CD4^+$ T cells, the addition of exogenous IL-2 markedly increased the secondary expansion of and IFN-γ production by $CD8^+$ T cells (44). The maximal expansion was achieved when using IL-2 concentrations between 10 and 100 ng/ml (44). This finding is in accordance with our data, because in our cocultures with LPS-stimulated TCAIM-BMDCs $CD4^+$, T cells were not activated compared with cocultures with LPS-stimulated Control-BMDCs, but the addition of 20 to 200 ng/ml IL-2 could normalize proliferation of $CD8^+$ T cells.

Considering that TCAIM-BMDCs showed a reduced Ca^{2+} influx and thereby a nearly abolished IL-2 secretion after LPS-stimulation, it is not surprising that it affected especially the priming of $CD8^+$ T cells and to a lower extent of $CD4^+$ T cells (45). Indeed, the addition of IL-2 to cocultures of TCAIM-BMDCs with allogeneic T cells is able to restore proliferation of $CD8^+$ T cells to nearly normal levels, whereas the effect was not as evident for $CD4^+$ T cells.

As pointed out earlier, Ca^{2+} influx accelerates the generation of mROS (30). Furthermore, triggering of certain TLRs in immune cells results in formation of mROS important for bactericidal activity and for regulation of redox-sensitive signaling pathways regulating cytokine release and Ag-presenting capacity (22, 26). It has been shown that ROS production in DCs upon communication with cocultured T cells determines their Ag-presentation capacity (46, 47). In those studies, scavenging of ROS by antioxidants,

