NFATc1 controls the cytotoxicity of CD8\(^+\) T cells

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Cytotoxic T lymphocytes are effector CD8\(^+\) T cells that eradicate infected and malignant cells. Here we show that the transcription factor NFATc1 controls the cytotoxicity of mouse cytotoxic T lymphocytes. Activation of \(\text{Nfatc1}^{-/-}\) cytotoxic T lymphocytes showed a defective cytoskeleton organization and recruitment of cytosolic organelles to immunological synapses. These cells have reduced cytotoxicity against tumor cells, and mice with NFATc1-deficient T cells are defective in controlling Listeria infection. Transcriptome analysis shows diminished RNA levels of numerous genes in \(\text{Nfatc1}^{-/-}\) CD8\(^+\) T cells, including \(\text{Tbx21}\), \(\text{Gzmb}\) and genes encoding cytokines and chemokines, and genes controlling glycolysis. \(\text{Nfatc1}^{-/-}\), but not \(\text{Nfatc2}^{-/-}\), CD8\(^+\) T cells have an impaired metabolic switch to glycolysis, which can be restored by IL-2. Genome-wide ChIP-seq shows that NFATc1 binds many genes that control cytotoxic T lymphocyte activity. Together these data indicate that NFATc1 is an important regulator of cytotoxic T lymphocyte effector functions.

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The primary function of CD8+ T cells is to eradicate infected and tumor cells. Upon activation and differentiation of naïve CD8+ T cells to effector CD8+ T cells, cytotoxic T lymphocytes (CTL) synthesize large amounts of the inflammatory cytokines IFNγ and TNF, and the cytotoxic effector molecules perforin and granzyme B, which are deposited in lytic granules in the cytoso. Upon contact of CTLs with target cells, the lytic granules are re-orientated and recruited to the immunological synapse (IS), along with the microtubule-organizing center (MTOC), the Golgi apparatus and mitochondria1, 2. At or near the immunological synapse, lytic granules fuse with the cell membrane and release perforins and granzymes to kill target cells3.

CD8+ T cell contact with cognate antigen leads to intracellular T cell receptor (TCR)-mediated signaling that, along with co-stimulatory signals, orchestrates gene expression programs to control the expansion and differentiation of CD8+ T cells to CTLs in peripheral lymphoid organs. Upon primary stimulation and the generation of effector cells, most of the activated CD8+ T cells die, but a small number of cells survive and develop into memory CD8+ T cells. According to surface expression and similar to CD4+ T cells, memory CD8+ T cells are classified into central memory CD8+ TCM cells and effector memory CD8+ TEM cells that differ in their homing capacity and effector function4, 5. However, the identification of tissue-resident memory TRM cell subsets suggests that a variety of other CD8+ memory T cells exist to ensure optimal immunity against infection and cancer6.

One prominent signaling network that has an important function in the generation and function of activated CD8+ T cells and CTLs is the Ca++/calcineurin/NFAT network. Activation of this network is initiated by the TCR-mediated release of Ca++ from endoplasmic stores, resulting in the multimerization of Stromal interaction molecules (STIM) that contact pore-forming ORAI proteins and activate Ca++ influx from the extracellular space through Ca++ release activated Ca++ channels (CRAC)7. The rise of intracellular Ca++ leads to the rapid activation of the Ser/Thr-specific phosphatase calcineurin that binds and dephosphorylates the highly phosphorylated cytosolic NFAT proteins, and stimulates their nuclear import8.

The family of NFAT transcription factors consists of five members that share a common DNA-binding domain of approximately 300 amino acid residues. There are only a few studies on NFAT transcription factors in CD8+ T cells. In one study, a defective nuclear translocation of NFATc1 has been described for NFATc1 in CD8+ T cells upon chronic infection9, whereas in another study a predominant nuclear localization of NFATc1 was reported for anergic CD8+ T cells10. The effect of NFATc1 (NFAT2) ablation on CD8+ T cell physiology has been reported11, but genome-wide assays on the effect of NFATc1 on gene expression in CTLs have not.

Here we show that upon TCR stimulation, ablation of NFATc1 results in an impaired formation of F-actin rings around the immunological synapse in CTLs, and poor recruitment of lytic granules and mitochondria to the synapse. Genome-wide transcriptome and chromatin immuno precipitation (ChIP) assays show that NFATc1 controls genes (including Il2 and Ifng) that orchestrate the activity of activated CD8+ T cells (aCD8+ T cells) and CTLs. Although NFATc1 and NFATc2 overlap in their binding to most of these genes, in most cases NFATc1 has a much stronger transcriptional effect than NFATc2 in aCD8+ T cells and CTLs. Upon activation, Nfatc1+/− aCD8+ T cells reduce the metabolic switch from oxidative phosphorylation (OXPHOS) to glycolysis, an effect that can be restored by IL-2. Taken together our data demonstrate that NFATc1 controls transcription of genes that direct the cytotoxicity of CD8+ T cells.

**Results**

**NFATc1 for cytoskeleton reorganization in activated CTL**

NFATc1 ablation alters the shape of CTLs. When WT CTLs adhere to a glass slide on which αCD3/CD28 have been attached they spread out in a velvet-like lamellipodium. By contrast, Nfatc1−/− CTLs form numerous spikes and filopodia-like structures which differ conspicuously from the shape of WT CTLs (Fig. 1a), and the area covered by Nfatc1−/− CTLs is reduced, compared to WT CTLs (Fig. 1b).

The rapid formation of IS at the TCR is accompanied by the depletion of cortical F-actin, the accumulation of MTOC, and the recruitment of mitochondria and lytic granules12. When we studied the formation of F-actin rings generated around the IS upon activation by total internal reflection fluorescence (TIRF) microscopy, we detected radially symmetric F-actin rings for WT cells whereas Nfatc1−/− CTLs generated no or smaller and irregular rings (Fig. 1b–d).

