Supplemental information

Mitochondrial metabolism coordinates stage-specific repair processes in macrophages during wound healing

Sebastian Willenborg, David E. Sanin, Alexander Jais, Xiaolei Ding, Thomas Ulas, Julian Nüchel, Milica Popović, Thomas MacVicar, Thomas Langer, Joachim L. Schultze, Alexander Gerbaulet, Axel Roers, Edward J. Pearce, Jens C. Brüning, Aleksandra Trifunovic, and Sabine A. Eming
Figure S1. Related to Figure 1

- **A**: CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> wildtype wound MΦs
  - 4 RNAseq 4 dpi
  - 5 RNAseq 14 dpi

- **B**: Enrichment score
  - PC #1 (38.6%)
  - PC #2 (10.8%)

- **C**: PCA
  - 4 dpi
  - 14 dpi

- **D**: Enrichment score
  - Glyceraldehyde-3-phosphate biosynthetic process
  - Heme metabolic process
  - Pos. regulation of oxidative phosphorylation uncoupler activity
  - Mitochondrial translational elongation
  - Neg. regulation of mitochondrial membrane permeability in apoptotic process
  - Mitochondrial ATP synthesis coupled proton transport
  - Regulation of oxidative phosphorylation uncoupler activity
  - Citrate metabolic process
  - Succinate metabolic process
  - Oxalacetate metabolic process
  - Carnitine metabolic process, CoA-linked
  - Transcription initiation from mitochondrial promoter
  - Regulation of pyruvate dehydrogenase activity
  - tRNA aminoacylation for mitochondrial protein translation
  - Mitochondrial RNA 3'-end processing
  - ATP transport
  - Carnitine metabolic process
  - Mitochondrial DNA replication
  - Mitochondrial acetyl-CoA biosynthetic process from pyruvate

- **E**: Gene expression [fold increase]
  - Hif1a
  - Slc2a1
  - Pfkfb3
  - Pdk1
  - Ldha
  - Lipa
  - Mgll
  - Acadm
  - Crot

- **ANOVA**
  - Fold change: >2
  - FDR P value: 0.05

- **differentially expressed genes**
- **PCA**
- **HC**
- **PEA**
- **QC/QA**

- **4 dpi**
- **14 dpi**
Figure S3. Related to Figure 3

A

![Graph of Dars2 expression fold change over days post injury.]

B

![Scatter plots of CD45^{+}CD11b^{+}F4/80^{+} cell gate for different days post injury.]

C

![Graph of fold change in RFP^{+} macrophages at 4 dpi and 14 dpi.]

D

![Images of tissue sections stained with H&E.](Day 4 post injury)

E

![Plots showing relative and absolute numbers of CD45^{+}CD11b^{+}F4/80^{+} cells per wound at 4 dpi and 14 dpi.]

---

Control | Dars2^{MKO}
---

**Note:** Figures A, B, C, D, and E illustrate the expression and distribution of Dars2 and other cell types over time post injury.
Figure S4. Related to Figure 4

A

B

4 dpi 14 dpi

Sod2 Gsr Gclc Gclm

-2 -1 0 1 2
Row Z-Score

4 dpi 14 dpi

Sdha Sdhb Sdhc Sdhd Sdha1 Sdha2 Sucl2 Suclg1 Suclg2
Figure S5. Related to Figure 6

A

Number of CD11b F4/80+ peritoneal cells [x 10^6]

|                | NaCl | IL-4c |
|----------------|------|-------|
| Control        | ![Control](image1) | ![Control](image2) |
| Dars2^MKO      | ![Dars2^MKO](image3) | ![Dars2^MKO](image4) |

** p < 0.01

B

- Gate: 7-AAD^+ single cells
- Gate: G1
- Gate: G2

| Condition       | MFI   | Events | Mean fluorescence intensity |
|-----------------|-------|--------|----------------------------|
| Vehicle         | 32954 | ![Vehicle](image5) | ![Vehicle](image6) |
| IL-4 + IL-13    | 48895 | ![IL-4 + IL-13](image7) | ![IL-4 + IL-13](image8) |

** p < 0.01

Control Dars2^MKO
Figure S6. Related to Figure 7

A

![PC1 vs PC2 plot](image)

- **Stimulus**:
  - M(−)
  - M(IL-4+IL-13)

