SUPPLEMENTAL INFORMATION

High salt reduces the activation of IL-4+IL-13 stimulated macrophages

Katrina J. Binger[1,11, 12], Matthias Gebhardt[1,11], Matthias Heinig[2], Carola Rintisch[2], Agnes Schroeder[3], Wolfgang Neuhofer[4], Karl Hilgers[3], Arndt Manzel[3], Christian Schwartz[3], Markus Kleinewietfeld[5], Jakob Voekl[6], Valentin Schatz[7], Ralf A. Linker[3], Florian Lang[6], David Voehringer[3], Mark D. Wright[8], Norbert Hubner[2], Ralf Dechend[1,9], Jonathan Jantsch[7], Jens Titze[3,10], Dominik N. Muller[1,2,12]

Containing:

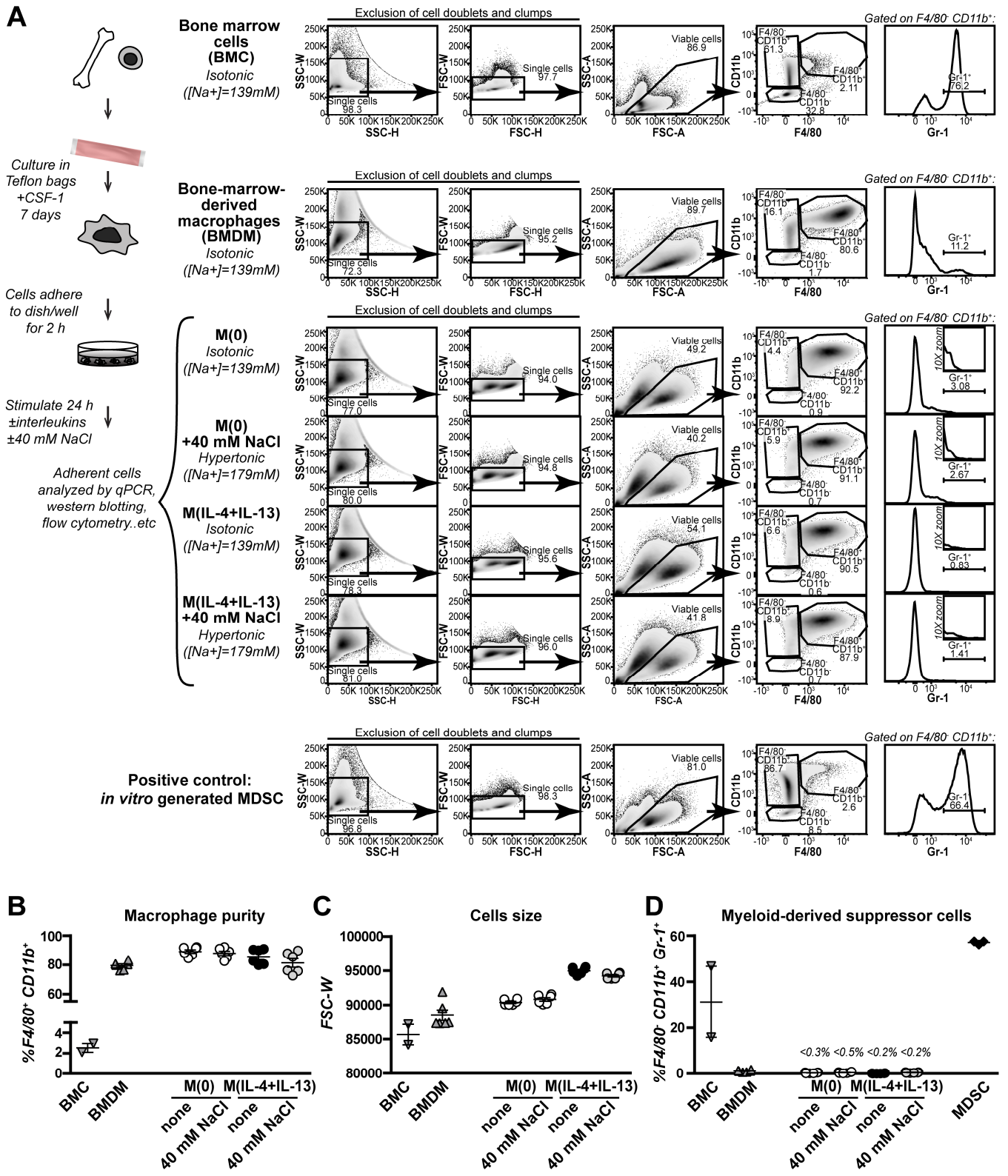
Supplemental Figure 1-11

Supplemental Table 1,3,4

Supplemental Experimental Procedures

Supplemental References

Supplemental Figure 1, *related to Figure 1*



Supplemental Figure 1. Characterisation of *in vitro* generated macrophages by flow cytometry.
A) Schematic for the experimental set-up. Bone marrow cells (BMC) from C57Bl/6 mice were differentiated into bone marrow-derived macrophages (BMDM) by incubating in the presence of CSF-1 for 7 days. This was performed in isotonic media. Preparations of BMDM were then plated into cell culture plates as required, allowed to adhere for 2 h before stimulation with IL-4+IL-13 in the absence (none) or presence of an additional 40 mM NaCl (or other condition as required). The final concentration of Na^+ during each step of the differentiation and activation procedure is indicated. Single cell preparations of each cell type were

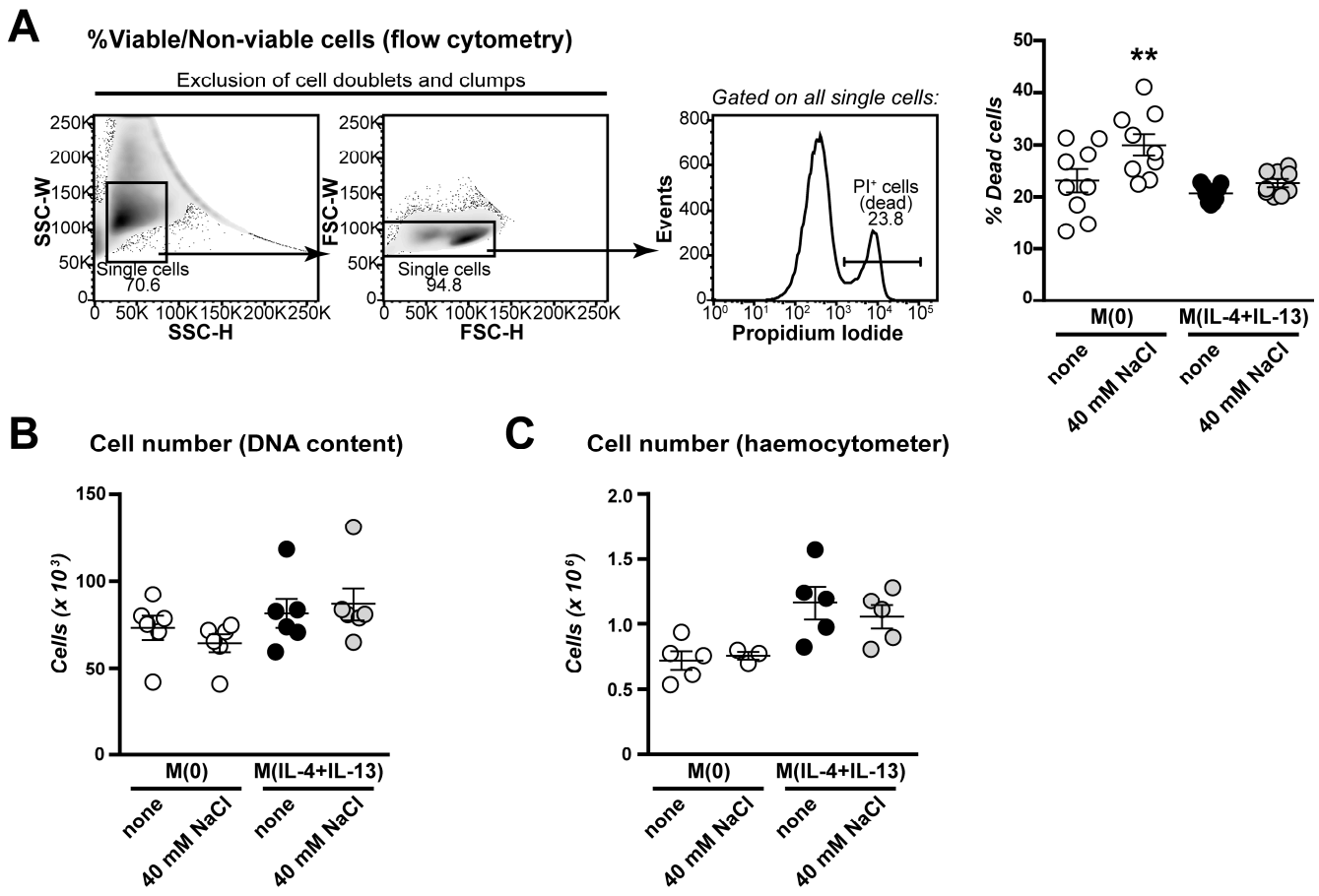
then analysed by flow cytometry to determine the cell size (FSC-W), macrophage purity (CD11b+F4/80+) and for the presence of myeloid-derived suppressor cells (MDSCs) ($F4/80^{-}CD11b^{+}Gr\text{-}1^{+}$). As a positive control for MDSC staining, BMC were incubated with IL-6 and GM-CSF for 4 days, to induce differentiation into MDSCs.

B) Quantification of macrophage ($CD11b^{+}F4/80^{+}$) purity from **A**.

C) Quantification of cell size (FSC-W) as from **A**.

D) Analysis of the proportion of MDSCs ($CD11b^{+}F4/80^{-}Gr1^{+}$) in the respective cell cultures as from **A**. A minor proportion of MDSCs (<0.2%) were detected in the IL-4+IL-13 stimulated macrophages used for the suppression assays.