The decrease of actin density in the center of IS is accompanied by the appearance of MTOC (Fig. 1d, e) consisting of microtubules and further cytosolic components13, including attached mitochondria and lytic granules14. Using TIRF, we did not observe in Nfatc1−/− CTLs a marked delay in MTOC accumulation (Fig. 1d). However, a defect was detected in the assembly of mitochondria and lytic granules at the IS in living cells using fluorescence microscopy. Upon staining of WT and Nfatc1−/− CTLs for mitochondria with MitoTracker® and lytic granules with LysoTracker® followed by incubation of cells with αCD3/CD28-coated beads we detected the recruitment of mitochondria and cytotoxic granules to the IS in living WT CTLs within 1-5 min. In Nfatc1−/− CTLs both types of organelles did not, or slowly accumulate at the IS (Fig. 1f and Supplementary Fig. 3b) revealed a 40% decrease in killing, compared to WT CTLs (Fig. 1a). A decrease in killing capacity was also observed for sorted CD62L+CD44+ CTLEm cells (Supplementary Fig. 3a). WT and Nfatc1−/− CTLs generated from CD8+ T cells of C57/BL6 mice by co-culture with irradiated Balb/c splenocytes for 6 d and A20F tumor cells as targets (see Supplementary Fig. 3b) revealed a 40% decrease in killing, compared to WT CTLs. Those Nfatc1−/− CTLs showed also a reduction in the surface expression of the degranulation marker CD107a whose induction is correlated with the cytotoxicity of cells (Fig. 2b). Moreover, they showed a reduction in the number of cells producing IFNγ, IL-2 and TNF whereas the number of CTLs expressing IL-17 and granzyme B was increased (Fig. 2c; Supplementary Fig. 3c). No or subtle differences were detected between WT and Nfatc1−/− CTLs regarding the surface expression of CD62L, CD44, CD25, FasL and CD69 (Supplementary Fig. 3d).

**Listeria monocytogenes** infection induces a strong CD8+ T cell response that combats infection15. To investigate the role of NFATc1 in CTL responses against *Listeria monocytogenes,*
**Fig. 1** NFATc1 controls the re-organization of cytoskeleton and polarization of cytosolic organelles. 

- **a** Spreading of WT and Nfatc1−/− (Nfatc1f/f x CD4-cre mice) CTLs on αCD3/CD28-coated glass slides within 5 min. Scanning electron microscopy. LP, lamellipodium. 
- **b** Quantification of data of spreading (above) and F-actin ring formation (below) of CTLs activated for 5 min (t-test, unpaired, p-value < 0.0001). Left: each dot/triangle corresponds to one cell. Right: mean of three independent experiments. 
- **c** Formation of F-actin rings in WT and Nfatc1−/− CTLs upon αCD3/CD28 activation within 5 min. TIRF microscopy. 
- **d** Appearance of MTOC-tubulin near the IS of CTLs upon activation for 5 min. TIRF microscopy. 
- **e** Organization of F-actin and MTOC-tubulin in CTLs upon activation for 20 min. Confocal microscopy. For scale bar see **d**. 
- **f** Repositioning of mitochondria and lytic granules to the IS in living WT but not Nfatc1−/− CTLs. Left: analysis by confocal microscopy; overlay of bright field and merged fluorescence images of lysosomes (red) and mitochondria (green). The dark dots correspond to beads coated with αCD3/CD28. Right: quantification by epifluorescence microscopy. The column presentation compiles the data of more than 15 cells from 3 mice each.
we infected WT and Nfatc1−/− x CD4-cre mice with the transgenic (tg) strain ΔActα Lm-Ova lacking protein Actα expression that induces actin assembly. Listeria monocytogenes bacteria were detected in the livers of 8 of the 14 Nfatc1−/− x CD4-cre mice infected with ΔActα Lm-Ova for 5 d whereas only two of the WT mice showed signs of infection (Fig. 2d). Infected Nfatc1−/− x CD4-cre mice showed an increase in number of CD62L−, CD44+ central and decrease of CD62L+, CD44+ effector memory cells, and a reduced number of cells producing TNF and IL-2, compared to WT mice (Fig. 2e, f; Supplementary Fig. 3e).

**NFAT expression in aCD8+ T cells and CTLs.** The effect of NFATc1 on CTL activity led us to study NFAT expression in CD8+ T cell cultures. To generate CTLs, splenic CD8+ T cells were stimulated first with plate-bound αCD3/CD28 for 3 d (resulting in aCD8+ T cells), followed by incubation with IL-2 for 5–6 d. To mimic contacts with target cells, finally CTLs were stimulated by TPA and Ionomycin (T + I) for 5 h (Fig. 3a). These culture conditions approximate CD8+ T cell responses in vivo and allowed us to generate large numbers of cells for molecular studies.

Stimulation of CD8+ T cells by αCD3/CD28 resulted in a strong increase of NFATc1 expression within 1 d. At the RNA level, this led to a moderate 3-fold increase in P1 promoter-directed Nfatc1 and a decrease in both P2-directed Nfatc1 and Nfatc2 transcripts (Fig. 3b, c). At the protein level, a strong increase in short isoform NFATc1/αA was observed (see asterisks in Fig. 3d). However, further incubation of aCD8+ Ts in IL-2 medium led to the disappearance of NFATc1/αA proteins. However, several of the newly synthesized NFATc1 proteins were smaller than NFATc1/αA. Since incubation of...
NFATc1/αA with protein extracts of CTLs resulted in peptides of the same size, they are most likely degradation products of NFATc1/αA that often lost the α-peptide (see lane 12 in Fig.3d, and compare Supplementary Fig.4a with Fig.3d). As described previously17, such cleavage was also detected for NFATc2 (Fig. 3e).

NFAT-mediated gene expression in aCD8+ T cells and CTLs. To determine the transcriptional program controlled by NFATc1 in CD8+T cells, we performed transcriptome analysis of aCD8+ Ts and CTLs generated from splenic CD8+ T cells of WT and Nfatc1+/− x CD4-cre mice that did not show any NFATc1 expression in their aCD8+ Ts and CTLs (Supplementary Fig. 4b). For comparison, we also determined the transcriptome of Nfatc2−/− mice by next generation sequencing (NGS). We sequenced mRNAs from (i) activated CD8+Ts (aCD8+ Ts), (ii) CTL − and (iii) CTL + cells.