- **Genotype**:
  - Control
  - Dars2MKO

B

|       | M(−)       | M(IL-4+IL-13) | Cluster |
|-------|------------|---------------|---------|
| Control | Dars2MKO   | Control       | Dars2MKO|
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |
|        |            |               |         |

Cluster 1-20
Figure S1. Related to Figure 1. Distinct metabolic transcriptional profiles in early and late phase wound MΦs

(A-D) Bulk RNA-seq analysis of sorted wound MΦs isolated from wild-type mice at 4 and 14 dpi. 1 technical replicate of \( n = 4 \) biological replicates at 4 dpi, 5 at 14 dpi.

(A) Schematic illustrating the workflow of the bulk RNA-seq analysis.

(B) PCA based on all identified genes.

(C) HC showing the z-transformed expression values of the 1,000 most variable genes.

(D) GOE analysis of genes encoding proteins with mitochondrial localization (based on MitoCarta 3.0 datasets) and being differentially expressed in early versus late phase wound MΦs.

(E) qRT-PCR in sorted wild-type wound MΦs at indicated time points normalized to wild-type blood monocytes. 1 technical replicate of \( n = 5 \) biological replicates per group for BM, 5 at 2 dpi, 4 at 4 dpi, 4 at 14 dpi. \(*P < 0.05, **P < 0.01, ***P < 0.001\) by 1-way ANOVA with Tukey's Multiple Comparison Test. BM, blood monocytes.

Figure S2. Related to Figure 3. Efficient Dars2 gene deletion in BMDMs isolated from \( Dars^{MKO} \) mice

(A) Scheme illustrating the Dars2 gene construct and the 2 \( loxP \) sites flanking exon 3 along with the PCR fragment length before and after successful recombination.

(B) PCR analysis of genomic DNA isolated from BMDMs cultured in growth medium. 1 technical replicate of \( n = 2 \) biological replicates per genotype.

(C) qRT-PCR analysis of Dars2 gene expression in BMDM cultured in growth medium. 1 technical replicate of \( n = 7 \) biological replicates (control), 6 (\( Dars^{MKO} \)).

(D) Western blot analysis of DARS2 and subunits of MRC complexes in BMDM cultured in growth medium. Dashed line indicates the use of different gels. 1 technical replicate of \( n = 2 \) biological replicates per genotype.
(E) Quantification of absolute numbers of BMDMs cultured in growth medium. 1 technical replicate of $n = 12$ biological replicates (control), 11 ($Dars2^{MKO}$).

(F) Quantification of cell viability of BMDMs cultured in growth medium analyzed by trypan blue staining. 1 technical replicate of $n = 12$ biological replicates (control), 11 ($Dars2^{MKO}$).

(G) EFA of ECARs in BMDMs cultured for 24 h in growth medium. $n = 8$ technical replicates of 1 biological replicate per genotype. The mean value ± SEM is represented.

(H) qRT-PCR analysis in BMDMs stimulated with LPS (50 ng/mL) and IFN-$\gamma$ (50 ng/mL) [M(LPS+IFN-$\gamma$)] for 5 h or with IL-4 (50 ng/mL) and IL-13 (50 ng/mL) [M(IL-4+IL-13)] for 24 h or with vehicle [M(-)]. 1 technical replicate of $n = 4$ biological replicates per group.

*P < 0.05, **P < 0.01, ***P < 0.001 by Student’s unpaired two-tailed $t$-test (C) or 1-way ANOVA with Tukey's Multiple Comparison Test (H).

**Figure S3. Related to Figure 3. Wound healing response in Dars2^{MKO} mice**

(A) qRT-PCR analysis of Dars2 gene expression in sorted wound MΦs normalized to blood monocytes. 1 technical replicate of $n = 3$ biological replicates per group for BM, 8 controls at 4 dpi, 6 $Dars2^{MKO}$ at 4 dpi, 4 per group at 14 dpi. BM, blood monocytes.

(B and C) Flow cytometric analysis of RFP$^+$ macrophages among wound MΦs at 4 or 14 dpi. RFP$^+$ monocytes were adoptively transferred either at 2 or at 12 dpi. Representative flow cytometry plots ($n = 5$ per group in total) (B) and quantification of the fold change of RFP$^+$ cells within total MΦs normalized to the group which received monocytes at 2 dpi and was analyzed at 4 dpi (C). 1 technical replicate of 5 biological replicates per group.