Supplemental Figure 2, *related to Figure 1*

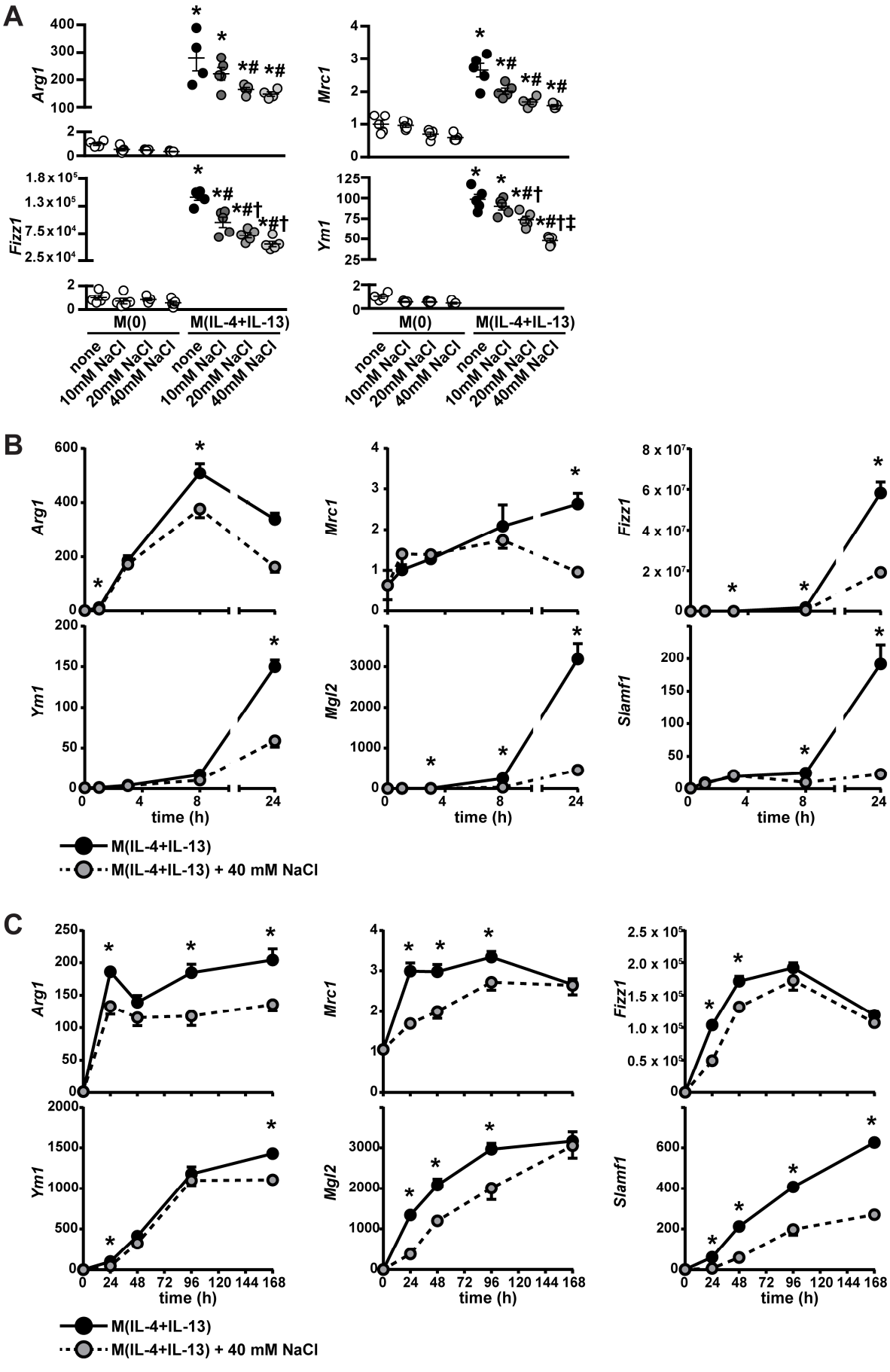


Supplemental Figure 2. Increased NaCl concentration of 40 mM is not associated with a decreased viability of IL-4+IL-13 stimulated macrophages.

A) The viability of unstimulated (M(0)) or IL-4+IL-13 stimulated macrophages alone or with an additional 40 mM NaCl was examined by monitoring the exclusion of a viability dye (Propodium Iodide; PI) by flow cytometry. The quantification is shown to the right. The experiment was repeated 3 times independently and then pooled. N=9 (technical). **p<0.01 vs M(0) none.

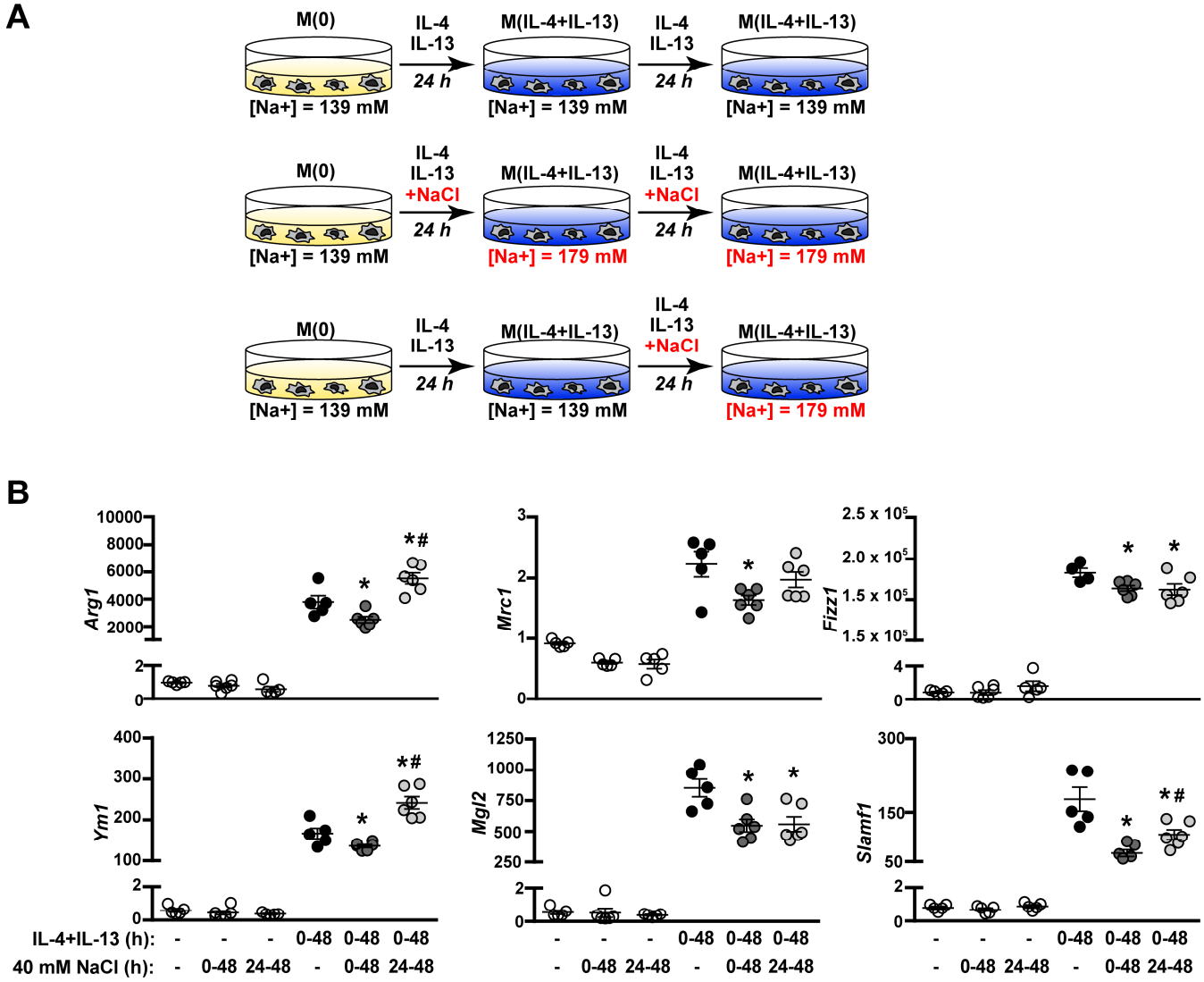
B&C) The number of cells following incubation for 24 h as in **A** was determined by measuring DNA content (CyQUANT) relative to a standard curve of known cell numbers (**B**), or by manually counting with a haemocytometer (**C**).

Supplemental Figure 3, ***related to Figure 1***



Supplemental Figure 3. Analysis of the dose- and time-dependency of reduced M(IL-4+IL-13) activation with high NaCl.
A) BMDMs were stimulated with IL-4 and IL-13 (M(IL-4+IL-13)) in the absence (none) or increasing concentrations of additional NaCl (10, 20 and 40 mM NaCl). Unstimulated (M(0)) macrophages were treated similarly. The expression of M(IL-4+IL-13) signature genes was determined by real-time PCR. The experiment was repeated at least three times independently. N=5 (technical). * $p<0.0001$ vs M(0); # $p<0.001$ vs M(IL-4+IL-13) none (no additional salt); †$p<0.05$ vs M(IL-4+IL-13) + 10 mM NaCl; ‡$p<0.05$ vs M(IL-4+IL-13) + 20 mM NaCl.
B&C) Macrophages were stimulated with IL-4+IL-13 alone or in the presence of an additional 40 mM NaCl. At the desired times, the expression of signature genes was determined by real-time PCR. The experiment was repeated twice independently. N=5 (technical). *$p<0.05$.

Supplemental Figure 4

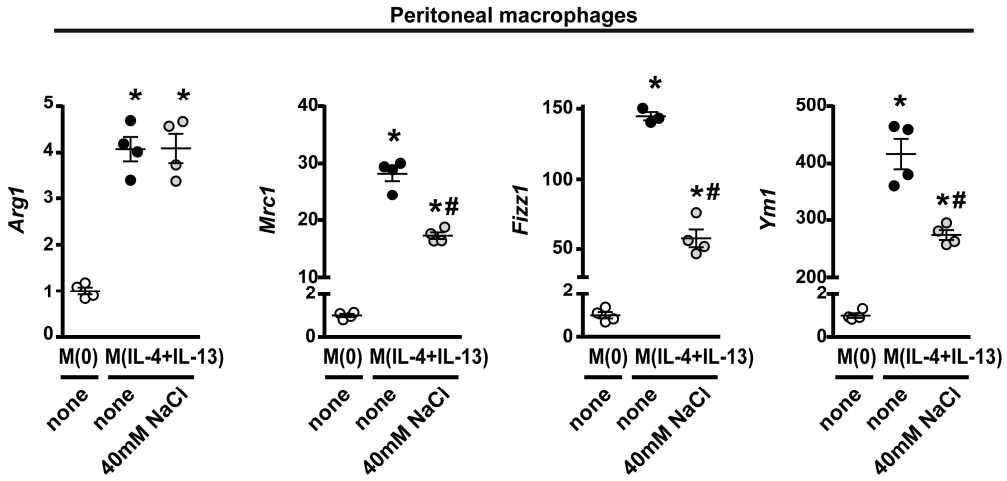


Supplemental Figure 4. Pre-stimulation to M(IL-4+IL-13) partially rescues the effect of salt on macrophage activation.