In the three types of CD8+ T cells shown in Fig. 3a, 6000–6500 genes were expressed in more than 4 reads per kilobase per million mapped reads (RPKMs). In the heat map, transcripts of 561 genes are listed (Fig.4a) whose expression was found to be changed 2 fold or more in at least one cell type. As expected from the high levels of nuclear NFATc1 in aCD8+ T cells and CTLs, we detected a 2.5-fold transcriptional alteration in 188 and 357 genes between WT and Nfatc1−− aCD8+ T cells and CTLs.
respectively. Only 14 and 57 genes were changed in Nfatc2−/− aCD8+ Ts and CTLs +. We observed only 6 genes that were affected in both Nfatc1−/− and Nfatc2−/− aCD8+ Ts, whereas in CTLs− the transcription of 13 and in CTLs + 26 genes were changed (Fig. 4b). Among them are the Ifng, Il3, Batf3 and Cd40l genes, and the genes encoding the chemokines Ccl3, Ccl4 and Xcl1.

The stronger effect of NFATc1 on gene expression in aCD8+ Ts and CTLs + is also visualized in scatterplots of NFAT-dependent genes. While ablating NFATc2 exerted a relative mild effect, NFATc1 ablation resulted in much stronger changes in gene expression in aCD8+ Ts and CTLs + (Fig. 4c). Functional annotation of NFATc1-dependent genes in aCD8+ Ts and CTLs + revealed that a large proportion of deregulated genes encoding enzymes in both cell types (Fig. 4d).

To identify the chromosomal sites and genes to which NFATc1/A binds in CTLs + we performed ChIP seq assays. Since ChIP-grade NFATc1 Abs are not available, we created a BAC tg mouse line in which the biotin-ligase BirA from E. coli and chimeric NFATc1/A-Bio proteins are co-expressed18 (Fig. 5a). From those mice, CD8+ T cells were isolated, differentiated into CTLs and stimulated with T + I for 5 h (to generate CTL + cells). Cross-linked NFATc1/A-chromatin complexes could be isolated with high specificity using streptavidin-coated magnetic beads and, upon removal of cross-links and DNA isolation, subjected to NGS of DNA. Similar to the endogenous Nfatc1 gene, the BAC Nfatc1 tg was induced upon induction of CTLs by T + I for 5 h (Fig. 5b). The results of ChIP seq assays revealed 19 759 DNA peaks upon annotation to the mm9 mouse genome. Motif analyses within these short DNA stretches resulted in the typical TGGAAA (or—

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**Fig. 4** Transcriptome assays of CD8+ T cells by next generation sequencing. a Heat map of 561 genes whose expression changed twofold in either Nfatc1−/− (Nfatc1−/− x CD4-cre mice) or Nfatc2−/− aCD8+ Ts and CTLs. b Number of genes whose expression was changed 2.5-fold in either aCD8+ Ts, CTL− or CTL + cells. Genes whose expression was specifically changed in Nfatc1−/− cells are shown in blue, those changed in Nfatc2−/− cells in orange, and those changed in both in gray. c Scatterplots of NFATc1- and NFATc2-dependent genes relative to Log2WT expression in aCD8+ Ts (upper panel) and CTL + cells (lower panel). d Functional annotation of genes changed 2.5-fold in RNA levels in NFATc1-deficient aCD8+ Ts and CTL + cells according to the PANTHER pathway analysis69.
on the reversed strand—TTTCCA) NFAT ‘core’ binding motif (Fig. 5c). Among the 357 genes whose RNA expression differed more than 2.5-fold between WT and Nfatc1−/− CTL cells stimulated by T + I for 5 h, 199 genes (56%) showed peaks for NFATc1/A-Bio. Therefore, they correspond to direct NFATc1 targets. According to published data19, 187 of the 357 genes correspond to NFATc2 binding.

Fig. 5 Analysis of DNA binding of NFATc1/A-Bio in CTLs by ChIP seq assays. a Scheme of the Nfatc1 BAC transgene expressing tagged NFATc1/A-Bio protein in mice. The two promoters P1 and P2 and the 11 exons of the gene are indicated. b Immune blots of whole cell protein extracts from CTLs expressing only the E. coli biotin-ligase BirA, or BirA and NFATc1/A-Bio. The upper blot was incubated with an Ab directed against streptavidin, the lower blot with the NFATc1-specific mAb 7A6. The star indicates a non-specific band. c Motif analysis of NFATc1 binding sites in the 19 759 NFATc1/A peaks identified with a p-value of −5 by MACS. The letter size indicates the frequency of nucleotides within those sites (presented as ‘Bits’). d Peaks for NFATc1/A-Bio and NFATc219 (p-value of −12 in MACS) within a distance of 100 kb to the 357 genes that were 2.5-fold differentially expressed in Nfatc1−/− CTL+ compared to WT CTLs. 161 genes show both NFATc1/A-Bio and NFATc2 binding. e Binding of NFATc1/A-Bio and NFATc2 to the prototypical NFAT target genes Ifng, Il2 and Il3 in CTLs + . Right, real-time PCR assays of Ifng, Il2 and Il3 RNA levels in WT, Nfatc1+/− and Nfatc2−/− CTL+ cells, normalized by Actb and relative to naïve CD8+ T cells.
showed binding for NFATc2, and 161 genes showed binding for both NFATc1 and NFATc2 (Fig. 5d).

The Ifng, Il2 and Il3 genes are prototypes of NFAT target genes. While NFATc1 and NFATc2 bind predominantly to the Il2 promoter and to a site 2.3 kb upstream, both NFAT factors bind to multiple sites around the Il3 and Ifng loci. NFATc1 ablation abolished RNA induction of all three genes in CTLs. Although NFATc2 binds to almost the same sites as NFATc1, the expression of these genes was not significantly affected.