(D) Representative H&E-stained wound sections at 4 dpi ($n = 13$ control, 14 $Dars2^{MKO}$ wounds in total) and quantification of fibrin-rich granulation tissue. d, dermis; e, epidermis; gt, granulation tissue; he, hyperproliferative epithelium; sm, skeletal muscle. Dotted line underlines the newly formed epithelium. White and black dashed lines indicate the gt and the
fibrin rich-area, respectively. Arrows indicate the epithelial tips. Scale bar, 500 µm. 1 technical replicate of 13 biological replicates (control), (14 Dars2^MKO).

(E) Relative and absolute numbers of MΦs per wound at indicated time points post injury. One technical replicate of n = 5 biological replicates per group at 4 dpi, 4 at 14 dpi.

*P < 0.05, **P < 0.01, ***P < 0.001 by Student’s unpaired two-tailed t-test (A, D) or 1-way ANOVA with Tukey's Multiple Comparison Test (B, E).

Figure S4. Related to Figure 4. High expression of genes involved in ROS detoxification and succinate metabolism in early phase wound MΦs

(A, B) Heatmaps of the bulk RNA-Seq analysis in sorted wound MΦs isolated from wild-type mice at 4 and 14 dpi showing the z-transformed expression values of indicated genes. 1 technical replicate of 4 biological replicates at 4 dpi, 5 at 14 dpi.

Figure S5. Related to Figure 6. Attenuated IL-4c-induced proliferation of tissue-resident MΦs in Dars2^MKO mice

(A) Quantification of CD11b^F4/80^ peritoneal MΦs in control and Dars2^MKO mice treated as indicated. 1 technical of n = 3 biological replicates per genotype in the NaCl group, 7 controls in the IL-4c group, 8 Dars2^MKO in the IL-4c group.

(B) Representative flow cytometry plots (n = 5 per group in total) of mitochondrial mass analysis by MTG fluorescent staining in CD11b^F4/80^ BMDMs isolated from control mice. 7-AAD/CD11b^F4/80^ single cells were gated (G2) and the mean fluorescence intensity (MFI) of MTG was analyzed. 1 technical replicate of 5 biological replicates per group.

**P < 0.01 by Student’s unpaired two-tailed t-test (A, B).

Figure S6. Related to Figure 7. RNA-seq analysis in BMDM isolated from control and Dars2^MKO mice
(A, B) Bulk RNA-seq analysis in BMDMs isolated from control and $Dars2^{MKO}$ mice. 1 technical replicate of $n = 2$ biological replicates per group.

(A) PCA based on all identified genes. (B) HC showing the z-transformed expression values of the 2,810 differentially expressed genes comparing M(IL-4 + IL-13) versus M(-) separately for control and $Dars2$-deficient BMDMs. $P$ value $< 0.01$ and Log2FC $> 0.5$ or $<-0.5$. 
Table S1: Oligonucleotides used for mouse genotyping and qRT-PCR. Related to Figure 3, Figure 4, Figure 6, Figure 7, Figure S1, Figure S2, Figure S3.

| Oligonucleotides | Genotyping |
|------------------|------------|
| **Genotyping**   |            |
| Primer 1 Dars2 flox | Sigma-Aldrich N/A |
| 5’TCTGGAATTCTAGGCCAGCCAC3’ |         |
| Primer 2 Dars2 flox | Sigma-Aldrich N/A |
| 5’TCTGGAATTCTAGGCCAGCCAC3’ |         |
| Primer 1 Lyz2-cre | Sigma-Aldrich N/A |
| 5’CTTGGGCTGCCAGAATTTC3’ |         |
| Primer 2 Lyz2-cre | Sigma-Aldrich N/A |
| 5’CTTGGGCTGCCAGAATTTC3’ |         |
| Primer 3 Lyz2-cre | Sigma-Aldrich N/A |
| 5’CCCAGAAATGCGCCAGATTACG3’ |         |
| Primer 1 Il4ra flox | Sigma-Aldrich N/A |
| 5’CCCTTCCTGGCCCTGAATTTC3’ |         |
| Primer 2 Il4ra flox | Sigma-Aldrich N/A |
| 5’CCCTTCCTGGCCCTGAATTTC3’ |         |
| Primer 1 Il4ra del | Sigma-Aldrich N/A |
| 5’GGCTGCGCTGGCCAGATTACG3’ |         |
| Primer 2 Il4ra del | Sigma-Aldrich N/A |
| 5’GGCTGCGCTGGCCAGATTACG3’ |         |

