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Monocyte progenitors give rise to multinucleated giant cells

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The immune response to mycobacteria is characterized by granuloma formation, which features multinucleated giant cells as a unique macrophage type. We previously found that multinucleated giant cells result from Toll-like receptor-induced DNA damage and cell autonomous cell cycle modifications. However, the giant cell progenitor identity remained unclear. Here, we show that the giant cell-forming potential is a particular trait of monocyte progenitors. Common monocyte progenitors potently produce cytokines in response to mycobacteria and their immune-active molecules. In addition, common monocyte progenitors accumulate cholesterol and lipids, which are prerequisites for giant cell transformation. Inducible monocyte progenitors are so far undescribed circulating common monocyte progenitor descendants with high giant cell-forming potential. Monocyte progenitors are induced in mycobacterial infections and localize to granulomas. Accordingly, they exhibit important immunological functions in mycobacterial infections. Moreover, their signature trait of high cholesterol metabolism may be piggy-backed by mycobacteria to create a permissive niche.
he development of the *Homo sapiens sapiens* has been in coevolution with mycobacteria. It can be safely assumed that this process is ongoing, since even in highly developed countries, humans are exposed to mycobacteria on a daily basis. Environmental mycobacteria with potential virulence for humans such as *Mycobacterium avium* are regularly found in drinking waters. Moreover, an estimated 2 billion people worldwide are infected with *M. tuberculosis* and most of these infections are latent, i.e. asymptomatic. In other words, tight immunological control is essential to prevent the individual from succumbing to mycobacterial infection, even after decades of coexistence. A well-appreciated site of mycobacterial latency is the unique multicellular granuloma reaction. Distinct types of macrophages (MΦ), particularly multinuclear giant cells (MGC), contribute to the core of granulomas. Although the first description of MGC in tuberculous granuloma was published 150 years ago and their origin from leukocytes has long been postulated, mechanisms underlying mycobacterial MGC formation have still been incompletely resolved. We have recently shown that the development of polyploid MGC involves cell-autonomous affiliation of DNA damage and impairment of p53 function by the potent antitubercular effector nitric oxide (NO), resulting in mitotic defects and multinucleation. Thus, much enlarged MΦ that avidly phagocytose mycobacteria and infected apoptotic cells arise. However, mycobacteria may override the antibacterial activity of MGC, turning them into dedicated mycobacterial niches within the human host. Despite the striking cell biological properties of MGC, their origin remains poorly understood. Dissection of the MGC pedigree seems particularly important, regarding the recent revolution in understanding MΦ development. In particular, it remains to be resolved, if MGC are the progeny of a specific cell lineage or subset, and what metabolic preconditions might determine the differentiation path towards an MGC. Mononuclear phagocytes, i.e., monocytes, MΦ, and dendritic cells (DC) share a common progenitor, the monocyte-MΦ-DC progenitor (MDP), although recent data rather indicate that MDP and GMP constitute independent progenitors of monocytes. The position of MDP in definitive hematopoiesis is based on experimental data from mice and receives further support by the recent identification of the human MDP analogue. An even more committed clonogenic common monocyte progenitor (cMoP), which renews monocytes and MΦ independently of DC, has been identified in the bone marrow and spleen of mice, as well as in human bone marrow and umbilical cord blood. Whereas the role of cMoP to monocyte and MΦ development is well established, biological functions beyond myeloid cell development have not been assigned to cMoP, yet.

Here, we introduce cMoP and its progeny, the circulating inducible monocyte progenitor (iMoP) as immune cells with a particular capacity for MGC transformation. The high cholesterol and lipid metabolism in cMoP is an essential prerequisite for MGC formation and may favor intracellular mycobacterial survival, since cholesterol serves as an energy source for *M. tb*. Together, we provide firm evidence for a so far unappreciated role of monocyte progenitors in mycobacterial infections. They inextricably integrate progenitor characteristics, such as high proliferative activity and low apoptosis, with specific functions in mycobacterial immunity.

