The effect of macrophage-targeted interventions on blood pressure – a systematic review and meta-analysis of preclinical studies

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An increasing body of evidence shows a role for macrophages and monocytes (as their precursors) in hypertension, but with conflicting results with regard to whether they are protective or harmful. Therefore, we systematically reviewed the effect of macrophage interventions on blood pressure in animal models, to explore which factors determine the blood pressure increasing vs. decreasing effect. A search in PubMed and EMBASE yielded 9620 records, 26 of which were included. Eighteen studies (involving 22 different experiments (k = 22)) performed macrophage depletion, whereas 12 studies specifically deleted certain macrophage proteins. The blood pressure effects of macrophage depletion were highly various and directed toward both directions, as expected, which could not be reduced to differences in animal species or methods of hypertension induction. Prespecified subgroup analysis did reveal a potential role for the route in which the macrophage-depleting agent is being administered (intraperitoneal vs intravenous subgroup difference of $P = 0.07$ (k = 22), or $P < 0.001$ in studies achieving considerable (ie, >50%) depletion (k = 18)). Along with findings from specific macrophage protein deletion studies—showing that deletion of one single macrophage protein (like TonEBP, endothelin-B, EP4, NOX-2 and the angiotensin II type 1 receptor) can alter blood pressure responses to hypertensive stimuli—the indication that each route has its specific depletion pattern regarding targeted tissues and macrophage phenotypes suggests a determinative role for these features. These hypothesis-generating results encourage more detailed depletion characterization of each technique by direct experimental comparisons, providing a chance to obtain more knowledge on which macrophages are beneficial versus detrimental in hypertension development. (Translational Research 2021; 230:123–138)

Abbreviations: $\alpha$1AMPK = AMP-activated protein kinase; DOCA = deoxycorticosterone acetate; EP$_4$ = prostaglandin E$_4$ type 4 receptor; IL-10 = interleukin 10; i.p. = intraperitoneal; i.v. = intravenous; L-NAME = L-N$^\text{G}$-Nitro arginine methyl ester; NOX-2 = NADPH oxidase 2; PBS =
phosphate buffered saline; PHD2 = prolyl hydroxylase domain protein 2; PPARγ = peroxisome proliferator–activated receptor-γ; TonEBP = tonicity-responsive enhancer-binding protein

AT A GLANCE COMMENTARY
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Background
An increasing body of animal studies shows a role for macrophages in hypertension. However, macrophage depletion leads to blood pressure increases in some studies, but decreases in others. In this meta-analysis, we found that the experimental technique used for macrophage depletion—more specifically, the route of administration of the depleting agent—may be of importance for determining the blood pressure effect.

Translational Significance
Since each technique is indicated to have a distinct depletion pattern in terms of tissues and phenotypes, a determinative role for these features is suggested. Direct experimental comparisons are however scarce, and highly warranted.

INTRODUCTION
Hypertension is highly prevalent throughout the world, and is regarded as the most important risk factor for global mortality. Its pathophysiology however, is still incompletely understood. In the last decades, the notion that monocytes and macrophages are involved in hypertension development came forward. Experimental hypertension appeared to induce monocyte infiltration in the vessel wall and organs like the kidney and the heart, and monocytes were shown to be increased in number and activation state in hypertensive animal models as well as hypertensive humans compared to healthy controls. Unraveling the causal relation between monocytes and macrophages and blood pressure regulation is essential for complete understanding of hypertension pathophysiology and might potentially result in new therapeutic targets.

To date, however, the involvement of macrophages and their monocyte precursors (further referred to as macrophages) in blood pressure regulation remains a complex matter that is not fully understood. Several animal studies have been performed to study the effect of macrophage depletion on blood pressure. Intriguingly, some of these studies report a protective role for macrophages in the development of hypertension (ie, blood pressure elevation after macrophage depletion) while others suggest a detrimental role (ie, blood pressure decrease after macrophage depletion). Potential reasons for these distinct outcomes are uncertain, but may relate to the way depletion is established.

Several experimental techniques for macrophage depletion are currently available. Nongenetic depletion is relatively straightforward compared to other methods, of which clodronate liposomes are the most widely used. When the liposomes are phagocytosed by macrophages, the phospholipid bilayers of the liposomes are degraded by lysosomal phospholipases and the clodronate (Cl2MBP) is released, which subsequently induces apoptosis of the macrophage.

An established genetic macrophage depletion model concerns transgenic mice that express the human diphtheria toxin receptor under the control of the mouse CD11b or LysM promotor. Since the human diphtheria toxin receptor is substantially more sensitive to diphtheria toxin than the mouse receptor, a certain administered dose of diphtheria toxin can specifically target the cells expressing the receptor without affecting other cells. Besides macrophage depletion models, genetically modified animal lines have been used to delete certain macrophage receptors or markers in various studies, using for example a Cre-Lox combination or adoptive transfer of monocytes from specific knock-out mice.

In this review, we therefore systematically assessed the effect of macrophage interventions on blood pressure in animal models, aiming to (1) explore potential reasons for the opposite effects of macrophage depletion on blood pressure in different studies and to (2) establish which macrophage proteins play a role in blood pressure regulation.

METHODS

Literature search. PubMed and MEDLINE were searched (until October 2019) for eligible studies. The electronic search strategy was designed by 2 authors (E.W. and C.H.) who were trained and experienced in systematic review searches for animal studies (SYRCLE, SYstematic Review Center for Laboratory animal Experimentation, Nijmegen, The Netherlands),
and can be found in the Supplemental Material. Additionally, reference lists of previously published narrative reviews and of included articles were checked for eligible studies that might have been missed by the electronic search. All animal species, publication dates, and languages were included in the search. Reporting of this review adheres to the PRISMA guidelines. The review protocol was registered at PROSPERO and can be accessed at www.crd.york.ac.uk/PROSPERO/display_record.asp?ID=CRD42019150488.