**Fig. 6** NFATc1 ablation affects the proliferation and differentiation of CD8+ T cells. a) CFSE labeling of aCD8+ T cells stimulated for 3 d by plate-bound αCD3/CD28 (0.05/2 μg/ml), and of CTLs which were stimulated by αCD3/CD28 (5/2 μg/ml) for 3 d followed by IL-2 for 5 d. b) Heat map of selected genes controlling the proliferation of Nfatc1−/− (Nfatc1f/f x CD4-cre mice) aCD8+ Ts. c) Heat map of selected genes affecting the fate of CD8+ Ts. d) Real-time PCR assays of Tbx21 and Eomes RNA expression in CTL + cells, normalized by Actb and relative to naïve CD8+ T cells. e) Flow cytometry of intracellular T-bet and Eomes expression in CTL + cells. f) ChIP seq assay of NFATc1/A-Bio and NFATc2 binding to the Tbx21 gene in CTL + cells. g) Real-time PCR assays of Ccr7, Il7r, Itgae and Irf4 RNA expression in CTLs, normalized by Actb and relative to naïve CD8+ T cells. Data from five PCR assays are shown as means ± SEM. Unpaired Student’s t-test was used for statistics.
Fig. 7 Reduced metabolic switch in Nfatc1−/− aCD8+ T cells. a Heat map of RNA expression from genes encoding enzymes of glycolysis in Nfatc1−/− (Nfatc1fl x CD4-cre mice) and Nfatc2−/− aCD8+ Ts. b Real-time PCRs of Slc2a1, Slc2a3 and Hk2 RNAs encoding the glucose transporters Glut1 and Glut3, and hexokinase 2, respectively, normalized by Actb and relative to naïve CD8+ cells. c Incorporation of 2-NBDG into aCD8+ Ts and CTLs upon incubation for 1 h at 37 °C. Data from 4 assays were compiled. d Extracellular flux analysis. Above, 4 × 10^5 WT and Nfatc1−/− CD8+ T cells were stimulated overnight with "low" concentrations of plate-bound αCD3 (0.05 μg/ml) and αCD28 (2 μg/ml), and subjected to extracellular flux analysis. Below, cells activated with "high" (standard) αCD3/CD28 concentrations (5/2 μg/ml) were assayed. e Mito stress test of aCD8+Ts treated as in d. f Below, incorporation of 2-NBDG (above) and extracellular flux analysis (below) of aCD8+ Ts from c.n.Nfatc1/A x Nfatc1fl x dlck-cre mice. Above, immune blots showing the lack of NFATc1 expression in cytoplasmic (CP) and nuclear (N) proteins of aCD8+ Ts from Nfatc1fl x dlck-cre mice (lanes 3 and 4), and the expression of c.n.NFATc1/A (point) in cells from c.n.Nfatc1/A x Nfatc1fl x dlck-cre mice (lanes 5 and 6). In lanes 1 and 2, proteins from WT cells were fractionated. Right, RT-PCRs showing Il2 and Myc RNA levels in aCD8+ Ts from WT, Nfatc1fl x dlck-cre and c.n.Nfatc1/A x Nfatc1fl x dlck-cre mice. Data of 5 PCR assays are shown, relative to naïve WT CD8+ T cells and normalized to Actb. Data are shown as means ± SEM. Unpaired Student’s t-test was used for statistics. g Extracellular flux analysis of CD8+ T cells from WT and Nfatc1fl x CD4-cre mice stimulated by αCD3/CD28 (5/2 μg/ml) without (above) or with 100 U/ml hIL2 (below) for 3 d. All assays were performed three times or more.
within and around the Il3, Ifng and Il2 genes, NFATc2 ablation resulted in very different effects on their expression. While NFATc2 ablation did not affect Il2 induction, it reduced Ifng induction to approximately 55% and inhibited almost completely Il3 induction in CTLs (Fig. 5e).

**NFATc1 affects CD8⁺ T cell differentiation.** Nfatc1⁻/⁻ aCD8⁺ T cells show a decrease in proliferation compared to WT and Nfatc2⁻/⁻ cells. Using a low concentration of plate-bound αCD3 (0.05 and 2 μg/ml αCD28) we detected a marked difference in proliferation between WT, Nfatc1⁻/⁻ and Nfatc2⁻/⁻ aCD8⁺ T cells. In contrast, all three types of CTLs did not display any difference in proliferation in medium containing high (100 U/ml) or low concentrations of IL-2 (10 U/ml; Fig. 6a). Among the numerous genes encoding cell cycle regulators, only the Cdkn1a gene encoding the cell cycle inhibitor p21WAF1/CIP1 was found as NFATc1 target in aCD8⁺ T cells. In aCD8⁺ T cells, NFATc1 inactivation led to a decrease in Cdkn1a RNA levels to ~60%, and NFATc1/A bound within and upstream from the Cdkn1a gene (Fig. 6b, Supplementary Figs. 4c, d).

Several genes whose expression controls the differentiation to effector and memory CTLs are direct NFAT targets. T-bet and Eomesoderm (Eomes) are key transcription factors controlling CD8⁺ T cell differentiation. In Nfatc1⁻/⁻ CTLs + the RNA level of the Tbxb21 gene encoding T-bet corresponds to approximately 30% of the WT level, and a lower T-bet protein expression was detected in Nfatc1⁻/⁻ (and Nfatc2⁻/⁻) CTL + cells (Fig. 6c,e). NFATc1 (and NFATc2) binds to two intronic sites and strongly to one site 11 kb upstream of the Tbxb21 gene (Fig. 6f). In contrast, the Eomes gene is not bound by NFATc1, and its expression was found to be strongly enhanced in Nfatc1⁻/⁻ CTLs (Fig. 6c–e) reflecting the reciprocal expression of both transcription factors.25, 26 NFATc1 also binds to and controls aCD8⁺ T cells the Il7rb1 and Il7rb2 genes (Supplementary Fig. 5a) encoding the IL-12 receptor that enhances Tbxb21 expression via mTOR.27

In Nfatc1⁻/⁻ aCD8⁺ Ts only one third of the Gzmb transcripts were generated compared to WT aCD8⁺ T cells, and NFATc1 binds to several sites of the Gzmb gene (Supplementary Fig. 5b). The transcript levels of Prf1 and Gzmb genes encoding the most prominent perforins and granzymes in CD8⁺ T cells increases 10-fold from aCD8⁺ Ts to CTLs but NFATc1 ablation led unaffected their expression in CTLs (Fig. 6c).