A) Schematic. Macrophages were left unstimulated, or stimulated with IL-4+IL-13 for a total period of 48 h. To test the effect of NaCl on the differentiation of macrophages, an additional 40 mM NaCl was added at t=0, and left for the duration of the experiment (0-48 h). To assess the effect of NaCl on pre-activated macrophages, cells were stimulated with IL-4+IL-13 in isotonic media for 24 h, before an additional 40 mM NaCl was added for the final 24 h of the experiment (24-48 h).

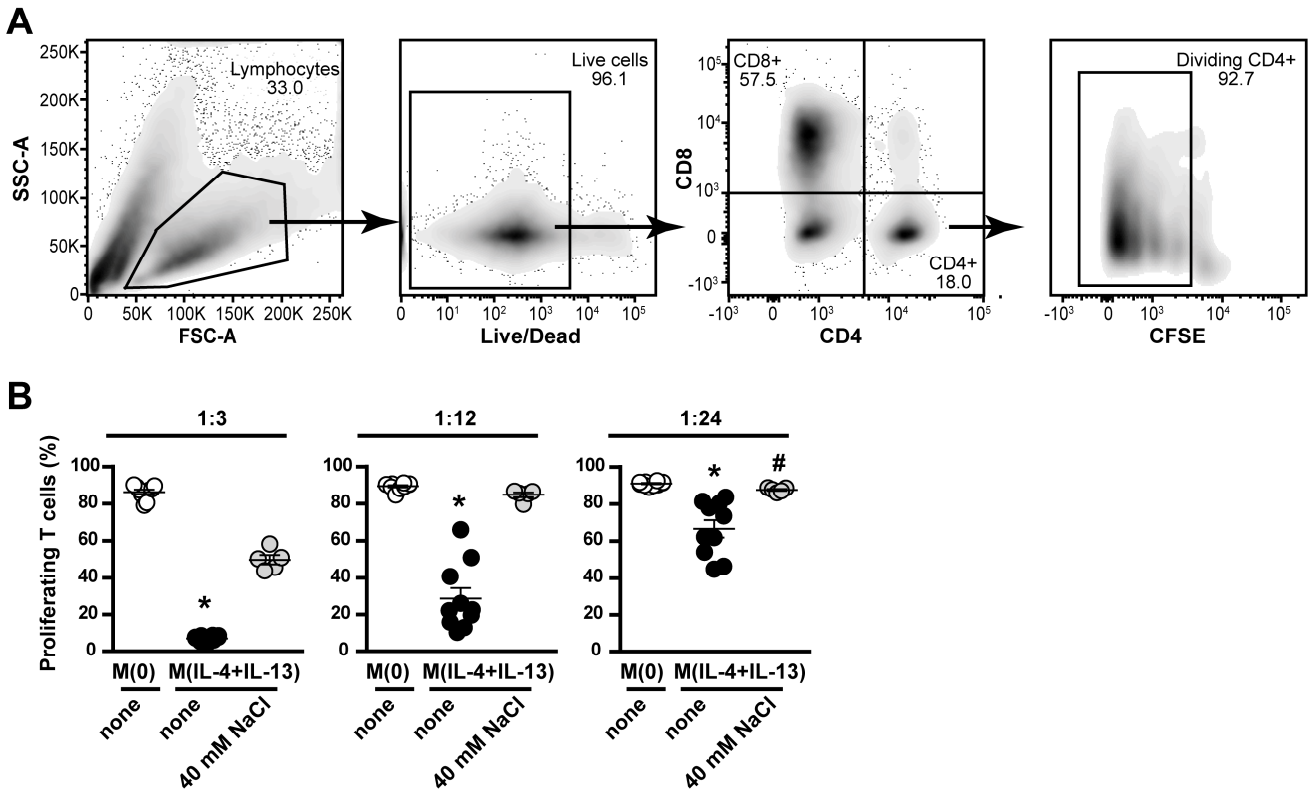
B) Signature gene expression of groups as in **A** was analysed by qPCR. The experiment was repeated twice independently. N=5 (technical). *p<0.05 vs M(IL-4+IL-13) alone; #p<0.05 vs M(IL-4+IL-13) + 40 mM NaCl 0-48 h.

Supplemental Figure 5



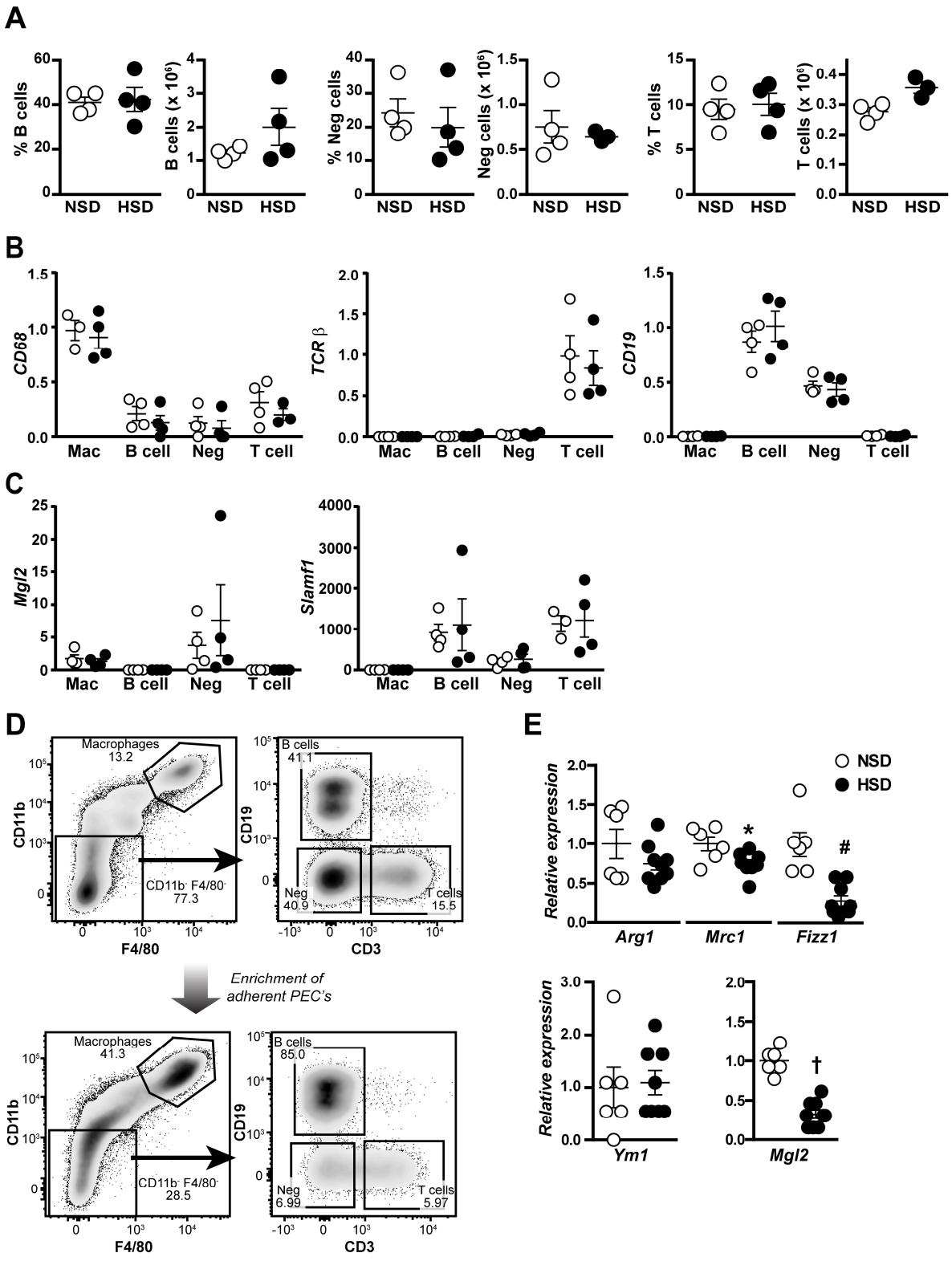
Supplemental Figure 5. High NaCl reduces the activation of IL-4+IL-13 stimulated peritoneal macrophages.
Peritoneal exudate cells (PEC) were isolated by lavage from 10 wildtype C57Bl/6 mice, pooled, and then allowed to adhere to 6-well tissue culture plates for 2 h, before washing of non-adherent cells and finally stimulation with IL-4+IL-13 alone (none) or in the presence of an additional 40 mM NaCl for 24 h. Adherent PECs consisted of approximately 42% macrophages (F4/80+CD11b+ by flow cytometry, data not shown). Following stimulation, M(IL-4+IL-13) signature gene expression was measured by real-time PCR. N=4 each group (technical). *p<0.001 vs M(0) none; #p<0.001 vs M(IL-4+IL-13) none.

Supplemental Figure 6, *related to Figure 4*



Supplemental Figure 6. Gating strategy for macrophage-T cell co-culture proliferation assay
A) Gating strategy for the analysis of proliferating CD4+ T cells (**Figure 4**)
B) Bars show the mean % of proliferating T cells incubated with macrophages at a ratio of 3:1, 12:1 or 24:1 splenocytes:macrophages (N=5 technical). The experiment was repeated at least three times independently. * $p<0.05$ vs M(0); # $p<0.05$ vs M(IL-4+IL-13) none.