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Study selection. In order to be included in this review all identified references were screened according to the following eligibility criteria: (1) the study needed to be a primary animal study with full-text available; (2) the intervention needed to specifically and systematically target monocyte/macrophages; (3) blood pressure outcomes had to be available in the main text or supplemental data. The screening process was performed by 2 independent reviewers (E.W. and T.v.C.) and consisted of 2 phases, the first of which was based on title and abstract and the second on the full text. Reviews and conference abstracts were excluded. The review was separated in 2 parts, with part I focusing on the effect of macrophage depletion, and part II focusing on the effect of macrophage-specific phenotype alterations.

Data extraction. Data extraction was done by 2 independent reviewers (E.W. and T.v.C.). In case of missing data in the article, authors were contacted to provide the information. We extracted data on animal species, animal strain, sex, age, number of animals (in treatment and control group), details of macrophage intervention (type of depleting agent, route of administration, dosage, timing), effectivity of macrophage intervention (% reduction in monocytes/macrophages (part I) or % reduction in the targeted macrophage marker (part II)), details of hypertension induction (animal model and/or type of agent, dosage, timing), method of blood pressure measurements, and blood pressure. One study can involve multiple experiments (k), data were extracted per experiment. For blood pressure, we extracted the mean blood pressure after macrophage depletion and the mean difference after-before macrophage depletion (if available) for part I, with according standard deviations (SD) and number of animals. For part II, we extracted the mean blood pressure of the animals with macrophage-specific deletion of specific markers and control animals, with according SD and number of animals. Data not reported numerically were extracted from graphs using a digital ruler.

Risk of bias assessment. Risk of bias was assessed using SYRCLE’s Risk of Bias tool by 2 independent reviewers (T.v.C. and E.W.). Two reporting aspects were added to this tool, involving “any form of blinding” and “any form of randomization.” Disagreements were resolved through consensus-oriented discussion or by consulting a third reviewer (C.H.). Similarity of treatment and control groups at baseline in studies was judged by baseline blood pressure (part I) or baseline age or weight (part II), in which a deviation of ±10% was deemed acceptable.

Data analysis. A quantitative synthesis (meta-analysis) was planned provided there were more than 2 studies with similar interventions (for at least 2 different markers in part II). Otherwise, data were summarized qualitatively. Data were analyzed using Comprehensive Meta-Analysis Version 3.3 (Biostat, Englewood, NJ 2013) using standardized mean differences (SMD; Hedges’s g). Effect sizes are presented as the SMD with 95% confidence intervals (CIs), because of species differences and heterogeneity in the data. Data were pooled using a random-effects model, assuming a common among-study variance component across subgroups (pooled within-group estimates of tau-squared). Heterogeneity was reported and assessed by the I² statistic. When the exact number of animals per group was unclear (eg, 3—9 animals), also after repeated attempt to contact the authors, we used the lowest number for the meta-analysis. Importantly, the effectiveness of macrophage depletion may differ substantially per species and strain, genetic background, and experimental condition.

Subgroup analyses. Potential sources of heterogeneity were determined a priori, and involved animal species, method of macrophage intervention (type of agent, route of administration), and method of hypertension induction. For subgroup analyses, at least 3 independent experiments had to be present. We adjusted significance levels according to the Bonferroni method to account for multiple analyses ($P^*$ number of comparisons).

Publication bias. Publication bias was assessed by funnel plots and the trim and fill method. Because SE-based precision estimates cause distortion of SMD funnel plots, $1/\sqrt{n}$ was used as the precision estimate in the trim and fill analysis.
RESULTS

Study selection. A flow-chart of the study selection process is depicted in Fig 1. A total of 8315 records was retrieved using the electronic search (after exclusion of duplicates). After initial screening of titles and abstracts, 52 articles were included for full-text review. Out of these, 26 studies were included; 18 studies concerning macrophage depletion (part I) and 12 concerning macrophage-specific targeting of certain proteins (part II) (4 studies were included in both part I and II).

Study characteristics. Details on study characteristics can be found in Table 1 (part I) and Supplemental Table 3 (part II). Of the 18 macrophage depletion studies (part I), 9 studies involved rat models, 8 involved mouse models, and one study involved both. The majority of studies (15 of 18) used clodronate liposomes as a macrophage depletion agent, other methods included administration of diphteria toxin to a transgenic mouse model (2 studies) or administration of gemcitabine or anti-Gr1 (one study). Most studies used liposomes filled with phosphate buffered saline (PBS) as a control treatment. The most used methods of hypertension induction were angiotensin-II administration (6 studies) and salt loading (5 studies). All studies measured blood pressure in a time period of minimally 1 week, with the exception of one study that measured blood pressure until day 3 of the experiment (Falkenham et al). The effectivity of macrophage depletion differed between studies, ranging from 40% to 90%.

For part II, all 12 studies used mice as the animal model of choice. Overall, the effect of deletion of 11 different macrophage receptors was investigated in these studies. The most used method of hypertension induction was angiotensin-II administration with or without the addition of L-NAME (2 studies and 5 studies, respectively). Ten studies used Cre-Lox recombination to delete specific macrophage markers or receptors. The mice expressed Cre recombinase under the control of either the LysM promotor (8 studies) or the CD11b promotor (1 study) or the CX3CR1 promotor (1 study). Two studies used adoptive transfer of monocytes of specific knock-out mice (Shah et al: adoptive transfer with myeloid derived suppressor cells from NOX2+/− mice; Wenzel et al: adoptive transfer with monocytes from Agtr1−/− and gp91phox−/− mice). The effectivity of deletion of the targeted proteins in macrophages differed between studies, ranging from 60% to 98% (Supplemental Table 3).