Among ‘fate markers’ for memory T cells, the Ccr7 and Il7r genes are NFATc1 targets. NFATc1 ablation led to a 4-5-fold decrease in Il7r transcripts in CTL + cells encoding the IL-7 receptor α chain CD127, a marker for long-living memory T cells.25, 28, 29 NFATc1 binds also to the Il7ra gene encoding the α chain of the IL-7 receptor, that was inhibited by rapamycin. However, NFATc1 ablation was without effect on 4E-BP1 phosphorylation (Supplementary Fig. 6b). This observation prompted us to study the role of NFATc1 in metabolism regulation of CD8⁺ T cells. Due to their high proliferation rate, activated T cells have a high demand for energy and cellular building blocks, as nucleotides and amino acids and, therefore, switch their metabolism from OXPHOS to glycolysis.30, 31 In RT-PCR assays we determined less than 30–35% of WT transcript level for the Scl2a1, Scl2a3 and Hk2 genes encoding the glucose transporters Glut1 and Glut3, respectively, and hexokinase 2 (Hk2), the enzyme responsible for the fixation of glucose in cells (Figs. 7a, b; Supplementary Fig. 8). Using the fluorescent glucose analog 2-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) we observed a 70% reduction in glucose uptake into Nfatc1⁻/⁻ aCD8⁺ Ts, compared to WT, whereas only a slight decrease was detected for Nfatc2⁻/⁻ cells (Fig. 7c). The decrease in glucose incorporation was accompanied by a decrease in extracellular acidification rate (ECAR) of Nfatc1⁻/⁻ aCD8⁺ Ts, as measured by extracellular flux analysis. For aCD8⁺ Ts generated with a low concentration of αCD3 (0.05 and 2 μg/ml αCD28) a 5-fold increase in glycolysis and glycolytic capacity was observed, while cells stimulated with standard concentrations (5 μg/ml αCD3, 2 μg/ml αCD28) displayed a three fold increase in both parameters. However, Nfatc1⁻/⁻ aCD8⁺ Ts stimulated with either low or high concentrations of αCD3/CD28 exhibited lower glycolysis and glycolytic capacity of 40–60% of WT level (Fig. 7d).

Contrary to the effect on glycolysis, NFATc1 ablation exerted only a moderate effect on mitochondrial respiration. In mito stress tests, the oxygen consumption rate (OCR) differed only slightly between WT and Nfatc1⁻/⁻ aCD8⁺ Ts, although Nfatc1⁻/⁻ aCD8⁺ Ts showed a tendency towards increased maximal respiration, compared to WT cells (Fig. 7e), and the RNA levels of genes encoding mitochondrial OXPHOS enzymes were slightly enhanced (Supplementary Fig. 9a).

In part, the decrease in glucose incorporation of Nfatc1⁻/⁻ aCD8⁺ Ts could be restored by overexpressing a constitutive nuclear (c.n.) version of NFATc1/αT. For this purpose we analyzed aCD8⁺ T cells from c.n.Nfatc1/αT x Nfatc1⁻/⁻ x dck-cre mice. Due to cre expression directed by the distal lck promoter30 these mice express c.n.Nfatc1/αT on removal of a ‘floxed’ STOP sequence from the Rosa26 locus31 in the absence of endogenous NFATc1. Those cells revealed a moderate, but reproducible increase in their glucose uptake, and in Il2 and Mryn RNA levels (Fig. 7f).

The effect of NFATc1 ablation on glycolysis led to the question of whether NFATc1 exerts a direct effect on glycolytic genes. However, in ChIP seq assays apart from the Hk2 and Gapdh genes (Supplementary Fig. 10a) no further genes of the glycolysis cascade showed distinct NFATc1 binding. One mediator of the NFATc1 effect could be IL-2 whose expression is strongly diminished in Nfatc1⁻/⁻ T cells (Fig. 5e). IL-2 is known to activate multiple metabolic and transcriptional programs in T cells, mainly through the Ser/Thr kinase mTORC1, a prominent IL-2 target in T cells.32 When we tested the effect of IL-2 in splenic CD8⁺ T cells on 4E-BP1, a direct target of mTOR signals, we observed a marked increase in TCR-mediated phosphorylation that was inhibited by rapamycin. However, NFATc1 ablation was without effect on 4E-BP1 phosphorylation (Supplementary Fig. 9b). Nevertheless, adding 100 U/ml IL-2 to CD8⁺ T cell cultures that were activated by αCD3/CD28 for 3 d revealed a marked increase in glycolysis of NFATc1-deficient cells (Fig. 7g). This finding is supported by similar RNA levels of glycolytic genes in WT and Nfatc1⁻/⁻ CTLs that were cultured in IL-2-containing medium for 6 d. Although we observed a drop in Slc2a1 and Hk2 RNA levels, we observed a similar glucose uptake and glycolysis of these cells (Supplementary Fig. 9c–f). Even upon re-stimulation of CTL- cells by αCD3/CD28 for 16 h, no
differences in glycolysis and glycolytic capacity were detected between both types of cells (Supplementary Fig. 9).

These findings do not exclude that NFATc1 affects also the metabolism of CTLs. The strong NFATc1 binding to the Slc7a5 gene (Supplementary Fig. 10a) encoding a leucine transporter that coordinates the metabolic reprogramming of CTLs\(^1\) suggests that in CTLs NFATc1 controls metabolic pathways other than glycolysis.

While IL-2 enhanced the level of glycolysis of Nfatc1\(^{-/-}\) aCD8\(^+\) Ts to that of Nfatc2\(^{-/-}\) cells, it did not completely rescue glycolysis to the level of WT cells. This suggests that genes in addition to Il2 that affect glycolysis might be controlled by NFATs. Candidates are the Irf4 and Myc genes. Both control the metabolic re-programming of T cells upon TCR stimulation\(^2\), and their RNA levels were markedly diminished in Nfatc1\(^{-/-}\) aCD8\(^+\) Ts, and the Irf4 gene showed NFATc1 binding (Fig. 6g; Supplementary Figs 6 and 10b).

**Discussion**

The results of our study show that NFATc1 controls the effector function of CD8\(^+\) T cells at multiple levels. Nfatc1\(^{-/-}\) aCD8\(^+\) T cells exhibit a decrease in proliferation and metabolic switch from OXPHOS to glycolysis, and—at the molecular level—in a poor RNA expression of genes coding for numerous glycolytic genes. This is mainly due to the missing IL-2 whose expression is induced by NFATc1. The diminished expression of Tbx21, encoding a key transcription factor of effector CD8\(^+\) T cells, and of Gzmmb gene are further molecular signs of defective function of Nfatc1\(^{-/-}\) CD8\(^+\) T cells. Upon activation, Nfatc1\(^{-/-}\) CTLs exhibit an impaired depletion of apical F-actin and recruitment of lytic granules and mitochondrial to the IS, and in a strong reduction in induction of numerous cytokine and chemokine genes. These include, apart from the Il2 gene, the genes encoding IFN-γ and IL-3, and the chemokine Ccl3, Ccl4, and Xcl1 genes (Fig. 4). These defects culminate in the defective eradication of infected and malignant cells.