**Results**

cMoP are dedicated progenitors for multinucleated giant cells. We previously found that MΦ progenitors have a higher potential than mature MΦ to undergo transformation into MGC upon infection with mycobacteria or TLR2 stimulation (Fig. 1a and Herrtwich et al.\(^9\)). However, since the progenitor characteristics remained undefined, we decided to systematically explore the identity of the MGC progenitor. Thus, we stringently sorted monocytes (MC), cMoP, MDP, and Lin\(^{-}\) c-kit\(^{+}\) progenitors from the bone marrow of *C57Bl/6* reporter mice (Fig. 1b). In agreement with the most widely accepted definitions (Auffray et al.\(^11\), Hettinger et al.\(^15\)), we identified MDP as CD11b\(^{-}\) C117\(^{+}\) CD115\(^{+}\) CX3CR1\(^{+}\) Ly6C\(^{-}\). cMoP differed from MDP with respect to Ly6C expression (CD11b\(^{-}\) C117\(^{+}\) CD115\(^{+}\) CX3CR1\(^{+}\) Ly6C\(^{-}\))\(^15\). MC were defined as CD11b\(^{-}\) CD117\(^{-}\) CD115\(^{+}\) CX3CR1\(^{+}\), and Lin\(^{-}\) c-kit\(^{+}\) progenitors as CD11b\(^{-}\) CD117\(^{-}\) CD115\(^{-}\) (Fig. 1c). The primary and most robust endpoint was the formation of MGC, which were defined as cells with at least three nuclei and an increased cytoplasmic area (Fig. 1a). Using this definition, cMoP exhibited an exceedingly high ability to form MGC in response to the mycobacterial glycolipid lipomannan (LM). Multinucleation and cell surface enlargement was evident already after 4 days, and increased further until day 6 (Figs. 1d and e). In contrast to cMoP, the quantitative MGC formation was only marginally above base line in MC and Lin\(^{-}\) c-kit\(^{+}\) cells under the same conditions (Fig. 1e). The recently disputed cMoP progenitor MDP\(^18\) showed some, albeit delayed and overall reduced capacity to form MGC, compared to cMoP (Fig. 1e). The high MGC-forming potential of cMoP was confirmed by in vitro infection with the *M. tb* complex species *M. bovis* Bacillus Calmette-Guérin (BCG) (Fig. 1f and Supplementary Figs. 1a and 1b). Under all these conditions, cMoP were not only potent, but also quite specific MGC progenitors, since besides absolute quantity, the relative proportion of cMoP-derived MGC was also increased compared to MC (Figs. 1d, g and Supplementary Fig. 1c). In extension of our relatively stringent definition of MGC as cells with at least 3 nuclei, we wondered if binucleation may serve as a marker for the transformation process into MGC. Indeed, we observed an absolute predominance of binucleated cells in cMoP compared to MC after exposure to BCG (Supplementary Fig. 1c). Yet, the total number of cells with two nuclei was highest in cMoP treated with M-CSF alone (Supplementary Fig. 1d). This was likely due to the complex balance of binucleated cells as an indicator of high multiplication rates, which resolves upon further proliferation (M-CSF control), and binucleation as an indicator of mitotic defects promoting polyploidy and multinucleation (BCG stimulation). More immature progenitors as MDP responded to stimulation with mycobacterial glycolipids predominantly with proliferation into mononuclear daughter cells (Supplementary Fig. 1e). Similar to monocytes from the bone marrow (MC), blood monocytes did not show MGC-forming properties, when stimulated with LM (Fig. 1g and Supplementary Fig. 1f).

cMoP combine macrophage and progenitor characteristics. In full agreement with their progenitor status and similar to previously published data\(^15\), cMoP showed rapid proliferation with total CFSE dilution already after 3 days in vitro (Fig. 2a). Stimulation with LM markedly decreased proliferation over the entire course of the experiment (Fig. 2b). These observations were confirmed by analysis of the cell density after stimulation with fixed *M. bovis* BCG (Fig. 2c). Next, we determined, whether cMoP differed from MC in their cell death response to mycobacteria. We found increased apoptosis in MC compared to cMoP, when stimulated with fixed *M. tb* (Fig. 2d). In line with these observations, MC stimulated with BCG showed a decreased mitochondrial potential compared to cMoP, which is indicative of apoptosis (Supplementary Fig. 1g). Accordingly, viability of cMoP exposed to mycobacteria and their glycolipids exceeded...
that of MC. Since cMoP were highly activated by the Toll-like receptor (TLR) 2 agonist LM (Fig. 1e), we wondered whether cMoP differed from other myeloid cell subsets in regard to TLR expression. Notably, we found cMoP to express TLR2 and TLR6, the cognate receptors of diacylated glycolipids\(^{19}\). In contrast, the expression of TLR13, the receptor for bacterial RNA, was very low in cMoP, yet high in MC (Fig. 2e). Since MDP and MC showed similar or higher expression of TLR2 and TLR6 than cMoP, we concluded that TLR expression was probably not a limiting factor in MGC formation by MC (Fig. 2e). These observations, together with those on survival and the superior MGC-forming properties of cMoP in the presence of mycobacteria, spurred the hypothesis that cMoP may be important in mycobacterial immunity. Thus, we measured induction of TNFα formation and nitric oxide (NO) as established components of mycobacterial immunity and found cMoP to be highly active for both parameters (Figs. 2f and g). High NO activity was of particular interest, since we previously found it to be one of the drivers of MGC formation via induction of DNA damage and impairment of p53\(^{10}\). In full accordance with a central role of NO in the mycobacterial response of cMoP, LM and BCG-induced MGC formation was abrogated in cMoP from inducible nitric oxide synthase-deficient (\textit{inos}\(^{-/-}\)) mice (Fig. 2h). Altogether, cMoP showed characteristic progenitor traits of high proliferative activity combined with signature properties for mycobacterial immunity.
cMoP-derived MΦ keep their potential to form MGC and show a characteristic metabolic profile. cMoP are an intermediate state between the multipotent progenitors for mononuclear phagocytes and monocytes. In view of the distinct immune cell functions of cMoP, we analyzed the transformation kinetics of cMoP in culture. We found the cMoP immunophenotype to be very transient, since already after 4 h in vitro almost two thirds of cMoP were negative for the progenitor marker CD117. In contrast, CD11b expression increased more slowly, with ~50% of cMoP expressing CD11b at 12 h, and thus acquired an immunophenotype reminiscent of monocytes (CD11b+ Ly6C+ CD117−), (Fig. 3a and Supplementary Fig. 2a). Stimulation with LM did not impact on the loss of CD117 expression, yet resulted in faster upregulation of CD11b (Supplementary Fig. 2b). After 48 h in culture with M-CSF, cMoP could no longer be distinguished in flow cytometry from monocyte-derived macrophages with respect to the typical MΦ-surface markers CD11b and F4/80 (Fig. 3b).