Risk of bias. Fig 2A+B (part I) and Fig 2C+D (part II) show the risk of bias assessment of the 26 included studies. Overall, several aspects of the SYRCLE risk of bias tool had to be scored unclear due to missing information regarding experimental details. For example, it was generally unclear whether the group allocation was blinded, the animals were housed randomly, and whether or not outcome assessment was blinded. Two reporting items were added to the SYRCLE risk of bias tool, with 6 of 18 (part I) and 3 of 12 (part II) studies reporting randomization at any levels, and 8 of 18 (part I) and 4 of 12 (part II) studies reporting blinding at any level.

Quantitative synthesis of results (part I). For part I, all 18 studies could be included in the meta-analysis regarding the absolute blood pressure after macrophage intervention, involving 22 experiments (k = 22). There were not enough studies that reported the mean difference (SD) in blood pressure after-before the macrophage intervention to pool the data (k = 1). The direction of the
Table 1. Summary of study characteristics (part I)

| Study (PMID) | Species and model | Sex | Age | N (T) | N (C) | Type of agent | Type of control | Dosage per day | Route | Interval | Hypertension induction | Macrophage / monocyte reduction (%) |
|-------------|-------------------|-----|-----|-------|-------|---------------|----------------|----------------|-------|----------|----------------------|----------------------------------|
| Czopek et al 1 (30657897) | M; CD11b-DTR and FVB/N (control) | M CD11b-DTR and FVB/N (control) | M | 6–10 wk | 9* | 13* | DT | CL liposomes | 0.0025 mg/kg | i.p. | Every 2 days | ET-1 | 90% monocytes 90% monocytes |
| Czopek et al 2 (30657897) | M; CD11b-DTR and FVB/N (control) | M | 8–10 wk | 4 | 4 | DT | PBS injection | 0.0025 mg/kg | i.p. | Every 2 days | Salt | 85.5% monocytes |
| Falkenham et al (25794704) | M; C57BL/6 (Wild type) | M | 6 wk | 10 wk | 9 | 13* | CL liposomes | PBS liposomes, free CL | 25 mg/kg | i.v. | Every day | Ang II | 90% monocytes |
| Fehrenbach et al (31215801) | R; Dahl SS (SS/JrHSDMcw) | M | 7–9 wk | 8 | 11 | CL liposomes | PBS liposomes, free CL | 3.6 mg/kg | i.v. | Weekly | Salt | 50% splenic mΦ, 50% monocytes** |
| Huang et al 1 (29867533) | M; C57BL/6 | M | 6 wk | 8 | 8 | CL liposomes | PBS liposomes | 16.7 mg/kg | i.v. | Every 3 days | Ang II | 62% kidney mΦ, 70% monocytes |
| Huang et al 2 (29867533) | R; Dahl SS | M | 6 wk | 8 | 8 | CL liposomes | PBS liposomes | 6.7 mg/kg | i.v. | Every 3 days | Salt | 67% monocytes |
| Kain et al (26539962) | R; Sabra hypertensive | M | 6 wk | 4 | 5 | CL liposomes | PBS liposomes | 10 mg/kg | i.v. | Every 2 days | Salt | 60% cardiac mΦ, 80% monocytes |
| Kriska et al (22467300) | M; C57BL/6 | M | 6 wk | 6* | 24* | CL liposomes | PBS liposomes | 25 mg/kg | i.v. | Every 2 days | L-NAME | / |
| Machnik et al. (2009) (19412173) | R; Sprague-Dawley | M | 8–9 wk | 16 | 9 | CL liposomes | PBS liposomes | 417 mg/kg | i.v. | Every 3 days | Salt | 70% ear mΦ |
| Machnik et al. (2010) 1 (20142563) | R; Sprague-Dawley | M | 8–9 wk | 12 | 12 | CL liposomes | PBS liposomes | 417 mg/kg | i.v. | Every 3 days | Salt | 44% ear mΦ** |
| Machnik et al. (2010) 2 (20142563) | R; Sprague-Dawley | M | 8–9 wk | 10 | 10 | CL liposomes | PBS liposomes | 417 mg/kg | i.v. | Every 3 days | Salt | 57% ear mΦ |
| Martin et al. (26730742) | R; Wistar | M | 5 wk | 5 | 5 | CL liposomes | PBS liposomes | 4 mg/kg | i.p. | Every 3 days | Ang II | 40% kidney mΦ** |
| Moore et al (26071547) | M; C57BL/6J | M | 7 wk | 7 | 7 | CL liposomes | PBS liposomes | 14 mg/kg | i.p. | Every 3 days | Ang II | 48% aortic mΦ, 65% monocytes** |
| Mui et al. (29351460) | R; Sprague-Dawley | R; Stroke-prone spontaneously hypertensive rats | M | 8 wk | 7–7 | 5–7 | CL liposomes | PBS liposomes | 3.6 mg/kg | i.v. | Weekly | Salt | 95% mesenteric arteries mΦ |
| Pires et al. (23647512) | R; Sprague-Dawley | M | 6–12 wk | 15 | 15 | CL liposomes | PBS liposomes | 12.5 mg/kg | i.v. | Every 3 or 4 days | Spontaneously hypertensive rat model | 53% cerebrum mΦ |