In CTLs, NFATc1 and NFATc2 overlap in their binding to almost all genes (and sites) but differ strongly in their transcriptional activity (Figs 4 and 5). These data seem to contradict published data on the role of NFATs, in particular of NFATc2 (NFAT1), in the exhaustion of CD8\(^+\) T cells\(^19\), an anergy-like state of activated CD8\(^+\) T cells chronically infected with viruses. However, they are not necessarily in conflict with a promoting role of NFATs in CD8\(^+\) T cell exhaustion. NFATc1 and NFATc2 differ in their expression in activated lymphocytes. While short immune receptor signals trigger the rapid translocation of both factors into the nucleus, more persistent signals induce the transcription of Nfat1 gene leading to the massive synthesis of NFATc1/αA within a few hours (Fig. 3d). Although within several days the levels of NFATc2 also increase by persistent TCR signals (Fig. 3e), signals other than TCR triggering result in the further increase of NFATc2 in peripheral T cells\(^34\). These and further data led us to conclude that the Nfat1 gene codes for (at least) two factors which differ remarkably in their transcriptional properties\(^35\). In our view (i) NFATc1/αA which is highly expressed in activated T cells (Fig. 3d) supports ‘immunity’, i.e. the effector function of lymphocytes, including glycolysis, whereas (ii) NFATc1/βC (and other longer NFATc1 isoforms) which is mainly expressed in resting T cells, supports the induction of ‘tolerance’ and ‘exhaustion’ of lymphocytes\(^35\). The latter shares peculiarities with NFATc2, such as two SUMOylation sites within the C-terminal domain that is missing in NFATc1/αA, and SUMOylation converts NFATc1/βC to a suppressor of IL-2 induction\(^36\).

In a study on the ablation of NFATc1 (NFAT2) in murine CD8\(^+\) T cells, Pachulec et al.\(^1\) described an increase in the percentage of CD8\(^+\)CD44\(^{hi}\)CD122\(^+\) cells in thymus, spleen and LNs of unchallenged Nfatc1\(^{-/-}\) x CD4-cre mice which the authors classified as innate-like CD8\(^+\) T cells with characteristics of conventional memory CD8\(^+\) T cells. In our Listeria infection assays, we observed a slight increase in the number of Nfatc1\(^{-/-}\) TCM, compared to WT cells, and a decrease of Nfatc1\(^{-/-}\) TEM cells (Fig. 2e). The latter showed a decrease in their cytotoxicity (Supplementary Fig. 3a), similar to the findings published by Pachulec et al.\(^1\) for Nfatc1\(^{-/-}\) CD8\(^+\) T cells.

We focused our attention on the molecular events controlled by NFATc1 (and NFATc2) during the activation and differentiation of CD8\(^+\) T cells to CTLs in vitro generated mostly from Nfatc1\(^{-/-}\) x CD4-cre mice as well. Those mice showed a normal development of peripheral CD4\(^+\)/CD8\(^+\) T cells\(^37\). Several of our data were also confirmed by using Nfatc1\(^{-/-}\) x dck-cre mice in parallel, in which the Nfat1 gene was inactivated in peripheral T cells, i.e. after thymic selection\(^30\). Taken together, our NGS and ChIP-seq data on the genome-wide gene control by NFATc1 in CD8\(^+\) T cells confirm and extend the data by Pachulec et al.\(^1\) on the NFATc1-mediated control of IFNγ production in CD8\(^+\) T cells.

NFATc1 binds to numerous genes controlling the activity and fate of CD8\(^+\) T cells, but several physiological events of CD8\(^+\) T cells are also indirectly controlled by NFATc1. One example is the metabolic switch from OXPHOS to glycolysis during T cell activation, others might be the diminished proliferation (Fig. 6a) and enhanced apoptosis of Nfatc1\(^{-/-}\) lymphocytes\(^38\). In Nfatc1\(^{-/-}\) CD8\(^+\) T cells, the expression of numerous genes encoding glycolytic enzymes are impaired, and glycolysis is decreased to less than 50% of normal level in WT cells. Adding IL-2 to aCD3/CD28-stimulated Nfatc1\(^{-/-}\) CD8\(^+\) T cells restored the defect to a large extent. In line with published studies\(^39\)-\(^41\) this implies that in addition to TCR signals IL-2 supports the metabolic switch from OXPHOS to glycolysis during primary T cell activation.

In ChIP-seq assays both NFATc1 and NFATc2 were found to bind at and near the promoter of the Il2 gene (Fig. 5e). However, NFATc2 ablation did not or very mildly affect Il2 expression, whereas NFATc1 ablation strongly impaired Il2 induction in both aCD8\(^+\) Ts and CTLs + . It is currently unclear why NFATc1—and not NFATc2—ablation exerts such a deleterious effect on Il2 transcription. One explanation might be the high concentrations of NFATc1 in aCD8\(^+\) T and CTL + cells upon TCR stimulation (Fig. 3d). It is known that the Il2 gene needs a certain threshold level for NFATs to be transcribed\(^42\),\(^43\) which might be reached by NFATc1, but not NFATc2 expression. In contrast to this all-or-none expression of the Il2 gene, the transcription of Ifng and Il3 genes seems to follow a gradual way that is affected by either NFATc1 or NFATc2 ablation (Fig. 5e).

In addition to IL-2, the diminished expression of other factors, such as of Irf4 and c-Myc, might also contribute to the impaired metabolic switch to glycolysis in Nfatc1\(^{-/-}\) aCD8\(^+\) Ts. The TCR-mediated expression of c-Myc was shown to bind to and to enhance the expression of numerous glycolytic genes\(^28\). A further candidate is the NFAT target gene Pldcl1 encoding the repressor PD-1 that was shown to affect glycolysis\(^45\). In a recent study high concentrations of intracellular l-arginine were reported to suppress glycolysis and to generate central memory-like T cells\(^46\).