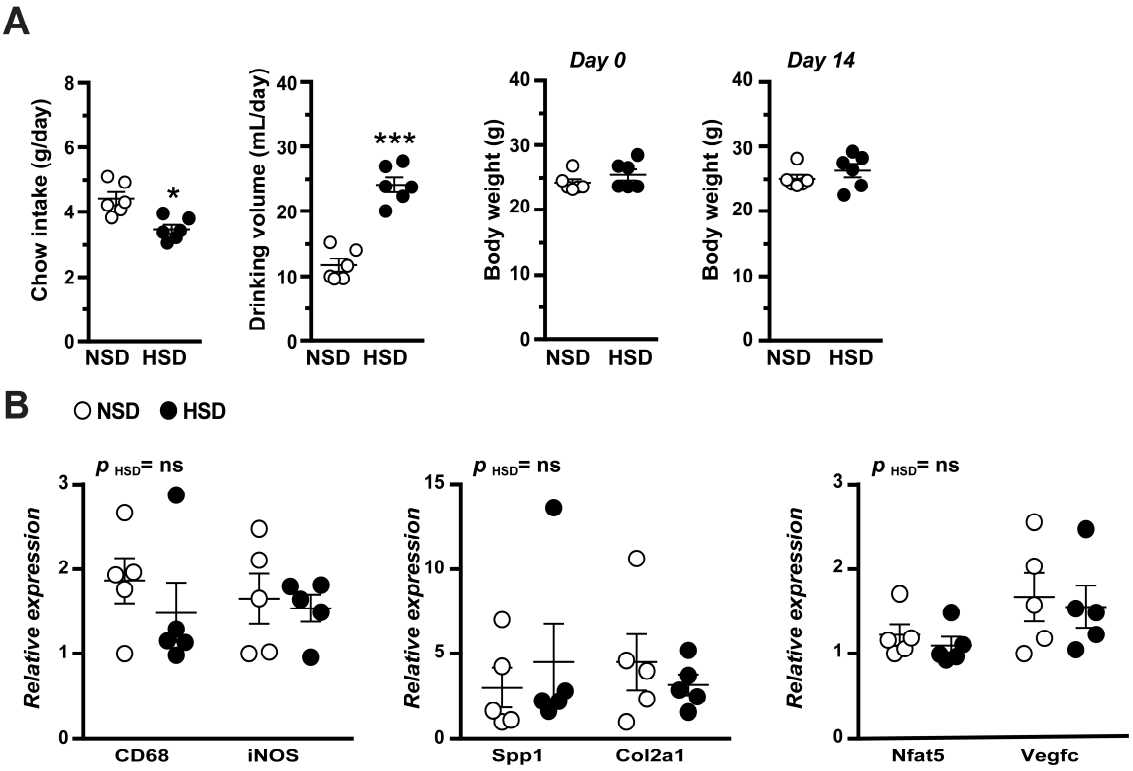
Supplemental Figure 7, *related to Figure 5*



Supplemental Figure 7. High salt diet reduces chitin-elucidated macrophage activation *in vivo*
A) The proportion and actual number of B cells (F4/80-CD11b-B220+), Negative cells (F4/80-CD11b-B220-CD3-) and T cells (F4/80-CD11b-CD3+) in PECs isolated from mice on a normal salt diet (NSD) or high salt diet (HSD) and injected with chitin as described in **Figure 5**.
B) The success of the FACS sorting of PECs as described in **Figure 5** was assessed by real-time qPCR of FACS-sorted populations for signature genes for macrophages (CD68), B cells (CD19) and T cells (T cell receptor β chain; Tcrb).

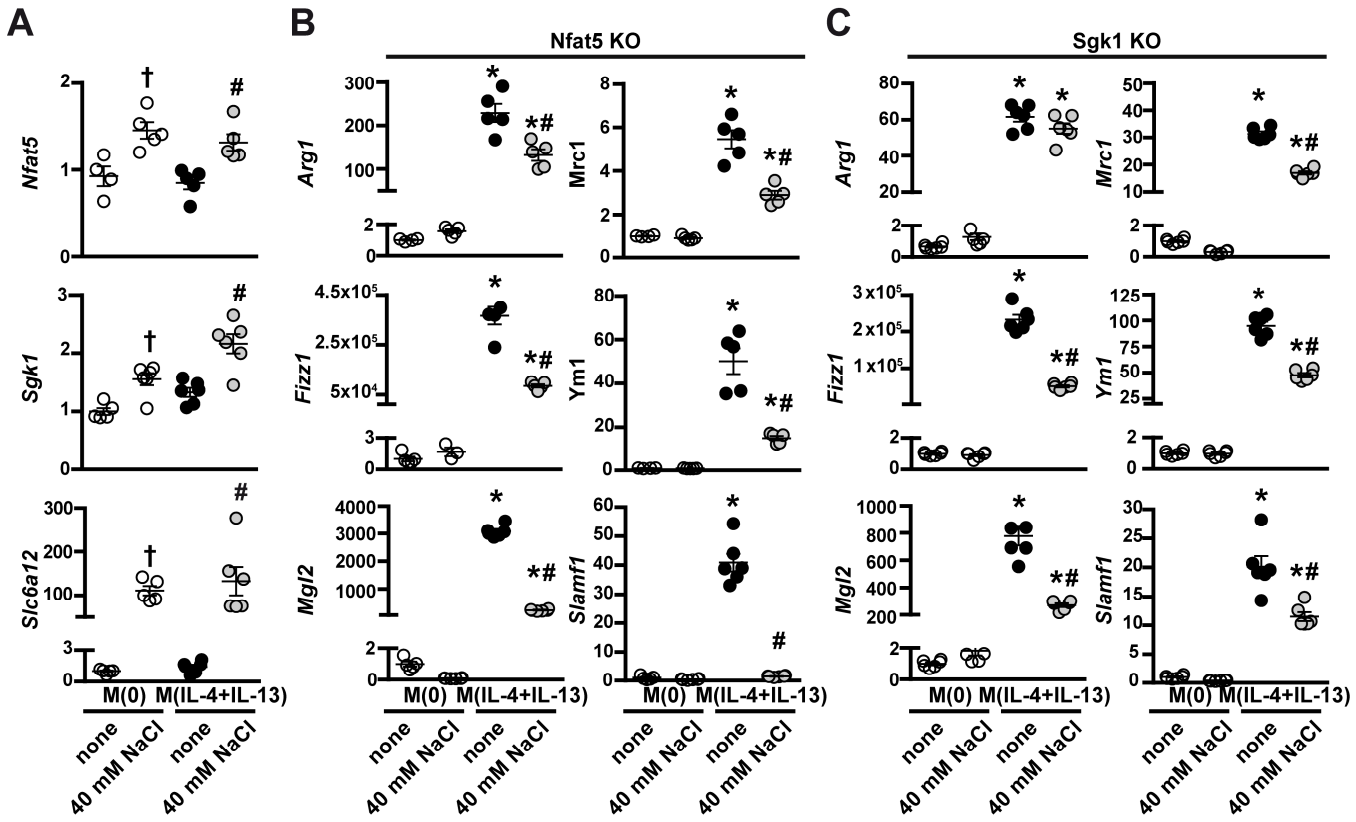
C) The expression of the M(IL-4+IL-13) signature genes *Mgl2* and *Slamf1* was analysed in FACS-sorted populations as described in **Figure 5**. (N=4 NSD vs. N=4 HSD).
D) Mice were fed a NSD or HSD for two weeks, at which point chitin was injected intraperitoneally (I.P.). After 2 days, PECs were collected by lavage and left to adhere to tissue culture plates for 2 h, before extensive washing and isolation of RNA for gene expression analysis by real-time qPCR. The proportion of macrophages (F4/80+CD11b+), B cells (F4/80-CD11b-B220+), T cells (F4/80-CD11b-CD3+) and "Neg" cells (F4/80-CD11b-B220-CD3-) was analysed by flow cytometry of the whole PEC lavage, and after adhesion to tissue culture plates for 2 h. After adhesion, a 3-fold enrichment of macrophages was routinely observed.
E) The expression of M(IL-4+IL-13) genes in adhered PECs as described in **D**. The experiment was repeated twice independently, and then pooled (N=6 NSD vs. N=9 HSD). *$p<0.05$, #$p<0.001$, †$p<0.0001$.

Supplemental Figure 8, *related to Figure 6*



Supplemental Figure 8. High salt diet delays wound-healing *in vivo*
A) The total chow intake and drinking volume of C57Bl/6 mice was measured over 14 days of wound-healing whilst on normal salt diet (NSD) and high salt diet (HSD). The body weight of the mice was also measured at day 0 and at the end of the time course. N=7 mice per group. *p<0.05; ***p<0.001.
B) Real-time qPCR analysis of macrophage signature genes (*CD68, Nos2),* fibroblast marker genes *(Spp1, Col2a1)* and tonicity responsive molecules *(Nfat5, Vegfc)* at wound sites from mice on a NSD and HSD. No significant difference was observed by 2-way ANOVA.