(continued on next page)
| Study (PMID)       | Species and model | Sex | Age | N (T) | N (C) | Type of agent | Type of control | Dosage per day | Route (i) | Interval | Hypertension induction | Macrophage / monocyte reduction (%) |
|-------------------|-------------------|-----|-----|-------|-------|---------------|----------------|----------------|-----------|----------|------------------------|-------------------------------------|
| Ruan et al        | CL                | M   | 8–10 wk | 6–15 | 6–15 | liposomes     | PBS liposomes   | 10.7 g/kg[^5] | i.p.     | Weekly (2 in 1st week) | Ang II                              |
| Shah et al 1      | M; RFP            | M   | 8–10 wk | 7    | 7    | gemcitibarine | Saline         | 0.125 mg[^6]   | i.p.     | Every 2 days | Ang II                              |
| Shah et al 2      | M; RFP            | M   | 8–10 wk | 7    | 7    | anti-Gr1      | Saline         | gemcitibarine | i.p.     | 30 mg/kg | Ang II                              |
| Shimada et al     | M; C57BL/6J       | M   | 8–10 wk | 22   | 23   | liposomes     | PBS liposomes   | /             | i.v.     | Once     | Salt                                 |
| Thang et al       | R; Sprague-Dawley | M   | /    | 6–8   | 6–8  | liposomes     | PBS liposomes   | 3.6 mg/kg[^7] | i.p.     | Weekly   | Salt                                 |
| Wenzel et al 1     | M; LysM-DTR (on C57BL/6 background) | M   | /    | 3–9   | 3–9  | DT            | DT to control mice | 0.005 mg/kg[^8] | i.v.     | Once daily | Ang II                              |
| Zandbergen et al  | R; Ren-2          | M   | 6 wk | 77    | 77   | liposomes     | PBS liposomes   | 2.9 mg/kg[^9] | i.v.     | Weekly   | Renin-2 mutation                    |

Species: M, mouse. R, rat. Sex: M, male. F, female. N (T): N (treatment group). N (C): N (control group). CL, clodronate. DT, diphtheria toxin. PBS, phosphate buffered saline. i.p., intraperitoneal. i.v., intravenous.

[^5]: First dose between brackets (if deviating from rest of the doses).
[^6]: Assuming a weight of approximately 20 g for each mouse.
[^7]: Not included in meta-analysis 2 because of <50% reduction.
[^8]: Assuming a weight of approximately 200 g for each rat. mφ, macrophage. Ang II, angiotensin II. ET-1, endothelin-1. L-NAME, L-NG-Nitro arginine methyl ester.
The effect of macrophage depletion on blood pressure differed considerably between studies, as expected (SMD (95% CI) −0.071 (−0.833−0.691); P = 0.86; I² 91%; k = 22), also when only including studies that achieved substantial (>50%) monocyte/macrophage reduction (−0.221 (−0.977−0.535); P = 0.57; I² 89%; k = 18; Supplemental Figs 1 and 2). Prespecified subgroup analyses did not reveal statistically significant differences between animal species (rats vs mice; P = 0.92), hypertension induction methods (salt loading vs. angiotensin-II vs other; P = 0.58), or route of administration, although the latter showed a trend toward a difference (P = 0.07) (intraperitoneal (i.p.): SMD (95% CI) 0.776 (−0.253−1.806); P = 0.14; I² 85%; k = 11, intravenous (i.v.): −0.850 (−2.060−0.359); P = 0.17; I² 93%; k = 8; Fig. 3 and 5, A). In the analysis only including studies that achieved substantial (>50%) monocyte/macrophage reduction (k = 18), i.p. administration of the macrophage depleting agent leads to a blood pressure increase (SMD (95% CI) 1.405 (0.472−2.338); P = 0.003; I² 69%; k = 8), and i.v. administration results in a blood pressure decrease (SMD (95% CI) −1.607 (−2.576 to −0.638; P = 0.001; I² 88%; k = 7; P < 0.001 for subgroup differences; Fig 4 and 5, B). The subgroup analyses on animal species (P = 0.40) and on method of hypertension induction (P = 0.93) did not reveal any differences (Fig 5, B).

**Publication bias.** Assessment of the funnel plot suggested no publication bias (Supplemental Fig 3).

**Qualitative synthesis of results (part II).** In the 12 studies that deleted specific macrophage proteins, a total of 10 different proteins were targeted. Since of only one (mineralocorticoid receptor) there were more than 2 independent experiments available, a qualitative rather than quantitative synthesis was carried out, summarized in Table 2. Of the total of 10 macrophage proteins, 3 were identified to have a protective role against hypertension development (ie, blood pressure increase with deletion), including TonEBP, endothelin-B, and EP4. NOX-2 and the angiotensin II type 1 receptor on the other hand, worsen hypertension, shown by attenuated angiotensin-II-induced blood pressure increases with adoptively transferred NOX-2−/− or angiotensin-II receptor type 1−/− monocytes compared to wild type monocytes. In contrast, NOX-2-negative myeloid-derived suppressor cells (involving monocyte subtypes but also granulocyte subtypes), increase blood pressure, indicating a protective role for this receptor in these cell types (in concordance with their apparent T-cell suppressive effect). Deletion of the macrophage mineralocorticoid receptor led to a blood pressure decrease in 2 studies (Bienvenu et al: L-NAME-induced hypertension, blood pressure at +4 weeks; Rickard et al.: DOCA-induced hypertension, blood...
pressure at +4 and +8 weeks), but an increase in one study (Usher et al: salt-induced and L-NAME-induced hypertension, blood pressure at +2 weeks and +3.5 weeks). Four proteins could not be demonstrated to have any effect on blood pressure, involving IL-10, PHD2, α1AMPK, and PPARγ (Supplemental Table 3).

DISCUSSION

The divergent and opposite results of macrophage depletion studies in terms of blood pressure underline the fine complexity of macrophage functionality in blood pressure regulation. We identified the route of administration of macrophage-depleting agents as well as the extent of macrophage depletion as potential determinants of the direction of the blood pressure effect. Together with the observation that specific and single macrophage proteins determine a blood pressure elevating vs. lowering effect, this points toward a decisive role for macrophage phenotype—since there are clear indications that different routes of administration target distinct tissues and phenotypes.

