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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: 000–000.

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; ECSIT, evolutionarily conserved mitogen-activated protein kinase (MAPK) signaling intermediate in Toll pathway; MHC, major histocompatibility complex; MHC-II, MHC class II; moDC, monocyte-derived DC; mROS, mitochondrial reactive oxygen species; mTMRM, tetramethylrhodamine-methyl ester.

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(TCAIM-BMDCs) as compared with control transduced BMDCs (Control-BMDCs). We showed that LPS- but not polyminosinic-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 up-regulation of MHC-II and CD86 expression or IL-6 production. In contrast, TCAIM abolished LPS-mediated Ca²⁺ influx, mROS formation and diminished II2 expression. LPS-mediated II2 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-2b) and C57BL/6 (H-2b) 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 Sents-verwaltung).

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.

Adenoviral transduction

Immature DCs were transduced on day 6 with a TCAIM encoding adeno-virus (TCAIM-BMDCs) or a control virus encoding Egfp (Control-BMDCs). To increase the transduction-rate 8 g/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. After 2 h, 2 g/ml Brefeldin A (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.

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 × 10⁵ cells was stained with 10 μM Cell Proliferation Dye eFlour 450 (CPD450; eBioscience) for 20 min at room temperature in the dark. Cells were washed once. After mixing BMDCs (4 × 10⁵) and CPD450-labeled responder cells (5 × 10⁵) 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 eFlour 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-PerCy7; 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-BV605 (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 Array from Bender MedSystem according to the FlowCytomix Mouse/Rat Basic Kit instructions. We measured TNF-α and IL-6 on a FACS Calibur (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 MilliMap MAP Kit protocol (Millipore) and were analyzed with the Bio-Plex200 System (Bio-Rad).

Measurement of mitochondrial membrane potential ΔΨ

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 μM 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 FACS Calibur (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 mM 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 FACS Calibur (BD Biosciences). Data were analyzed with FlowJo software.

Ca²⁺ influx

BMDCs were resuspended in prewarmed cell loading medium (HBSS containing 1 mM 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 FACS Calibur (BD Biosciences). Data were analyzed with FlowJo software.

Detection of apoptotic cells

After a surface staining step (CD11c-allophycocyanin), BMDCs were washed twice with cold PBS. Cell pellets were resuspended in 1/1 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 LSR Fortessa (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 H2O. Diluted cDNA was mixed with additional H2O 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 the Qiagen System Software Version 1.3.1.2.1 (ABI). Mouse hypoxanthine phosphoribosyltransferase (Hprt) 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 Hprt as housekeeping gene. The resulting ΔCt values of all target genes present on the arrays were used in a principle component analysis. 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. Variance over all samples was 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 sortend 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 Hprt (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’-TGCCGGCTAAGGAGCTTCTCAGCAAGCA-3’, anti-sense 5’-CATGCCAGAGGTCAGAACTAC-3’ (MWG Biotech AG). Primers applied for the detection of Hprt 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 CCAT/enhancer binding proteins (C/EBP) followed by a luciferase reporter gene (Signoss) 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 ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 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
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.
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 $\Delta \Psi$. To determine whether LPS-stimulated TCAIM-BMDCs show an altered $\Delta \Psi$, we used TMRM. TMRM is a lipophilic cation that accumulates $\Delta \Psi$-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-$\kappa$B/AP1 acti-
vation; therefore, we analyzed whether TCAIM overexpression affects LPS-induced Ca2+ signaling. Fig. 6E shows the intracellular Ca2+ 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 Ca2+ after ionomycin stimulation, which was lower in TCAIM-BMDCs. TCAIM-BMDCs were completely unable to increase free cytosolic Ca2+ 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
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 assay constructs with transcription factor binding sites for NFAT, NF-κB, API, and C/EBPβ. The following day, BMDCs were stimulated with LPS for 30 min and 3 h, and transcription factor binding activity was determined using the 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 API 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+ T cells, 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 Ca2+ 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 API as well as mitochondria are indispensable for Ca2+ signaling (22, 26–29).

Mitochondria are efficient Ca2+ buffers (30). Because of close proximity to the plasma membrane, mitochondria can take up large amounts of inflowing Ca2+, which prevents the inactivation of calcium release activated Ca2+ (CRAC) channels (31). Ca2+ 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 Ca2+ channels in the plasma membrane. Ca2+ 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 Ca2+ 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 ΔΨ and leads to the production of mROS (30). Inhibition of the prolonged Ca2+ uptake after LPS-stimulation in TCAIM-BMDCs by disturbing the Ca2+ transport into mitochondria could explain the reduced mROS production. Furthermore, mitochondria play an active part in regulating inositol triphosphate–mediated Ca2+ release from the ER (36, 37). Mitochondrial Ca2+ uptake exerts positive feedback effects on the inositol triphosphate receptor-mediated ER Ca2+ mobilization (38). Thus, mitochondria can affect both Ca2+ release from the ER and Ca2+ entry across the plasma membrane, thereby shaping the
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.
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 Ca2+ concentration rose within DCs. In contrast, when the DC formed a synapse with alloreactive CD4+ T cells, only an increase of Ca2+ 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 Ca2+ 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, Ca2+ 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,
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

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References