Supplemental Figure 9

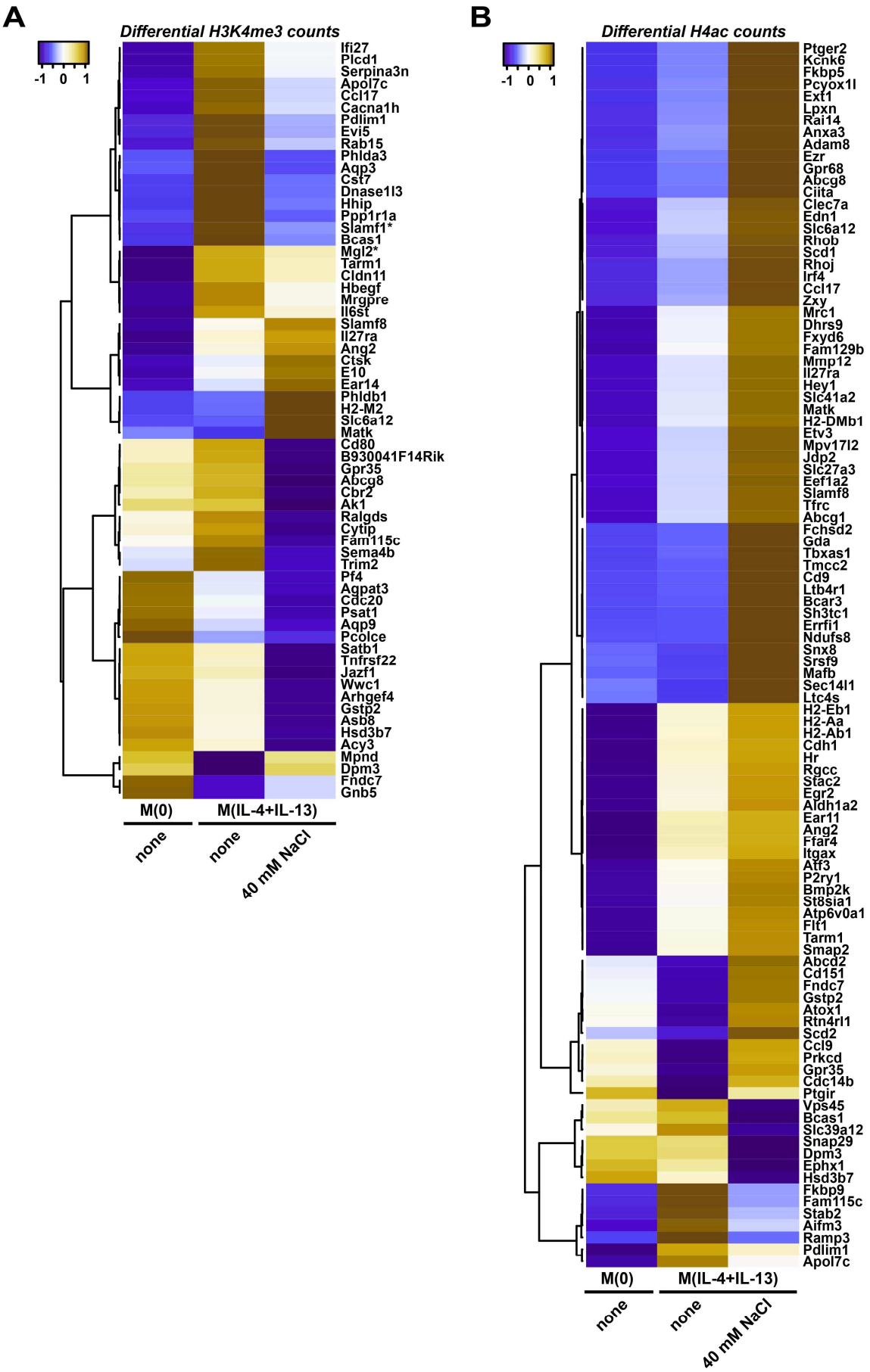


Supplemental Figure 9. Deletion of the tonicity molecules Sgk1 or Nfat5 does not rescue the effect of salt on M(IL-4+IL-13) activation

A) Macrophages were stimulated with IL-4 and IL-13 (M(IL-4+IL-13)) in the absence (none) or with high salt for 24 h. Unstimulated (M(0)) macrophages were treated similarly. The expressions of genes shown to mediate cellular responses to tonicity were determined by qPCR. The experiment was repeated at least three times independently. N=5 (technical).†$p<0.05$ vs none; #$p<0.05$ vs M(IL-4+IL-13) none.

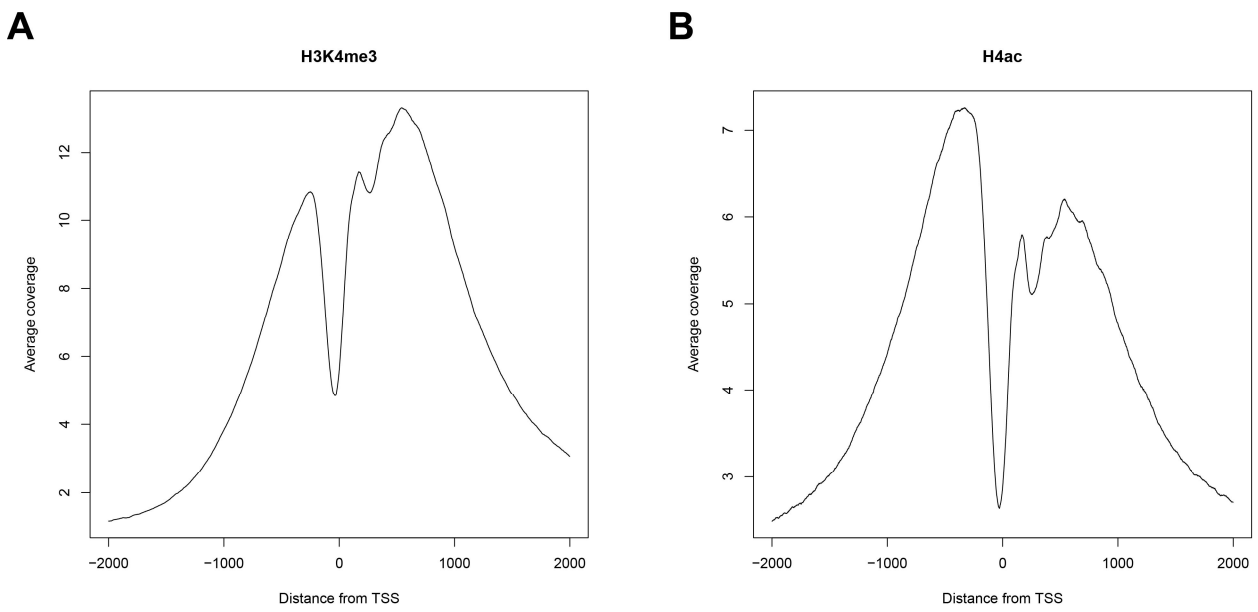
B&C) Cells were isolated from the bone marrow of *Nfat5* myeloid-specific knockout mice or *Sgk1* total knockout mice, and differentiated into macrophages. The yield and differentiation of *Nfat5* and *Sgk1* KO BMDMs was identical to wildtype (data not shown). Nfat5 KO (**B**) or Sgk1 KO (**C**) macrophages were then stimulated with IL-4 and IL-13 (M(IL-4+IL-13)) in the absence (none) or with high salt as described in **A**. The expression of M(IL-4+IL-13) signature genes was determined by real-time PCR. The experiment was repeated at least twice independently. N=6 (technical). *$p<0.0001$ vs M(0); #$p<0.001$ vs M(IL-4+IL-13) none (no additional salt).

Supplemental Figure 10, *related to Figure 7*



Supplemental Figure 10. Differential heat maps of H3K4me3 and H4ac modifications
A&B) Heat map of the differential H3K4me3 (**A**) and H4ac (**B**) marks between M(0), M(IL-4+IL-13) and M(IL-4+IL-13) activated in high salt (as identified in **Figure 7**). Presented is the mean of two independent biological replicates. The full list of gene names is shown. Data were scaled and centered around 0 row-wise.

Supplemental Figure 11



Supplemental Figure 11. Quality control of ChIP-seq data
A&B) The average coverage of histone modification ChIP-seq reads around all annotated TSS for H3K4me3 (**A**) and H4ac (**B**). Presented is the mean from two independent biological replicates.

Supplemental Table 1. Definition of gene clusters with/without changes in expression, H3K4me3, H4Ac and all possible combinations after polarization to M(IL-4+IL-13).
The number of genes assigned to each cluster is also shown. ↑ (up arrow), indicates an increase in expression/modification of more than 1.3 fold; ↓ (down arrow), indicates a decrease in expression/modification of less than 0.7 fold; nc, indicates no change.

Cluster	Expression	H3K4me3	H4Ac	Nr. Genes
1	↑	↑	↑	52
2	↑	↑	nc	29
3	↑	↑	↓	0
4	↑	nc	↑	67
5	↑	nc	nc	626
6	↑	nc	↓	4
7	↑	↓	↑	3
8	↑	↓	nc	21
9	↑	↓	↓	1
10	nc	↑	↑	66
11	nc	↑	nc	20
12	nc	↑	↓	0
13	nc	nc	↑	68
14	nc	nc	nc	631
15	nc	nc	↓	21
16	nc	↓	↑	18
17	nc	↓	nc	209
18	nc	↓	↓	103
19	↓	↑	↑	0
20	↓	↑	nc	1
21	↓	↑	↓	0
22	↓	nc	↑	6
23	↓	nc	nc	387
24	↓	nc	↓	21
25	↓	↓	↑	0
26	↓	↓	nc	135
27	↓	↓	↓	79

Supplemental Table 3. Sequences of primers (5'-3') for real-time PCR (all specific for mouse gene sequences)