This has best been investigated for clodronate liposomes. Both i.p. and i.v. clodronate liposomes deplete macrophages or their precursors in for example the circulation, bone marrow, spleen and liver, but i.p. administration additionally depletes macrophages in the peritoneal cavity and (some) lymph nodes. The extent of macrophage depletion within each organ also differs; for example for the colon, i.v. administration is more efficient compared to i.p. administration (90% vs. 50% macrophage depletion).23 With regard to phenotype, it becomes more complicated, since (to the best of our knowledge), this has not been determined with experiments directly comparing different routes of administration.

| Group by Route of administration | Study name | Statistics for each study | Hedges's g and 95% CI |
|---------------------------------|------------|---------------------------|-----------------------|
|                                |            | Hedges's g | Lower limit | Upper limit |          |          |          |
| i.p.                            | Martin et al. | -2.652    | -4.266     | -1.038     |          |          |
| i.p.                            | Macht et al. 2010 (1) | 1.530    | 0.645      | 2.416      |          |          |
| i.p.                            | Moore et al. | -2.127    | -3.385     | -0.869     |          |          |
| i.p.                            | Macht et al. 2010 (2) | 1.052    | 0.151      | 1.952      |          |          |
| i.p.                            | Wenzel et al. | -1.079    | -2.498     | 0.339      |          |          |
| i.p.                            | Macht et al. 2009 | 0.933    | 0.102      | 1.764      |          |          |
| i.p.                            | Ruan et al.     | 1.928    | 0.630      | 3.227      |          |          |
| i.p.                            | Czopek et al. (1) | 3.527    | 1.771      | 5.282      |          |          |
| i.p.                            | Czopek et al. (2) | 3.114    | 1.169      | 5.058      |          |          |
| i.p.                            | Shah et al. (1) | 1.487    | 0.285      | 2.689      |          |          |
| i.p.                            | Shah et al. (2) | 1.074    | 0.016      | 2.133      |          |          |
| i.p.                            |              | 0.776    | -0.253     | 1.806      |          |          |
| i.p. (i.v.)                     | Pires et al.     | -0.080   | -0.777     | 0.616      |          |          |
| i.p. (i.v.)                     | Thang et al.     | -1.950   | -3.168     | -0.732     |          |          |
| i.p. (i.v.)                     | Mui et al.       | -1.383   | -2.656     | -0.110     |          |          |
| i.p. (i.v.)                     |              | -1.104   | -3.029     | 0.821      |          |          |
| i.v.                            | Fehrenbach et al. | 7.309    | 4.828      | 9.791      |          |          |
| i.v.                            | Kain et al.      | -0.833  | -2.063     | 0.398      |          |          |
| i.v.                            | Huang et al. (1) | -3.488  | -5.011     | -1.965     |          |          |
| i.v.                            | Zandbergen et al. | -0.441  | -1.435     | 0.554      |          |          |
| i.v.                            | Huang et al. (2) | -2.629  | -3.484     | -1.054     |          |          |
| i.v.                            | Fahnhanem et al. | 0.402  | -0.424     | 1.229      |          |          |
| i.v.                            | Kriska et al.    | -4.746  | -6.230     | -3.263     |          |          |
| i.v.                            | Shimada et al.   | -0.885  | -1.488     | -0.283     |          |          |
| i.v.                            |              | -0.850  | -2.060     | 0.359      |          |          |

Fig 3. Part I: meta-analysis 1 (including all studies). Data were analyzed using standardized mean differences (Hedges’s g). Effect sizes are presented as the standardized mean difference (SMD) with 95% confidence intervals, because of species differences and heterogeneity in the data. Data were pooled using a random-effects model, assuming a common among-study variance component across subgroups (pooled within-group estimates of tair-squared).
### Table

| Route of administration | Study name                      | Hedges's g | Lower limit | Upper limit |
|-------------------------|--------------------------------|------------|-------------|-------------|
| i.p.                    | Machnik et al. 2010 (2)        | 1.052      | 0.151       | 1.952       |
| i.p.                    | Wenzel et al.                  | -1.079     | -2.498      | 0.339       |
| i.p.                    | Machnik et al. 2009            | 0.933      | 0.102       | 1.764       |
| i.p.                    | Ruan et al.                    | 1.928      | 0.630       | 3.227       |
| i.p.                    | Czopek et al. (1)              | 3.527      | 1.771       | 5.282       |
| i.p.                    | Czopek et al. (2)              | 3.114      | 1.169       | 5.058       |
| i.p.                    | Shah et al. (1)                | 1.487      | 0.285       | 2.689       |
| i.p.                    | Shah et al. (2)                | 1.074      | 0.016       | 2.133       |
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| i.p.                    | Czopek et al. (2)              | 3.114      | 1.169       | 5.058       |
| i.p.                    | Shah et al. (1)                | 1.487      | 0.285       | 2.689       |
| i.p.                    | Shah et al. (2)                | 1.074      | 0.016       | 2.133       |
| i.p.                    | Czopek et al. (1)              | 3.527      | 1.771       | 5.282       |
| i.p.                    | Czopek et al. (2)              | 3.114      | 1.169       | 5.058       |
| i.p.                    | Shah et al. (1)                | 1.487      | 0.285       | 2.689       |
| i.p.                    | Shah et al. (2)                | 1.074      | 0.016       | 2.133       |
| i.p.                    | Czopek et al. (1)              | 3.527      | 1.771       | 5.282       |
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| i.p.                    | Shah et al. (1)                | 1.487      | 0.285       | 2.689       |
| i.p.                    | Shah et al. (2)                | 1.074      | 0.016       | 2.133       |
| i.p.                    | Czopek et al. (1)              | 3.527      | 1.771       | 5.282       |
| i.p.                    | Czopek et al. (2)              | 3.114      | 1.169       | 5.058       |
| i.p.                    | Shah et al. (1)                | 1.487      | 0.285       | 2.689       |
| i.p.                    | Shah et al. (2)                | 1.074      | 0.016       | 2.133       |

**Fig 4.** Part II: meta-analysis 2 (including only studies achieving substantial ($\geq 50\%$) macrophage depletion). Data were analyzed using standardized mean differences (Hedges’s $g$). Effect sizes are presented as the standardized mean difference (SMD) with 95% confidence intervals, because of species differences and heterogeneity in the data. Data were pooled using a random-effects model, assuming a common among-study variance component across subgroups (pooled within-group estimates of tau-squared).