| qRT-PCR          |            |
|------------------|------------|
| Aft4 forward     | Sigma-Aldrich Kaspar et al., 2021 |
| 5’ AACATCAATCTCGTCCCG 3’ |         |
| Aft4 reverse     | Sigma-Aldrich Kaspar et al., 2021 |
| 5’ GTTCTTCAGCGAACAGGC 3’ |         |
| Arg1 forward     | Sigma-Aldrich N/A |
| 5’ GCCCGAAGCACTCAACGGGAGG 3’ |         |
| Arg1 reverse     | Sigma-Aldrich N/A |
| 5’ ACCAGAAGGAAGCAGTGGAGATAAC 3’ |         |
| Acadm forward    | Sigma-Aldrich PrimerBank ID: 158508465c1 |
| 5’ AACACAAACTCGAAGCAGG 3’ |         |
| Acadm reverse    | Sigma-Aldrich PrimerBank ID: 158508465c1 |
| 5’ TTCTGCTGTCGCAACTCA 3’ |         |
| Ccl22 forward    | Sigma-Aldrich PrimerBank ID: 154240695c1 |
| 5’ CTCTGGCATCAGCTTTAGTGA 3’ |         |
| Ccl22 reverse    | Sigma-Aldrich PrimerBank ID: 154240695c1 |
| 5’ GAGGGTATCAAAACACGCGC 3’ |         |
| Crot forward     | Sigma-Aldrich PrimerBank ID: 142387204c2 |
| 5’ GGTGGCTCAATGTTGCCTAC 3’ |         |
| Crot reverse     | Sigma-Aldrich PrimerBank ID: 142387204c2 |
| 5’ GGTGGCTCAATGTTGCCTAC 3’ |         |
| Ddit3 forward    | Sigma-Aldrich Kaspar et al., 2021 |
| 5’ TGCCCTTTACCCGAGACGG 3’ |         |
| Ddit3 reverse    | Sigma-Aldrich Kaspar et al., 2021 |
| 5’ CGCAGGGCTCAAGTAGTGAAGG 3’ |         |
| Dars2 forward    | Sigma-Aldrich PrimerBank ID: 141801674c3 |
| 5’ AGCTCTAAGAGATTGCCAGG 3’ |         |
| Dars2 reverse    | Sigma-Aldrich PrimerBank ID: 141801674c3 |
| 5’ GACCGGAGATTACTGTTCCAGG 3’ |         |
| Il6 forward      | Sigma-Aldrich N/A |
| 5’ ACACATGTCTCGGAAATC 3’ |         |
| Il6 reverse      | Sigma-Aldrich N/A |
| 5’ AAGTGCCATCATCGTTGCCATAC 3’ |         |
| Gclc forward     | Sigma-Aldrich PrimerBank ID: 324710985c2 |
| 5’ GGACAAACCAACCAACCATCC 3’ |         |
| Gclc reverse     | Sigma-Aldrich PrimerBank ID: 324710985c2 |
| 5’ GGACAAACCAACCAACCATCC 3’ |         |
| Gene | Forward | Reverse | Supplier | PrimerBank ID |
|------|---------|---------|----------|--------------|
| Gclm | 5´ GGACAAACCCCAACCATCC 3´ |  | Sigma-Aldrich | 142373116c3 |
| Gclm | 5´ CCTGCTCTTCACGATGACCG 3´ |  | Sigma-Aldrich | 142373116c3 |
| Gsr  | 5´ GACACCTCTTCTCGACTACC 3´ |  | Sigma-Aldrich | 160298212c1 |
| Gsr  | 5´ CACATCCAACATTCACGCAAG 3´ |  | Sigma-Aldrich | 160298212c1 |
| Hif1a| 5´ GGGGAGGACGATGAACATCAA 3´ |  | Sigma-Aldrich | 226061947c3 |