Numerous cytokine and chemokine genes are direct NFATc1 targets, and by regulating those genes NFATc1 seems to control the mobility and motility of CTLs and, thereby, their cytotoxicity. It is unknown whether such NFATc1-dependent key molecules control also the cytoskeleton re-organization and recruitment of
mitochondria and lytic granules to the IS. The interplay between mitochondria and Orai1 channels at the IS is highly relevant for the generation of local (at the IS) and global Ca2+ signals (in the cytosol and in the nucleus). Mitochondria take up Ca2+ and reduce local Ca2+ domains at the IS, which in turn inhibit Ca2+ dependent inactivation of Orai1 channels.48, 49. Thus, mitochondria control local and global Ca2+ signals following TCR induced Ca2+ entry by Orai1 channels. By controlling Ca2+ signals, mitochondria regulate NFAT translocation into the nucleus in CD4+ lymphocytes.48

While we did not detect defects in the expression of numerous formin genes that orchestrate the formation of cytoskeleton,49 in CTLs a strong reduction was observed in the expression of NFATc1-target genes Actn1 and Plek (Supplementary Fig. 6). The expression of Actn1 gene encoding α-actinin 1 contributes to T cell activation by forming multiprotein complexes with Lck and Rack1 that couple antigen with TCR triggering, and it was shown to participate in T cell migration.47, 51

Taken together, our study revealed numerous novel NFATc1 targets in cytotoxic T cells. The detailed knowledge of their NFAT-mediated control will help to interfere with the molecular mechanisms that control the activity of cytotoxic T cells to eradicate infected and malignant cells.

Methods

Mice and T cell activation. All mice used in the experiments were 8-12 weeks old, at C57/B6 background and sex- and age-matched. The T cell-specific CD4−/− mice have been described.52 Two Nfatc1−/− mice lines (also designated as Nfat2−/− mice,31, 37, 35) and conventional Nfat1−/− mice53 were used. The c.n:Nfat1α x Nfatc1−/− dclk−/− mice express a constitutively active version of NFATc1/αα from the Rosas2 locus upon removal of a ‘floxed’ STOP sequence in peripheral T cells. Animal experiments were performed according to protocols approved by local licenses (55.2-3331.01-5/310B and -76/14), which are approved by the Regierung von Unterfranken, Würzburg. For CD8+ T cell isolation, the mouse CD8 (Ly2) microbeads kit (Milteny Biotech) was used according to the manufacturer’s protocol. After purification, the purity of CD8+ T cells was checked by flow cytometry. Cells were suspended in X-vivo medium to a final concentration of 2 x 10^6/ml. For CD3/CD28 stimulation, if not stated otherwise 5 μg CD3e (clone 37.51) and 2 μg CD28 (clone 145-2C11) (both BD Pharmingen) were diluted in 1 ml PBS which was used to coat multi well plates. T cells were also stimulated with 10 ng/ml TPA and 0.5 μM ionomycin (normally for 5 h). To generate CTLs, CD8+ T cells were transduced by CD3/CD28 for 2-3 h followed by incubation of (5-6) d in medium containing 100 μl/ml IL-2. In some assays, CD8+ T cells were co-cultured for 6 d with splenocytes from a Balb/c mouse that were irradiated (by 30 Gy, 5-, 16 kV).

Cytotoxicity assays. In the first assay (Fig. 2a), the mineral oil induced murine MOPC-315.BMP2.FUGL55 plasmacytoma cell line (H2b) expressing eGFP and luciferase was used as target cell line for killing assays. This cell line originates from an allogeneic donor and, therefore, allo-reactive B6 CTLs recognizing the MHC I and II molecules were generated. The mineral oil induced murine MOPC-315.BMP2.FUGL55 plasmacytoma cell line (H2b) expressing eGFP and luciferase was used as target cell line for killing assays. This cell line originates from an allogeneic donor and, therefore, allo-reactive B6 CTLs recognizing the MHC I and II molecules were generated. The strain is defective in the mission of replicated bacteria, and thereby increases virulence up to 1000 fold.25

Infections with Listeria monocytogenes. For Listeria infections in vivo, an attenuated Listeria strain Lm-Ova ΔActa was used. The strain is defective in the mission of replicated bacteria, and thereby increases virulence up to 1000 fold.25

Immunoblotting and PCR assays. Immune blots were performed with whole protein extracts on PAGE-SDS gels followed by detection of NFATc1 using 7A6 mAb #556602, or for NFATc2 mAb #506574 (both BD Pharmingen). For detecting NFATc1α/α, a pAb raised against the NFATc1α peptide Ab #5G-45 (Biorbyt, Cambridge) was used. As an actin loading control, filters were stained with 0.1% Ponceau Red (PR in Fig. 7) and/or re-probed with the mAb αb8227 specific for β-actin (Abcam). Signals were developed using a chemiluminescence detection system (Thermo Fisher Scientific).

For RT-PCR assays, RNA was isolated from washed and deep-frozen cells by the TRIzol/isopropanol protocol. cDNAs were synthesized using the iScript cDNA synthesis kit according to the manufacturer’s instructions (Bio-Rad). Real-time PCR assays were performed using the SYBR Green I detection system (Bio-Rad). Primers for the amplification of the target genes were designed according to the published data (National Center for Biotechnology Information, National Library of Medicine) and are listed in Supplementary Table 1.
green master mix (Applied Biosystem) using the primers presented in Supplementary Table 1, and as described previously.8

Flow cytometry. T cells were washed once in cold PBS containing 0.1% BSA (FACS buffer) before blocking with anti-FcγRII/III (2.4G2, BD Pharmingen, San Diego, CA). Stainings were performed on ice using conjugated mAbs (eBioscience, San Diego, CA, if not stated otherwise), diluted 1:300 (1:200 for intracellular cytokines) in FACS buffer followed by incubation for 20 min. After washing with FACS buffer, cells were analyzed on a FACS Canto II (BD) and FlowJo software (Tree star, Ashland, OR). The following Abs were used: CD8-FTTC (#11-0081-82), IL-2-APC (#17-7021-81), IFN-γ-APC (#17-3111-82), CD62L-PE (#12-0621-82), CD44-APC (#17-0441-81), Perforin-APC (#17-9392-80), Granzyme B-PE (#12-8898-80). Abs against TNF-PE (#130-092-245), T-bet-PE (#130-092-246) and CD44-APC (#17-0441-81) were from Miltenyi Biotec. For intracellular staining, the fixation and permeabilization kit (Plus Brefeldin A; eBioscience, Cat. no. 88-8823-88) was used according to manufacturer’s recommendation.