**Fig 5.** Summary estimates of subgroup analysis of meta-analysis 1 (A) and 2 (B). A. Meta-analysis 1 ($k = 22$). There was no significant difference between subgroups on species ($P = 0.92$) or method of hypertension induction ($P = 0.58$). There was a trend toward a difference between subgroups on route of administration ($P = 0.07$). B. Meta-analysis 2 ($k = 18$). There was no significant difference between subgroups on species ($P = 0.40$) or method of hypertension induction ($P = 0.93$). There was a significant difference between subgroups on route of administration ($P < 0.001$). i.p., intraperitoneal; i.v., intravenous; SMD, standardized mean difference; SEM, standard error of the mean.
administration using the same agent. Ferenbach et al did reveal important information, by showing considerable differences between i.v. clodronate liposomes and i.p. diphtheria toxin in CD11b-diphtheria toxin receptor mice. I.v. administration of clodronate liposomes left “M2-like” CD206+ renal macrophages unaltered, in contrast to i.p. administration of diphtheria toxin. Also, i.p. diphtheria toxin had a more profound effect on circulating monocytes and renal macrophages. Only i.v. clodronate liposomes appeared to protect against kidney damage. This is in concordance with the finding in the present review that also in terms of blood pressure, the beneficial effect is only achieved with i.v. administration of clodronate liposomes and not with i.p. Potentially, but speculatively, macrophage depletion is only beneficial to a certain extent, ensuring that enough “beneficial” macrophages are left behind. Importantly, different types of macrophage-depleting agents almost certainly target distinct tissues and phenotypes (e.g., clodronate liposomes vs diphtheria toxin, the most used methods in this review), which raises the question whether these studies should be pooled at all. Since the goal of all these interventions is similar, that is, “macrophage depletion,” we chose to start with a full overview including all studies. While this imposes a risk of comparing pears and oranges, it can actually also reveal important information by subsequently looking into blood pressure effects of subgroups. This full overview also included one study that used gemcitabine and anti-Gr1 as a depletion agent, which should be noted is the most precarious to include since these agents target myeloid-derived suppressor cells, which involve monocyte subtypes but also granulocyte subtypes. However, since actually probably none of the macrophage-depletion methods currently available are perfectly specific for macrophages (discussed further below in more detail), and for example for gemcitabine there are indications that in fact preferentially monocytes are targeted, we chose to include this study, while checking that repeating the analyses excluding this study did not materially affect our results. We emphasize that pre-specified subgroup analysis on type of agent could not be performed due to the limited amount of studies using other methods than clodronate liposomes. Control treatments mainly consisted of liposomes filled with PBS (for the majority

| Macrophage proteins | Proposed mechanism |
|---------------------|--------------------|
| Endothelin B receptor$^{13}$ | Protective against hypertension development |
| TonEBP$^{21}$ | Macrophage TonEBP regulates VEGF-C-mediated lymphangiogenesis, which appears to protect against excessive salt-induced blood pressure rises (albeit by mechanisms not fully elucidated) |
| PGE$_2$ type 4 (EP$_4$)$^{18}$ | COX-2 via EP$_4$ plays a role in VEGF-C production and lymphangiogenesis |
| Angiotensin II type 1 receptor$^{14}$ | The angiotensin II type 1 receptor contributes to reactive oxygen species formation and vascular dysfunction |
| NOX-2 (NADPH oxidase)$^{14}$ | The NOX-2 pathway contributes to reactive oxygen species formation and vascular dysfunction |
| Mineralocorticoid receptor$^{36-38}$ | Contributes to hypertension$^{56,57}$ |

Table 2. Role of macrophage proteins in blood pressure regulation (part II). A qualitative synthesis is depicted for the 6 macrophage proteins that were identified to play a role in hypertension development, as derived from the 12 studies included in part II of this review.
of studies that used clodronate liposomes) which seems to be a good control experiment. Although it has been suggested that also PBS liposomes may temporarily inhibit macrophage function, Falkenham et al directly assessed the effect of PBS liposomes on different leucocyte subsets and did not observe indications for this. In the case that an inhibitory effect of PBS liposomes on macrophages does exist, this may have resulted in an underestimation of the blood pressure effect in our meta-analysis rather than an overestimation (since substantial macrophage depletion would be compared to subtle macrophage depletion instead of no macrophage depletion at all). The notion that the phenotype of macrophages is determinative is supported by Harwani et al, who describes that in general, pro-inflammatory (M1-like) macrophages are the culprits in hypertension while anti-inflammatory (M2-like) macrophages are able to reduce blood pressure. Amongst others, spontaneously hypertensive rats are shown to have higher “M1/M2” ratios than normotensive Wistar-Kyoto rats, and after reducing this ratio by heme oxygenase-1 induction, blood pressure decreases. On the other hand, this notion does not exclusively comply with the effects of the described receptors in this review. For example, despite its beneficial role (shown by aggravated blood pressure increases with deletion), TonEBP has been associated with the “M1” phenotype rather than the “beneficial” “M2” phenotype. Of note, the fact that macrophages are not static but rather highly versatile and able to switch between phenotypes should be taken in consideration when assessing the role of macrophage phenotypes and hypertension.