Gene name	Forward sequence	Reverse sequence	Probe sequence
Arg1	CCACAGTCTGGCAG TTGGAA	GCATCCACCCAAAT GACACA	TGGCCACGCCAGGGT CCAC
Mrc1	AATACCTTGAACCC ATTTATCATTCC	GCATAGGGCCACCA CTGATT	CGATGTGCCTACCGG CTGCCC
Fizz1	CGTGGAGAATAAGG TCAAGGAACT	CACTAGTGCAAGAG AGAGTCTTCGTT	TTGCCAATCCAGCTA ACTATCCCTCCACTG
Ym1	TCCTACTGGAAGGA CCATGGAGCA	TCCTGGTGGGCCAG TACTAATTGT	
Mgl2	GAGACAGACTTGAA GGCCTTGAC	GCCACTTCCGAGCC ATTG	
Slamf1	TGGCTAATGGATCC CAAAGGA	CCATCACACCTCCA CCTGTT	
Nfat5	AGCTGGAAATGGAA CATTGGA	CGCACAACATAGGG CTCTTCT	
Sgk1	CGGTGGACTGGTGG TGTCTT	GTCGTACATCTCAG CCGTGTTC	
Slc6a12	AGAACTTTACCTCG CCTGTCATG	CGTGGATGCCCGAT GTAATAC	
Klf4	AAACCTATACCAAG AGTTCTCATCTCAA	CCGTCCCAGTCACA GTGGTAA	
Irf4	CGGGCAAGCAGGAC TACAA	TCGGAACTTGCCTTT AAACAATG	
Cd68	TTCTGCTGTGGAAAT GCAAG	CAATGATGAGAGGC AGCAAG	
Nos2	GGGCAGCCTGTGAG ACCTT	TGCATTGGAAGTGA AGCGTTT	TCCGAAGCAAACATC ACATTCAGATCCC
Il6	GTTGCCTTCTTGGGA CTGATG	GGGAGTGGTATCCT CTGTGAAGTCT	TGGTGACAACCACGG CCTTCCC
Tnfa	GGTCCCCAAAGGGA TGAGAA	TGAGGGTCTGGGCC ATAGAA	TTCCCAAATGGCCTCC CTCTCATCA
Nfkbia	CTGCACACCCCAGC ATCTC	CAGACACGTGTGGC CATTGT	ACTCCGTCCTGCAGG CCACCAA
Tcrb	GGCACAATCCTCGA AACCA	CCACTTGTCCTCCTC TGAAAGC	TTCCGCTGCCAAGTGC AGTTCCA
Cd19	GGTTTCTCTGGTGGC TTTTCTC	CATTCGCTTCCTTTT CCTTCTC	
Spp1	GAGGTGATAGCTTG GCTTATGGA	TGTGGCATCAGGAT ACTGTTCATC	
Coll1a2	CTA CTG GTG AAA CCT GCA TCC	GGG CGC GGC TGT ATG AG	CCC AAC CTG TAA ACA CCC CAG CGA AG
Vegfc	TCA GCA AGA CGT TGT TTG AAA TTA C	TGA TTG GCA AAA CTG ATT GTG ACT	
Gapdh	CACCGACCTTCACC ATTTTGT	GGGCCACGCTAATC TCATTTT	

MyoD	GACGCCGCCCCTCAGT	TGCAGTCGATCTCTCAAAGCA	

Supplemental Table 4. ChIP-seq quality metrics computer with the SPP tool as defined by the ENCODE consortium (Ref 50).

	Filename	numReads	estFragLen	corr_estFragLen	NSC[1]	RSC[2]	QualityTag
none	Sample_H3K4me3-con1-bio1-tech1.bam	48958784	150	0.5059	1.849	3.552	2
none	Sample_H3K4me3-con1-bio3-tech1.bam	41636837	150	0.5103	1.964	4.287	2
40 mM NaCl	Sample_H3K4me3-con2-bio1-tech1.bam	60490146	145	0.5192	1.815	2.992	2
40 mM NaCl	Sample_H3K4me3-con2-bio3-tech1.bam	46633261	150	0.5109	1.930	3.957	2
IL-4+IL-13	Sample_H3K4me3-con3-bio1-tech1.bam	47525779	150	0.5116	1,848	3,502	2
IL-4+IL-13	Sample_H3K4me3-con3-bio3-tech1.bam	37248332	150	0.5006	1,968	4,512	2
IL-4+IL-13+40 mM NaCl	Sample_H3K4me3-con6-bio1-tech1.bam	25987469	130.145	0.478/0.474	2,174	3,811	2
IL-4+IL-13+40 mM NaCl	Sample_H3K4me3-con6-bio3-tech1.bam	42147226	150	0.5006	1,894	4,244	2
none	Sample_H4ac-con1-bio1-tech1.bam	78999383	145	0.5368	1.276	6.575	2
none	Sample_H4ac-con1-bio3-tech1.bam	45050178	150	0.4626	1.330	15.963	2
40 mM NaCl	Sample_H4ac-con2-bio1-tech1.bam	81529474	130,145	0.5480/0.5463	1.257	4.539	2
40 mM NaCl	Sample_H4ac-con2-bio2-tech1.bam	50115023	145	0.4628	1.204	14.004	2
IL-4+IL-13	Sample_H4ac-con3-bio1-tech1.bam	74815658	145	0.5326	1,279	6,027	2
IL-4+IL-13	Sample_H4ac-con3-bio3-tech1.bam	43557150	150	0.4613	1,350	9,784	2
IL-4+IL-13+40 mM NaCl	Sample_H4ac-con6-bio1-tech1.bam	48880525	145	0.4565	1,392	7,303	2
IL-4+IL-13+40 mM NaCl	Sample_H4ac-con6-bio3-tech1.bam	41021039	150	0.4553	1,367	9,696	2
	Sample_input-mix-bio1-tech1.bam	62502414	150,340,530	0.4718/0.4611/0.4598	1.039	2.470	2

[1] NSC: normalized strand cross correlation
[2] RSC: relative strand cross correlation

Supplemental Experimental Procedures

Preparation and activation of bone marrow-derived macrophages

Bone marrow cells were isolated from the femur and tibia of freshly euthanized mice, by flushing with ~10 mL of activation media (RPMI1640 containing L-Glutamine (21875-034, Gibco), 10% (v/v) Fetal calf serum (FCS) (S0615, Biochrom), 10 mM HEPES (15630-056, Gibco), 50 μM β-mercaptoethanol (M3148, Sigma), 1% (v/v) Penicillin-Streptomycin (15140-122, Gibco), without colony stimulating factor (CSF)-1. Cells were then pelleted, and resuspended into monocyte differentiation media containing CSF-1 (DMEM (41966-029, Gibco), 10 % (v/v) FCS, 5% (v/v) Adult horse serum (S-HEU03-I, Cell Concepts), 1:100 Non-essential amino acids (M7145, Sigma), 50 μM β-mercaptoethanol (M3148, Sigma), with 20% (v/v) L929 conditioned media containing CSF-1). Conditioned media containing CSF-1 was generated by collecting the media from L929 cells (ATCC, Wesel, Germany) cultured for 14 days in DMEM containing 10 % (v/v) FCS, 1:100 Non-essential amino acids, HEPES and 1% (v/v) Penicillin-Streptomycin. Conditioned media from L929 cells were routinely assessed for contamination and were mycoplasma-free (PCR Mycoplasma Test Kit I/C, PromoKine, Heidelberg, Germany). For macrophage differentiation, 10 x 10^6 bone-marrow cells were then cultivated in 50 mL of differentiation media for 7 days in sealed, hydrophobic Teflon® bags (FT FEP 100 C (DuPont), American Durafilm) at 37ºC and 10% CO_2. The yield of bone-marrow derived macrophages (BMDM) from one bag was consistently ~70-100 x 10^6 cells with a purity of 90-95% (determined as F4/80$^+$ CD11b$^+$ cells by flow cytometry (data not shown, see experimental details in Flow Cytometry section below)). For activation experiments, the differentiated macrophages were harvested from Teflon bags, pelleted and resuspended into activation media. No CSF-1 was present for activation experiments. For analysis by qPCR and western blotting, 2 x 10^6 differentiated macrophages were plated per well of 6-well plates. For T cell suppression functional assays, 20 x 10^6 differentiated

macrophages were plated into 75 cm^2 flasks. For ChIP-seq, 40 x 10^6 differentiated macrophages were plated into 150 cm^2 flasks. In all cases, the differentiated macrophages were first allowed to rest and adhere for 2 hrs. Activation to M(IL-4+IL-13) was performed by the addition of recombinant mouse IL-4 (404-ML-010, R&D Systems) and recombinant mouse IL-13 (PMC0134, Invitrogen) to the activation media, to a final concentration of 10 ng/mL each. Activation to M(LPS) was by the addition of LPS (*E. coli* O111:B4, Sigma-Aldrich) to the activation media to a final concentration of 10 ng/mL. For the analysis of the effect of salt on macrophage activation, an additional 40 mM NaCl (Carl Roth GmbH) was also added to the culture media. Tonicity experiments were performed the same where an additional 80 mM urea (which has an equivalent increase in osmolality and passes through the cell membrane) or 80 mM mannitol (which has an equivalent increase in tonicity but does not enter the cell) (both from Sigma). Akt inhibitor LY294002 was used at a concentration of 50 nM (Cell Signaling, #9901). Unless otherwise noted, cells were activated for 24 h at 37ºC and 5% CO2.

Preparation and activation of MyrAkt BMDM

Bone marrow cells were isolated from the femur and tibia of transgenic mice which express a constitutively active Akt (NH(2)-terminall myristoylation signal-attached Akt; "MyrAkt"). These mice have a *loxP* STOP sequence followed by the MyrAkt1 gene, inserted into the *Rosa26* locus. When either bred to mice expressing Cre recombinase, or by incubating with recombinant Cre enzyme, the STOP sequence is deleted and MyrAkt expression is induced. We utilized the inducibility of this model and first differentiated (under normal, isotonic conditions) bone marrow cells from MyrAkt mice into BMDM as before, in the absence of any recombinase, to ensure that the development of macrophages from MyrAkt mice was identical to WT controls. We have name these "$MyrAkt^{inactive}$", as there is no activation of the

MyrAkt transgene in these conditions. Following the 7-day differentiation procedure, recombinant Cre enzyme (TAT-Cre, kind gift of Rajewsky MDC, Berlin, Germany) was added to induce excision of the STOP cassette and thus, induce MyrAkt expression ("MyrAkt^{active}"). Following incubation with TAT-Cre, BMDM were left for 2 days to allow for full recombination and MyrAkt expression. BMDM from wildtype C57BL/6 mice were treated with TAT-Cre and incubated for 2 days as per transgenic cells. Finally, MyrAkt^{active} and WT BMDM were left unstimulated, or stimulated with IL-4+IL-13 alone or with an additional 40 mM NaCl for 24 h. Expression of M(IL-4+IL-13) signature genes was measured by qPCR.