| Hif1a| 5´ GGGTGTTTCTTGTACCCACA 3´ |  | Sigma-Aldrich | 226061947c3 |
| Il10 | 5´ AGCCGGGAAGACAATAACTG 3´ |  | Sigma-Aldrich | N/A          |
| Il10 | 5´ CATTTCCGATAAGGCTTGG 3´ |  | Sigma-Aldrich | N/A          |
| Il1b | 5´ CGACCCCAAAAGATGAAGGGCTGC 3´ |  | Sigma-Aldrich | N/A          |
| Il1b | 5´ GCTCTTGTTGATGTGCTGCTGCG 3´ |  | Sigma-Aldrich | N/A          |
| Ldha | 5´ CAAAGACTCTGTGTAACTGCGA 3´ |  | Sigma-Aldrich | 257743038c1 |
| Ldha | 5´ TGGACTGTACTTGACAATGTTGG 3´ |  | Sigma-Aldrich | 257743038c1 |
| Lipa | 5´ CTGGTGAGGAACACTCGGTC 3´ |  | Sigma-Aldrich | 162287342c2 |
| Lipa | 5´ AGCCGACTAGGATGGAGATG 3´ |  | Sigma-Aldrich | 162287342c2 |
| Mgll | 5´ CGGACTTCCAAGTTTTTGTCAGA 3´ |  | Sigma-Aldrich | 6754690a1   |
| Mgll | 5´ GCAGCCACTAGGATGGAGATG 3´ |  | Sigma-Aldrich | 6754690a1   |
| Mrc1 | 5´ TGCCGACATGCCAGGACGAAA 3´ |  | Sigma-Aldrich | N/A          |
| Mrc1 | 5´ GTGGGCTCTGTTGGCGAGT 3´ |  | Sigma-Aldrich | N/A          |
| Nos2 | 5´ CCACCTTGGTGAAGAGACTGACTGCT 3´ |  | Sigma-Aldrich | N/A          |
| Nos2 | 5´ AGGGGCAAGCCATGTCTGAGACTGACT 3´ |  | Sigma-Aldrich | N/A          |
| Pdk1 | 5´ GGAATCTCCTGGTCAATGGC 3´ |  | Sigma-Aldrich | 227908810c1 |
| Pdk1 | 5´ TCCTGAGAGATTGCTCGGGGA 3´ |  | Sigma-Aldrich | 227908810c1 |
| Pklfb3 | 5´ AAAATCCCAACCTGTGATTGT 3´ |  | Sigma-Aldrich | 295293219c1 |
| Pklfb3 | 5´ TGAGGTAGCGAGTCAGCTTCT 3´ |  | Sigma-Aldrich | 295293219c1 |
| Retnl | 5´ TATGAACAGATGGGGCCTCCT 3´ |  | Sigma-Aldrich | N/A          |
| Retnl | 5´ GGCAGTTGCAAGATTGCCCTCAC 3´ |  | Sigma-Aldrich | N/A          |
| Slc2a1 | 5´ GCTGTGCTTCTGAGCTTC 3´ |  | Sigma-Aldrich | N/A          |
| Slc2a1 | 5´ CACATACATGGGCACAAAGC 3´ |  | Sigma-Aldrich | N/A          |
| Sod2 | 5´ AGAGAGCTCCTTCAGATATGG 3´ |  | Sigma-Aldrich | 31980762a1   |
| Sod2 | 5´ CTCGGGGCGTTGAGATTGT 3´ |  | Sigma-Aldrich | 31980762a1   |
| Gene   | Primer  | Supplier | Cat. No. |
|--------|---------|----------|----------|
| Tnfa   | forward | 5´GACCCTCACACTCAGATCATCTTCT 3´ | Sigma-Aldrich | N/A |
| Tnfa   | reverse | 5´CCTCCACTTGGTTGTTGCT 3´ | Sigma-Aldrich | N/A |
| Vegfa  | forward | 5´TGTACCTCCACCATGCCAAGT 3´ | Sigma-Aldrich | N/A |
| Vegfa  | reverse | 5´CGCTGGTAGACGTCATGAA 3´ | Sigma-Aldrich | N/A |
| Chil3  | reverse | 5´TACCAGTTGGCTAAGGACAGGCC 3´ | Sigma-Aldrich | N/A |
| Chil3  | forward | 5´ACTGAACGGGGCAGGTCAAACT 3´ | Sigma-Aldrich | N/A |