As said above, an essential remark with regard to both macrophage depletion studies and macrophage protein alteration studies is that probably none of the methods used are perfectly specific for macrophages. Theoretically, clodronate liposomes may additionally target neutrophils and dendritic cells—in concordance with their ability for phagocytosis—although in practice, several studies demonstrated no effect on circulating neutrophil counts or (renal and splenic) dendritic cells. With diphtheria toxin, it depends on to which promotor the receptor is coupled, which involved LysM14 and CD11b13 in the studies in this review. LysM is also expressed in granulocytes and some dendritic cells, and CD11b is expressed by all myeloid cells. Indeed, there was a reduction in circulating dendritic cells with CD11b-targeted depletion, although no reduction in neutrophils was observed with both methods. When more immune cells are targeted, adoptive transfer of each type can help to distinguish cell-specific roles. Wenzel et al14 and Czopek et al13 performed adoptive transfer of CD11b+Gr1+ monocytes and were able to confirm that the observed effect of depletion was truly due to monocytes and not to other potentially depleted cell types. However, dendritic cells remain a relatively ignored cell type, despite that they are targeted with many macrophage depletion methods and there is increasing evidence that they play a role in blood pressure regulation as well. For example, when dendritic cells are triggered by in vitro sodium excess and subsequently adoptively transferred into naive mice, hypertension is aggravated, with mechanisms likely involving promotion of renal oxidative stress, inflammation, and fluid retention. Also, besides the macrophage depletion methods used in the studies that could be included in this review, more (and newer) methods exist and evolve rapidly. A detailed overview is given by other authors and involves among others macrophage Fas-induced apoptosis and CX3CR1-DTR models. Taken together, the potential role of other possibly depleted immune cell types than macrophages with each method needs to be kept in mind, and further scrutinized.

The question remains which mechanisms underlie the relation between macrophages and hypertension. Protective actions that have been currently identified involve clearance of vasoconstricting agents (endothelin-1), induction of lymphangiogenesis, and suppression of T-cell activation, but many more may be yet undiscovered. Cytokine excretion is assumed to have a key role in the harmful effects of macrophages and hypertension, leading to vascular dysfunction and renal sodium retention. Specifically, typical “M1” or pro-inflammatory macrophage cytokines like TNF-α and IL-1β have been implicated in the development of hypertension, supporting the idea of macrophage phenotype as a determinant feature. The process of clinical translation is ongoing, with the CANTOS trial showing a beneficial effect of anti-IL-1β on cardiovascular disease but not on blood pressure. Also, the effect of anti-TNF-α on blood pressure is not univocal in humans. Interpretation of negative findings with regard to the presumed importance of inflammation in hypertension is hampered by the lack of sufficiently precise characterization of inflammation, which is still a broad term encompassing a multiplicity of distinct pathways. A detailed narrative description of underlying mechanisms of macrophages and hypertension as well as the role of cytokines fall beyond the scope of this review, and for this we refer to several comprehensive literature reviews from other authors. It may be pointed out that, besides having a causal role in hypertension development, monocytes and macrophages can also be affected as a consequence of hypertension, subsequently attenuating or worsening further blood pressure increases.
Although our literature search involved all animal species, the included studies in the present review only involved rats and mice. Separating rat studies from mouse studies did not result in a clear distinction in outcome. Mouse and rat strains were too variable to perform subgroup analyses on. The immune system of rats and mice bear some important differences compared to humans, complicating translation of our results to the human hypertension setting. Among others, lymphocytes are the most abundant leukocytes in rats and mice, while in humans neutrophils form the majority. For translation to the human situation, human haematopoietic system mice may be an interesting alternative, although they involve quite complicated models and some have poor reconstitution of mononuclear phagocyte system cell types. Also complicating translation to the human situation, is the lack of existence of a perfect animal model for primary human hypertension (ie, the most abundant form of hypertension in humans), largely due to the still incomplete pathophysiological knowledge of primary hypertension. Each experimental model of hypertension comes with its own pitfalls and advantages, which has been reviewed in detail by other authors. Our meta-analysis did not reveal considerable differences in macrophage depletion between salt-induced hypertension and angiotensin-II-induced hypertension; other methods like L-NAME or endothelin-I administration were not applied in enough included studies to reliably compare.

In humans, the role of macrophages in blood pressure regulation remains difficult to study. Among the first evidence in humans, Czopek et al demonstrated that blood pressure increased more in vasculitis patients who received a treatment that also affected monocytes and macrophages (cyclophosphamide) compared to patients who received a treatment without an effect on monocytes and macrophages (mycophenolate mofetil). Furthermore, a salt-sensitive blood pressure increase in 11 healthy human volunteers was shown to coincide with an increased pro-inflammatory monocyte phenotype (increased CCR2 expression) and macrophage phenotype (increased HLA-DR expression and reduced CD206 expression; and increased pro-inflammatory cytokine secretion in vitro). Successful translation to humans, however, requires very specific interventions (since general macrophage interventions are unfeasible), which necessitates more detailed knowledge on when and which macrophages are protective vs. harmful in hypertension development.