Isolation and stimulation of peritoneal macrophages

Male wildtype mice (C57BL/6JOlaHsd; Harlan Laboratories, Rossdorf, Germany) were euthanized and 5ml of ice-cold PBS, 3% (v/v) FCS (S0615, Biochrom) was injected into the peritoneal cavity. After gentle massage the lavage fluid was recovered using a syringe and needle. Peritoneal exudate cells (PEC) were then pelleted by centrifugation and resuspended in activation media. Peritoneal cells were allowed to attach for 1h to enrich for plastic-adherent cells, before washing once with media. Cells were then left unactivated (M(0)) or stimulated with IL-4+IL-13 alone or with an additional 40 mM NaCl for 24 h. Signature gene expression was by qPCR.

qPCR

Activated macrophages were washed once with PBS and then resuspended in Qiazol for the isolation of total RNA using a RNeasy RNA isolation kit (Qiagen), according to the manufacturer's protocol. The synthesis of cDNA and quantitative analysis of mRNA expressions by real-time PCR was performed as described previously (1). The expression

levels of target genes for were normalized by the expression of 18S, unless indicated otherwise. Primer sequences are detailed in **Supplemental Table 3.**

Western blotting

Activated macrophages were washed once with cold PBS on ice and then scraped into RIPA buffer (9806S, Cell Signaling) containing protease and phosphatase inhibitors. Protein was extracted by freeze-thawing in RIPA buffer over night at -20ºC, followed by centrifugation to remove insoluble cellular debris. Total protein was determined by a Bradford assay and an equal amount of total protein was loaded per lane. Samples were run on SDS-PAGE and then transferred onto nitrocellulose membranes for western blotting. Quantification of bands was performed with the program ImageJ(2). Antibodies used are: Hsp60 (#12165S, Cell Signaling), Arginase 1 (#610708, BD Biosciences), phospho-STAT6^{Tyr641} (#9361, Cell Signaling), STAT6 (#9362, Cell Signaling), β-actin (#NB600-501, Novus Biologicals), phospho-Akt^{S473} (#4060, Cell Signaling), Akt (#9272, Cell Signaling), phopho-p70S6K^{Thr389} (#9234, Cell Signaling), p70S6K (#2708, Cell Signaling), phospho-IκBα (#9246, Cell Signaling), IκBα (#sc-371, Santa Cruz).

Flow cytometry

Adherent macrophages were prepared into single-cell suspensions by incubation in ice-cold PBS/2 mM EDTA for 15 min, before gentle scraping. For cell viability analysis, cells were first counted manually using a haemocytomer, and then propidium iodide (PI) staining solution (51-66211E, BD Pharmingen) was added to a final of 1µg/ml prior to analysis by flow cytometry. For other analyses, following preparation of single-cell suspensions, cells were then pelleted and first incubated with an Fc receptor blocking antibody (#14-0161-85, BD Biosciences), before staining with the following diluted antibodies: PE-labelled α-PD-L2

(#12-9972-82, BD Biosciences), APC-labelled α-F4/80 (#17-4801-1631, BD Biosciences), FITC-labelled α-CD11b (#553310, BD Biosciences), Pacific Blue-labelled α-F4/80 (#48-4801-80, eBiosciences), APC-labelled α-CD38 (#130-103-030, Miltenyi Biotec), and PerCP-Vio700 labelled Gr-1 (#130-102-171, Miltenyi Biotec). Stained macrophages were then washed and data was acquired on a BD FACSCanto II instrument (BD Biosciences). Analysis was performed with the FlowJo software version 10 (TreeStar). Cell doublets were excluded by analysis of FSC-H vs FSC-A. Cell size was measured as the mean FSC-W signal.

Cell viability assays

BMDM were plated in 96-well plates at a density of 50,000 cells per well, and activated to M(0) or M(IL-4+IL-13) alone or in the presence of an additional 40 mM NaCl. After 24h the culture media was removed, cells were washed once with PBS, and the DNA content of each well was determined using the CyQUANT® Cell Proliferation Assay Kit (#C7026, Life Technologies). Total cell numbers were derived from comparing to a standard curve of serially diluted M(0) macrophages.

Leishmania major infection and nitrate production

Infection, viability counting and nitrate production was performed exactly as described previously (3). Briefly, BMDM were infected with *Leishmania major* and then activated to M(0), M(IL-4+IL-13) or M(LPS) in the absence (none) or presence of an additional 40 mM NaCl, and the percentage of BMDM infected was measured microscopically after 72 h. Nitrate levels were measured by a Griess assay.*L. major* parasites (MHOM/IL/81/FE/BNI) were a kind gift of Dr. Uwe Ritter (University Hospital Regensburg, Germany), which were then propagated as described previously (3).

T cell proliferation/suppression assays

Single-cell splenocyte suspensions were prepared by pushing spleens from male WT C57BL/6JOla mice through a 70 µm cell strainer (BD Biosciences). Splenocytes were labelled with 1 µM carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) according to the manufacturer's instructions. Labelled cells (250,000/well) were first stimulated alone (in isotonic media) with plate-bound α-CD3 (0.5 µg/mL; BD Biosciences) and α-CD28 (0.2 µg/mL; BD Biosciences) for 24h. Simultaneously (and separately), BMDM were left unstimulated (M(0)) or activated to M(IL-4+IL-13) alone or with an additional 40 mM NaCl for 24 h. On the next day, M(0), M(IL-4+IL-13), and M(IL-4+IL-13+40 mM NaCl) activated macrophages were detached from flasks with PBS/2 mM EDTA and gentle scraping. As we have previously shown that salt affects T cell activation and function(4), M(IL-4+IL-13) macrophages activated in high NaCl were washed thoroughly (3 X PBS) to remove excess salt (i.e the T cells did not experience hypertonic conditions). M(0) and M(IL-4+IL-13) macrophages stimulated in isotonic media were treated similarly. After washing, macrophages were added to 96-well plates containing the CFSE-labelled splenocytes at macrophage:splenocyte ratios of 1:3, 1:6, 1:12, 1:24, and co-cultured for an additional 3.5 days. The proliferation of $CD4^+$ T cells after 4.5 days was determined by staining with antibodies against CD4 (#558107, BD Biosciences) and CD8a (#553033, BD Biosciences), and then assessing the dilution of CFSE of CD4 gated T cells by flow cytometry on a BD FACSCanto II instrument (BD Biosciences). Analysis was performed with the FlowJo software version 10 (TreeStar). Dead cells and doublets were excluded from the analysis.

Chitin administration and FACS sorting of peritoneal immune cells

Prior to chitin injection, male age-matched C57BL/6JOlaHsd mice (Harlan Laboratories, Rossdorf, Germany) were randomly assigned to receive a high salt diet (4% NaCl in chow

plus 1% in the drinking water; HSD) or normal-salt diet (0.4 % in chow plus tap water; NSD) for 14 days. Diets were obtained from Ssniff (Soest, Germany). Chitin (C9752, Sigma) was prepared as described before (5). Large particles were removed by straining through a 100µm cell strainer (BD Biosciences) before I.P. injection (200 µl/mouse). Mice were housed for an additional two days with HSD or NSD, sacrificed and peritoneal exudate cells (PEC) were collected by injection of 5ml of ice-cold PBS, 3% (v/v) FCS (S0615, Biochrom) into the peritoneal cavity. After gentle massage the lavage fluid was recovered using a syringe and needle. Peritoneal exudate cells (PEC) were then pelleted by centrifugation and resuspended into FACS buffer (0.5% FCS, 2 mM EDTA in PBS). PECs were then stained with the following diluted antibodies: FITC-labelled α-CD11b (#553310, BD Biosciences), Pacific Blue-labelled α-F4/80 (#48-4801-80, eBiosciences), eFluor660-labelled αCD3 (#50-0032-82, eBiosciences), APC-Cy7-labelled B220 (#552094, BD Biosciences). Stained PECs were then washed, analysed and sorted on a FACSAria (BD Biosciences). To sort, doublets were first excluded by FSC-A vs. FSC-H analysis, and then gated as desired. All sorted samples were re-analysed and macrophages had a purity >85%, whilst all other populations had purities >90%. Sorted cells were immediately put into Qiazol solution and stored at -80ºC for RNA extraction and subsequent gene analysis by qPCR. Analysis was performed with the FlowJo software version 10 (TreeStar).

We also analysed the expression of M(IL-4+IL-13) signature genes in crude, unsorted PECs, based on a published protocol (5). Briefly, PECs were isolated by lavage from mice on a NSD and HSD diet as before, and then pelleted and resuspended in media (RPMI, 1% (v/v) Penicillin-Streptomycin) and then allowed to adhere to 6-well plates for 1h at 37°C, 5% CO_2. Non-adherent cells were removed by extensive washing with PBS and adherent PECs were then lysed in Qiazol (Qiagen) RNA isolation and qPCR. Investigators had no knowledge of the treatment group assignment.

Wound preparation, treatment and measurement

Prior to wounding, 7 male age-matched C57BL/6JOlaHsd mice (Harlan Laboratories, Rossdorf, Germany) were randomly assigned to receive a HSD or NSD (as described above) for 14 days. Each mouse was subjected to two 8-mm excisional wounds, one on each side of the midline of the back, followed by the same treatment regime. Wounds (length × width) were measured on day 0, 3, 5, 7, 10, 12 and 14, and the % total wound area was calculated as the percentage of wound area compared to day 0 of the respective animal. One wound in each group was excluded from analysis, as a piece of the bedding stuck to the wound and affected the healing process. Investigators had no knowledge of the treatment group assignment. At the end of the assay (14 d), skin samples were taken from site of the wounds and RNA extracted for gene expression analysis as described above.

Gene expression profiling by microarray

Total RNA was extracted as described above from M(0), M(IL-4+IL-13), and M(LPS) macrophages activated with and without salt (N=4), and then analyzed using the Illumina Mouse ref 8v2.0 array. Data was processed on the Illumina GenomeStudio V2011.1 Platform (Gene Expression Module 1.9.0), quantile normalized on a probe level (25,697), without background correction. The data was then $\log_2$ transformed, and quality control using principle component analysis did not highlight any batch effects (data not shown). Differential gene expression analysis was performed using analysis of variance in linear models. We modeled the gene expression levels using: 1) macrophage polarization and, 2) application of salt, as explanatory variables. We controlled the false discovery rate using the method of Benjamini and Hochberg(6). Fold-changes were computed from mean log expression values in each condition relative to M0 without salt. A list containing 259 genes

were then identified as having a difference in expression of greater than 1.2-fold between M(IL-4+IL-13) stimulated in the absence (none) or presence of an additional 40 mM NaCl (see **Supplemental Table 2**). This relatively low fold-change limit was set so as to account for subtle differences expected with this small perturbation in environment, in comparison to large differences usually expected when comparing knockout models or pharmacological inhibitors. The list of salt-sensitive M(IL-4+IL-13) genes was then analyzed for the enrichment of gene ontology (GO) pathways with the package GOstats(7). Multiple testing was corrected for using the Benjamini Hochberg method(6).