RNA seq assays. RNA from deep-frozen CD8+ T cells was extracted using Trizol. Quantity of total RNA was assessed with the Qubit 2.0 and quality was checked using a RNA 6000 Nano chip on Agilent’s bioanalyzer. Sequencing libraries were prepared from 600 ng total RNA using a NEBnext ultra RNA library prep kit following the manufacturer’s instruction. The resulting barcoded CDNA libraries were sequenced on an Illumina HiSeq 2500 platform for 50 nucleotides (single end).

Imaging of cytoskeleton dynamics in CTLs. CTLs in warm RPMI/0.5% BSA medium were activated on glass bottom chamber slides (μ-slides, Ibidi, Germany) coated with collagen I and stimulated with irradiated BALB/c splenocytes. After 5 min, cells were immediately stopped by covering cells with a pre-warmed fixation solution of 4% formaldehyde and 0.05% glutaraldehyde in PBS for 10 min at 37°C. Samples were rinsed in PBS, permeablized with 0.1% Triton X-100 for 5 min, treated with 50 mM NH4Cl for 10 min and blocked with 5% BSA. For visualization of filamentous F-actin cells were stained with phalloidin 488 (Actin-stain, Cytokeleton) and for MTOC reorientation with an anti-β-tubulin primary Ab (clone 9F3, Cell Signaling) overnight at 4°C followed by incubation with anti-rabbit-Alexa 568 secondary Ab (Invitrogen) for 45 min at RT and a final step with DAPI for nuclear staining. Preparations were stored in PBS for total reflection (TIRF) microscopy and data were obtained using a Leica AM TIRF system with software AF 6000 LX, equipped with a 100x oil objective (HCX PL APO, NA 1.47) and laser lines 488 and 561 nm. For confocal laser scanning microscopy (CLSM) samples were stored with mounting medium (Ibidi). Images were acquired on a Zeiss LSM 780, provided with 40x and 63x oil objectives (Plan-Apochromat, NA 1.4) and laser excitation at 405, 488 and 561 nm and processed with software ZEN 2012.

To quantify the size of F-actin depletion zone imaged by TIRF microscopy and the increase in cellular spreading taken by CLSM on Ab-coated surfaces, cell area was calculated after setting a manual threshold and measured in square micrometer using the software package Fiji.[59] For morphological examination under a field scanning electron microscope (SEM), CTLs were co-stimulated using 12 mm glass slices in a 24-well plate, fixed in pre-warmed 6.25% glutaraldehyde in 50 mM phosphate buffer (pH 7.2) for 15 min at RT and subsequently overnight at 4°C. After three washing steps in PBS, samples were prepared by stepwise dehydration in acetone, then critical point dried, sputtered with gold/palladium and imaged on an FEI Quanta 200 scanning electron microscope.

Imaging of recruitment of lytic granules and mitochondria. C57/B6 CD8+ T cells were purified and stimulated with irradiated BALB/c splenocytes as described above. On 5 d after isolation, CTLs were sorted on a BD FACSAria TMIII (70 μm nozzle; gating on FSC and SSC, cooled reservoir) to remove dead cells and debris. CTLs were cultured overnight in X-vivo medium, loaded with either 400 nM LysoTracker Green DND-26 and 100 nM MitoTracker Deep Red FM or 400 nM Lysotracker Red DND-99 and 100 nM MitoTracker Green FM for 30 min at 37°C, washed and transferred into a black clear-bottom imaging microwell plate (Greiner 781096, 384 wells) in X-vivo medium containing either 400 nM LysoTracker Green DND-26 or 400nM LysoTracker Red DND-99 (13×10^5 CTL/well). Shortly before the start, 2×10^5 Dynabeads (Mouse T-Activator CD3/CD28 beads; Life Technologies, 11456D) were added to each well. Time-lapse imaging was performed on a Zeiss AxioObserver at 37°C and 5% CO2 with a Fluor 20 ×0.75 objective, using transmitted light and epifluorescence with filter set 38 HE (Ex 470/40, Em 525/50) and a custom filter set (Ex 620/60, Em 700/ 75). Background fluorescence was subtracted, and the polarization of mitochondria and lysosomes was quantified (assuming stable polarization when the fluorescence signal was close to the contact zone for more than 3 images (or 60 s)). For the intensity profiles shown in Supplementary Fig. 1c, cells in stable contact with beads for at least 10 frames (200 s) were analyzed if cell location was plane to the contacting bead (the cell did not lie above or below the bead), no labeled organelles were located above or below the bead and the cell diameter was larger than 5 μm in order to faithfully localize the proximal and distal part of the cell. The selected cells at the chosen time points were analyzed with Fuji ImageJ 2.0 using the “Line Tool” (for round or elongated cell morphology) or the “Segmented Line Tool” (for cells with non-symmetric morphology). A line was drawn from the contacting bead towards the center of the IS and further along the midline of the cell body to the rear of the cell and intensity values were measured along this line.

Metabolic assays. In extracellular flux assays, ECAR of CD8+ T cells was measured with a XF96 analyzer (Seahorse Bioscience). 4× 10^5 freshly prepared CD8+ T cells from WT, Nfatc2−/− x CD4-cre and Nfatc2−/− mice were maintained overnight without stimulation or stimulated by αCD3/CD28. In parallel, the same number of CTL- cells were stimulated for a further day by αCD3/CD28. Upon confluence, cells were seeded in a 24-well plate coated with poly-D-lysine (Sigma; 50 μg/ml) in XF Seahorse medium with glutamine alone. Then, 10 mM glucose was injected, followed by 1 μM oligomycin and 20 mM 2-deoxyglucose. Mito stress test assays for the determination of oxygen consumption rate (OCR) were performed in a similar way according the manufacturers protocol (Seahorse Bioscience). 2- NBDG (Cayman Chemical) assays were performed by incubation of cells with 50 μM 2-NBDG for 1 h at 37°C followed by flow cytometry.

Statistical analysis. Statistical analyses were performed using GraphPad (Prism) software, version 6.0. Data presented as mean and error bars in figures represent ± SEM. Unpaired t-tests were performed to evaluate the statistical significance of the data set. Statistical significances were calculated and indicated (***p<0.001, **p<0.005 and *p<0.05).

Data availability. Sequence data that support this study have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE98726. For analysis of NFATc2 binding, data from GSE64409 were used. All other data supporting the findings of this study are available with the article, and can also be obtained from the authors.

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Author contributions
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