Roughly, there may be 2 general types of approach for future studies (Table 3). The first approach, total macrophage depletion (reviewed in part I), was the major starting point of this research area and significantly contributed to the knowledge on macrophages and hypertension. However, as this review points out, major but also subtle differences exist between different methods (and even potentially between different routes of administration of the same agent), leading to opposite effects on blood pressure in some cases. Rather than regarding this as a nuisance, future direct comparisons that closely characterize targeted phenotypes/locations with each depletion method along with its effect on blood pressure can shed more light on when and which macrophages are beneficial vs. detrimental in hypertension development (for now, no definitive conclusion can be made yet since there is a paucity of direct comparisons). The second approach consists of targeting specific macrophage proteins (reviewed in part II). Mechanistically, this approach may be more interesting, and therefore provides a suitable way to obtain more knowledge on pathophysiology. However, since an abundance of macrophage proteins exists, this approach may be complementary to the first approach. From the proteins that are currently identified, it can be deduced that the pro-angiogenic function of macrophages may be important for protection against hypertension, and that pro-inflammatory phenotypes contribute to hypertension. Especially pro- vs. anti-inflammatory is still a broad term, and should be reduced to specific processes. With each approach, potential side effects or off-target effects should be closely scrutinized, as well as potential differences in hypertension induction methods (preferentially making use of more than one type of induction).

The main strength of this review is that we are—to the best of our knowledge—the first to address this issue in a systematic manner. The impact of systematic reviews of animal studies is increasing because of their value in pointing out flaws in methodologic quality in current animal research and improving translation to clinical care by specifically uncover directions for future research, amongst others. Nevertheless, certain limitations need to be considered. It should be emphasized that our finding with regard to the relevance of the route of administration of macrophage depleting agents, derived from subgroup analysis, is hypothesis-generating rather than hypothesis-testing, especially since certain important prespecified subgroup analyses (ie, on type of agent) could not be performed. However, the hypothesis-generating nature of this meta-analysis does guide future research in this area. For instance, our results warrant a direct comparison between different macrophage depleting techniques along with more close characterization of depleted phenotypes and tissues. Second, there was substantial heterogeneity between studies. However, heterogeneity in animal research is expected due to the
Table 3. Hypotheses generated by the results of this systematic review. Motivations for each hypothesis are given, and suggestions are made for future studies.

| Hypotheses generated | Based on | Suggestions for future hypothesis-testing research |
|-----------------------|----------|--------------------------------------------------|
| i) pro-inflammatory* macrophage phenotypes contribute to hypertension and anti-inflammatory *macrophage phenotypes may alleviate hypertension |
| *this may not necessarily mean phenotypes associated with the classical “M1” vs “M2” phenotype |
| 1) i.v. administration of macrophage depleting agent → lowers blood pressure → is suggested to preferentially target pro-inflammatory phenotypes |
| 2) i.p. administration of macrophage depleting agent → increases blood pressure → is suggested to have a more rigorous effect on all phenotypes |
| 2) Deletion of one singular macrophage protein can change the blood pressure response to hypertension induction |
| - Direct comparisons between i.v. and i.p. administration in terms of targeted macrophage phenotype and blood pressure |
| - Targeting of specific macrophage proteins according to groups of mechanistic processes (eg, lymphangiogenesis, pro-inflammatory priming, clearance of vasoconstricting agents) to uncover underly pathophysiology. |
| ii) location of macrophages may determine their role in blood pressure regulation |
| Opposite blood pressure effect between i.v. and i.p. administration of macrophage depleting agent, along with indications for different depletion patterns in terms of targeted tissues. |
| Direct comparisons between i.v. and i.p. administration in terms of targeted tissues and blood pressure. All studies need to report macrophage reduction in several tissues. |
| iii) the extent/rigour of macrophage depletion might determine the effect on blood pressure |
| 1) selection of studies based on the extent of macrophage depletion affected the results (studies showing less than <50% depletion appeared to have divergent results) |
| 2) i.p. seems more rigorous than i.v.; potentially also beneficial (ie, anti-inflammatory) macrophages are being targeted |
| Direct comparisons between different extents of macrophage depletion and their associated effect on blood pressure. All studies need to report the percentage of circulating monocyte and tissue macrophage (preferably several tissues) reduction. |

explorative nature of most studies, and, exploration of sources of heterogeneity can even be regarded as one of the most added values in meta-analyses of animal studies. Among others, the large differences in effectiveness of macrophage depletion between studies may be an important contributor to the large heterogeneity that was observed between studies in this meta-analysis. To address this, we performed an analysis that excluded studies reporting 50% or less monocyte or macrophage reduction, but the chosen cut-off is arbitrary. This analysis does reveal that the extent of macrophage depletion may also determine the effect on blood pressure, since the studies with less substantial depletion appeared to have divergent effects. Therefore, the extent of macrophage depletion in the circulation as well as in different tissues needs to be specifically considered and measured (and ideally, compared) in future experiments. Third, the risk of bias of the included studies was scored unclear in the majority of studies due to poor reporting of essential methodological details. Last, and perhaps most importantly, the fact that macrophage interventions are not 100% specific and may also target other immune cell types (mostly neutrophils and dendritic cells), deserves consideration in making definitive conclusions, despite that we were conservative with regard to selection of eligible interventions. We excluded depletion methods that were shown to have very clear off-target effects on tissues or other subsets of immune cells (besides dendritic cells, which may be targeted with every method currently available). Excluded interventions involve for example altered vascular development with monocytic macrophage colony stimulating factor-deficient osteopetrotic mice (op/op), endothelial alterations with CCR2 knockout mice, or interventions targeting macrophage phenotype by administration of agents that can have a variety of side effects. Also, interventions with antibodies targeting receptors (eg, CCR2 or CD161a) or cytokines (eg, IL-6 or CCL2/MCP-1) were deemed too unspecific, since
most receptors and cytokines are either present on or produced by more immune cells than solely monocytes or macrophages. Although the choice on in- versus exclusion for specific macrophage depletion techniques was done with great caution by 2 independent authors (as described above), this choice may not be black and white, since specificity of methods is often not completely known and may even differ between different reports or with new insights. In general, it should be noted that only 2 of the 26 included studies comprised female animals. Since there is evidence for considerable differences in the immune system and hypertension

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Supplementary materials

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