ChIP-seq and data analysis

We used a modified protocol described previously (8). Briefly, activated macrophages were detached from flasks with EDTA and nuclei were isolated by centrifugation through a sucrose cushion. Samples were digested with micrococcal nuclease (Sigma-Aldrich) and chromatin mononucleosomes were subsequently precipitated over night at 4°C with α-H3K4me3 (9751 S, Cell Signaling), or α-H4ac (06-866, Millipore) bound to Dynabeads® Protein A (10002D, Invitrogen). A MinElute PCR Purification Kit (Qiagen) was used to purify immunoprecipitated DNA after proteinase K (Sigma) digestion, where the specificity of immunoprecipitation was confirmed with known active (*Gapdh*) and inactive (*Myod1*) genes by qPCR. Then, 50 ng of purified DNA was used to construct ChIP-seq libraries according to the manufacturer's protocol (Illumina/Solexa). After cluster generation, sequencing was performed using the Illumina HiSeq 2000 platform. Short reads were mapped to the mouse reference genome (mm9) using the Bowtie algorithm, retaining only reads which mapped uniquely. Duplicate artefact reads were removed using SAMtools(9). The quality of our ChIP-seq data was assessed using metrics developed by the ENCODE consortium ((10); **Supplemental Table 4**). For further quality control, read coverage plots in

4 kb regions around annotated transcription start sites (TSS) (**Supplemental Figure 11**) were generated(8). We quantified the ChIP-seq promoter occupancy in TSS regions (±2kb) based on gene annotation from Ensembl release 62, for M(0) and M(IL-4+IL-13) polarized macrophages with and without stimulation with salt in two biological replicates per condition (in total 8 samples). Differential occupancy analysis was performed on the raw read counts using DESeq. We obtained differential occupancy results for macrophage polarization and for salt treatment using DESeq's negative binomial regression model. The false discovery rate was controlled using the Benjamini Hochberg method(6). For further analysis and visualization, read counts were normalized using a quantile based scaling factor(11) and log transformed (natural logarithm). Fold-changes were computed from the mean count values. Finally the data was integrated with the gene expression data using the probeset to Ensembl mapping from the bioconductor package illuminaMousev2.db. To generate a M(IL-4+IL-13) signature, a significant differential result was defined for either gene expression, H3K4m3 or H4Ac (FDR < 0.05) between M(0) and M(IL-4+IL-13). A pool of 2568 genes was identified, which were then sorted further into clusters with respect to the three variables (**Supplemental Tables 1&2**). A fold-change of 1.2 was set for comparison of the difference between M(IL-4+IL-13) macrophages activated in normal media (none) or in the presence of additional 40 mM NaCl.

Metabolic studies

Extracellular flux analysis was performed to determine the effect of NaCl on the mitochondrial respiratory capacity (oxidative phosphorylation, OXPHOS) and glycolysis. To do so, mitochondrial and glycolytic stress tests were performed with an XF-24 Extracellular Flux Analyzer (Seahorse Biosciences). Macrophages were first seeded at a density of 1 x 10^5 cells/well of an XF-24 microplate, and stimulated with or without IL-4+IL-13, in the absence

or presence of 40 mM NaCl for 24 h. For analysis of OXPHOS, a XF Cell Mito Stress Test Kit was used, according to the manufacturer's directions (#103015-100, Seahorse Biosciences). Briefly, cells were exchanged into XF Base media (#102353-100, Seahorse Biosciences) containing 11mM glucose and 1 % FCS. Five consecutive measurements of the oxygen consumption rate (OCR) were then obtained under basal conditions, after which the following compounds were added sequentially (five measurements each): 1 μM oligomycin, to inhibit mitochrondrial respiration; 1.5 μM fluoro-carbonyl cyanide phenylhydrazone (FCCP), a mitochondrial uncoupler to elucidate maximal respiration; 100 nM rotenone plus 1 μM antimycin A, to inhibit the electron transport chain and measure the amount of non-mitochondrial respiration. For analysis of glycolysis, a XF Glycolysis Stress Test Kit was used, according to the manufacturer's directions (#103020-100, Seahorse Biosciences). Briefly, cells were exchanged into XF Assay media (#102365-100, Seahorse Biosciences) with no glucose and only 1 % FCS. Three consecutive measurements of the extracellular acidification rate (ECAR) were then obtained under basal conditions, after which the following compounds were added sequentially (5 measurements each): 10 mM glucose, to initiate glycolysis; 1 μM oligomycin, to inhibit mitochrondrial respiration and 50 mM 2-deoxy-glucose (2-DG), to inhibit glycolysis. Basal OCR, ECAR, SRC and glycolysis were calculated as per the manufacturer's directions.

Mitochondrial content and mass analyses

For analysis of mitochondrial mass, single-cell suspensions of activated macrophages were prepared as detailed before, and then incubated with MitoTracker Deep Red (#8778, Cell Signaling) at a concentration of 50 nM for 1 h at 37ºC, before washing and analysis by flow cytometry.

To determine the effect of NaCl on mitochondrial content, a real-time qPCR assay

was employed (12). Briefly, macrophages were activated to M(0) or M(IL-4+IL-13) alone or with an additional 40 mM NaCl for 24 h, after which total DNA was extracted with a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's directions. qPCR was then performed with primers for a mitochondrially encoded gene (cytochrome c oxidase subunit I; CO1) and a nuclear encoded gene (NADH dehydrogenase (ubiquinone) flavoprotein 1; NDUFV1) (12). The ratio of mitochondrial DNA to nuclear DNA expression was used as a measured of relative mitochondrial content.

Glucose uptake

Bone marrow derived macrophages were activated as described. After 24 h, cells were exchanged into glucose-free media (also without interleukins and excess NaCl) but containing 30 μM 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG). Cells were then incubated for 1 h at 37ºC, before being washed twice with PBS and analysed by flow cytometry.

Lactate production

Bone marrow derived macrophages were activated as described. After 24 h cells were washed twice with FCS- and interleukin -free isotonic media (RPMI1640 containing L-Glutamine (21875-034, Gibco), 0.5% (w/v) BSA (A-2153, Sigma), 10 mM HEPES (15630-056, Gibco), 50 μM β-mercaptoethanol (M3148, Sigma), 1% (v/v) Penicillin-Streptomycin (15140-122, Gibco) to remove residual lactate. Cells were then incubated in this FCS- and interleukin-free media for a further 1 h, and supernatants collected for lactate measurement with an EnzyFluo L-lactate assay kit (EFLLC-100, BioAssay Systems) according to the manufacturer's instructions. For data analysis lactate concentrations were normalized sample-wise to total protein concentrations, determined by Bradford measurements of recovered cell lysates.

Further statistical information

The number of animals for statistical power was chosen based on analysis of previous studies investigating the influence of dietary salt on immune cell function ((4); used N=12), activation of M2 in vivo by Chitin administration ((5); used N=5), or assessment of wound healing ((13); N=9). *In vivo* experiments were repeated independently twice and then pooled. For *in vitro* experiments, an appropriate number of technical replicates were used to obtain statistical power. This depended on the number of groups analysed and was determined based on previous experience. All *in vitro* experiments were repeated at least three times independently (biological replicates).

References

1. Marko, L., Kvakan, H., Park, J.K., Qadri, F., Spallek, B., Binger, K.J., Bowman, E.P., Kleinewietfeld, M., Fokuhl, V., Dechend, R., et al. 2012. Interferon-gamma signaling inhibition ameliorates angiotensin II-induced cardiac damage. *Hypertension* 60:1430-1436.

2. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671-675.

3. Jantsch, J., Schatz, V., Friedrich, D., Schroder, A., Kopp, C., Siegert, I., Maronna, A., Wendelborn, D., Linz, P., Binger, K.J., et al. 2015. Cutaneous Na+ storage strengthens the antimicrobial barrier function of the skin and boosts macrophage-driven host defense. *Cell Metab* 21:493-501.

4. Kleinewietfeld, M., Manzel, A., Titze, J., Kvakan, H., Yosef, N., Linker, R.A., Muller, D.N., and Hafler, D.A. 2013. Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature* 496:518-522.

5. Byles, V., Covarrubias, A.J., Ben-Sahra, I., Lamming, D.W., Sabatini, D.M., Manning, B.D., and Horng, T. 2013. The TSC-mTOR pathway regulates macrophage polarization. *Nat Commun* 4:2834.

6. Benjamini, Y., Drai, D., Elmer, G., Kafkafi, N., and Golani, I. 2001. Controlling the false discovery rate in behavior genetics research. *Behav Brain Res* 125:279-284.

7. Falcon, S., and Gentleman, R. 2007. Using GOstats to test gene lists for GO term association. *Bioinformatics* 23:257-258.

8. Barski, A., Cuddapah, S., Cui, K., Roh, T.Y., Schones, D.E., Wang, Z., Wei, G., Chepelev, I., and Zhao, K. 2007. High-resolution profiling of histone methylations in the human genome. *Cell* 129:823-837.

9. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25:2078-2079.

10. Landt, S.G., Marinov, G.K., Kundaje, A., Kheradpour, P., Pauli, F., Batzoglou, S., Bernstein, B.E., Bickel, P., Brown, J.B., Cayting, P., et al. 2012. ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. *Genome Res* 22:1813-1831.

11. Schulte, J.H., Marschall, T., Martin, M., Rosenstiel, P., Mestdagh, P., Schlierf, S., Thor, T., Vandesompele, J., Eggert, A., Schreiber, S., et al. 2010. Deep sequencing reveals differential expression of microRNAs in favorable versus unfavorable neuroblastoma. *Nucleic Acids Res* 38:5919-5928.

12. Guo, W., Jiang, L., Bhasin, S., Khan, S.M., and Swerdlow, R.H. 2009. DNA extraction procedures meaningfully influence qPCR-based mtDNA copy number determination. *Mitochondrion* 9:261-265.

13. Liao, X., Sharma, N., Kapadia, F., Zhou, G., Lu, Y., Hong, H., Paruchuri, K., Mahabeleshwar, G.H., Dalmas, E., Venteclef, N., et al. 2011. Kruppel-like factor 4 regulates macrophage polarization. *J Clin Invest* 121:2736-2749.