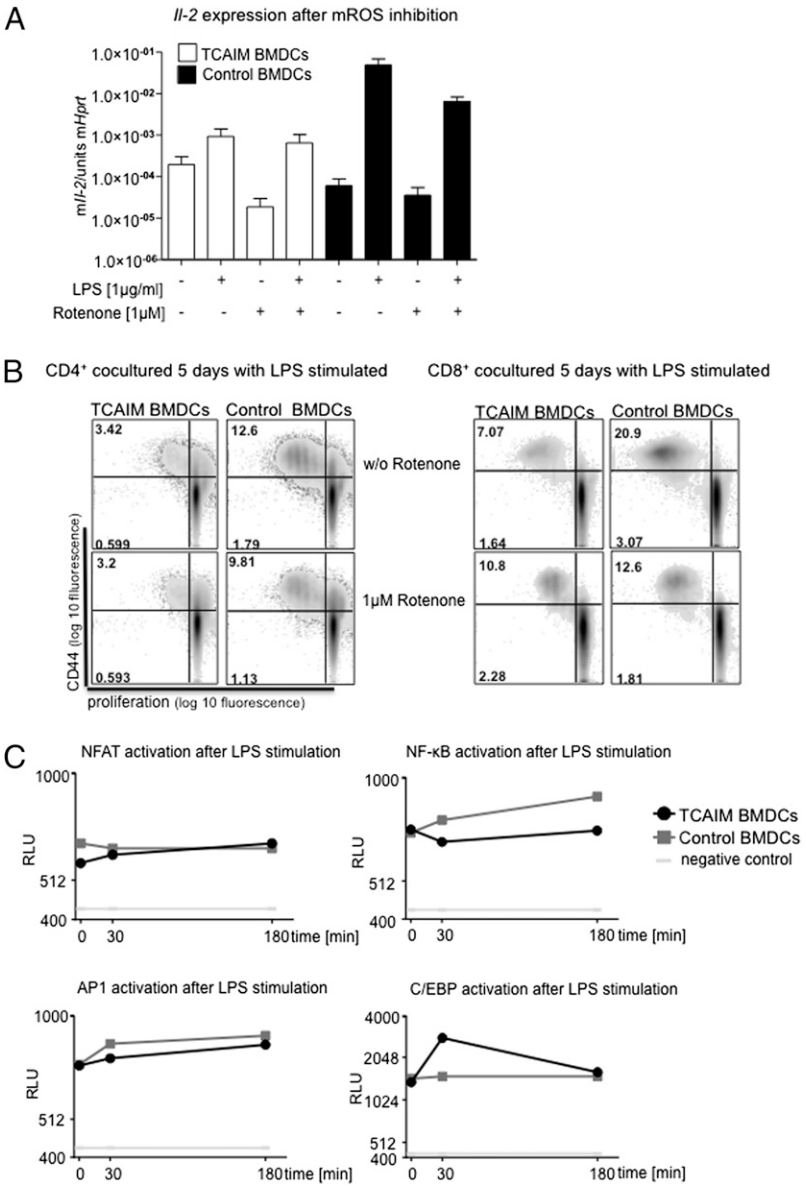


FIGURE 8. Inhibition of mROS generating complex I reduces *Il2* expression and T cell priming capacity of LPS-stimulated BMDCs. (**A**) Expression of *Il2* mRNA after overnight Rotenone (1 μM) treatment in unstimulated BMDCs and following 1 h LPS (1 μg/ml) stimulation. Mean ± SD from five independent experiments are shown. (**B**) Dot plots of proliferation and CD44 expression of alloreactive $CD4^+$ and $CD8^+$ lymph node cells cocultured for 5 d with overnight Rotenone-treated (1 μM) and 24 h LPS-matured (1 μg/ml) TCAIM-BMDCs and Control-BMDCs. The cells are pregated for living $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells. (**C**) TCAIM alters transcription factor binding activity of AP1, NF-κB, and C/EBPβ. TCAIM- and Control-BMDCs were incubated with baculoviruses encoding a construct of a consensus sequence for one of the following transcription factors: NF-κB, NFAT, AP1, or C/BEP followed by a luciferase reporter gene. Twenty-four hours later, cells were left untreated or were stimulated for 30 min and 3 h with 1 μg/ml LPS and luciferase activity was measured. Shown are the means of two independent experiments.

resulted in reduced release of inflammatory cytokines such as IL-6, but also abrogated induction of MHC and costimulatory molecules. We showed that the *Tcaim* overexpression in BMDCs, which completely abolished LPS-induced Ca^{2+} influx and impaired mROS production, was associated with diminished T cell priming capacity. In contrast to the findings above, this finding was not associated with reduced surface expression of MHC and costimulatory molecules or production of IL-6. This may be due to a more specific action of TCAIM on mROS, whereas scavenging antioxidants can also act on other enzymes generating ROS.

The effects of *Tcaim* overexpression on the T cell priming capacity were limited to LPS stimulation and were not observed upon poly-IC stimulation. This result further supports our hypothesis that TCAIM effects are mediated by inhibiting Ca^{2+} influx and mROS production, as we and others have observed that stimulation of BMDCs with TLR3 ligands such as poly-IC do not elicit either Ca^{2+} influx or mROS production (4, 15, 48, 49).

In conjunction with the diminished LPS-mediated Ca^{2+} influx and mROS production, we observed a reduced NF-κB but increased C/EBPβ binding activity. This observation might explain, on the molecular level, why TCAIM-BMDCs show reduced LPS-induced IL-2 expression, because NF-κB is well known to control *Il2* transcription positively (50, 51). In contrast, C/EBPβ was shown to influence *Il2* transcription negatively, at least in T cells (52).

Our investigations did not completely explain how increased *Tcaim* expression abolishes the priming capacity of BMDCs for cocultured allogeneic $CD4^+$ T cells, as addition of IL-2 did enhance but not normalize T cell proliferation. In addition to testing IL-2, we tested other γ-chain signaling cytokines, such as IL-15. We could neither detect an LPS-induced increase of IL-15 expression nor did addition of IL-15 restore T cell proliferation (data not shown).

Thus, the mitochondrial protein TCAIM diminishes the T cell priming capacity of BMDCs, in particular for $CD8^+$ T cells, by inhibiting LPS-mediated Ca^{2+} influx, mROS formation, NF-κB, and especially *Il2* expression.

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Disclosures

The authors have no financial conflicts of interest.

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