These findings resulted in the conundrum that cMoP, which rapidly acquired a monocyte immunophenotype in vitro, but not MC, were capable of transforming into MGC. Thus, we asked, whether cMoP retained the MGC-forming capability, even when the expression of identity-defining surface markers had turned into that of inflammatory monocytes. To address this question, we cultured cMoP in M-CSF for 48 h, which resulted in an early macrophage phenotype as outlined above (Fig. 3b), and then added LM for 4 days. Subsequently, we determined MGC formation and compared this with MGC generated from cMoP, which were stimulated with LM for 4 days directly after sorting. Intriguingly, both preparations did not quantitatively differ in giant cell formation (Fig. 3c). These observations indicated that surface marker expression alone was not sufficient to predict the MGC-forming potential of MΦ species. Therefore, we analyzed the transcriptional program of cMoP, MC, and their respective descendants after 48 h of in vitro differentiation. We cultured cMoP and MC in M-CSF only, or additionally with LM for 48 h, a time point when both cell types became very similar with respect to the expression of CD117, CD11b, and Ly6C. Notably, after 48 h most of the cells originating from cMoP or MC expressed F4/80, thereby qualifying as in vitro generated MΦ (Fig. 3b). Transcriptomic analysis revealed a multitude of differentially regulated genes between cMoP and MC after LM stimulation (Supplementary Figs. 2c and 2d). In particular, MC down-regulated genes of key enzymes for cholesterol and fatty acid metabolism upon TLR2 activation (Fig. 3d). In contrast, the respective lipid metabolism genes were not downregulated when cMoP were stimulated in a similar fashion, indicating that metabolic changes were connected with MGC transformation. Yet, the expression of the dehydrocholesterol reductase 24, which catalyzes the production of cholesterol from desmosterol20, as well as HMG-CoA-reductase, the rate-limiting enzyme for cholesterol synthesis and squalene epoxidase21, were upregulated in LM-stimulated cMoP compared to LM-stimulated MC (Figs. 3d and e). Corresponding to this, Msms1 and Lss, two genes involved in steroid metabolism and terpenoid backbone biosynthesis22, were upregulated in LM-stimulated cMoP (Fig. 3d). The same regulatory mechanisms were observed for key enzymes of fatty acid synthesis, such as fatty acid synthase and fatty acid desaturase (Figs. 3d and e). In contrast, the carnitine o-palmitoyltransferase 1, which mediates β-oxidation23 and therefore degradation of fatty acids, was transcriptionally downregulated in activated cMoP compared to MC. In addition, lipid binding molecules and transporters were differentially regulated in cMoP and MC (Fig. 3d and Supplementary Fig. 2e). Pathway analysis also revealed an upregulation of cholesterol biosynthesis in cMoP stimulated with LM compared to similarly treated MC. In contrast, the latter showed an upregulation of apoptosis signaling, toll receptor signaling, and chemokine/cytokine-mediated inflammatory signaling (Fig. 3f). Together, these findings linked the regulation of lipid metabolism to MGC formation, and in particular to the transformation potential of cMoP.

Cholesterol is an integral component of cell membranes and cholesterol synthesis is therefore essential in single-cell growth and proliferation20. High cholesterol metabolism in cMoP corresponded to their strong proliferative activity (Fig. 2a), as determined by the CFSE assay. In line with this, we found upregulation of the proliferation marker Mki-67 in cMoP with, and without LM stimulation, compared to MC (Supplementary Fig. 2f).

Further differences in lipid biosynthesis between cMoP and MC concerned prostanoids, which are well-established intermediates in the regulation of acute and chronic inflammation24. Whereas Pitgs, Pitgs1 and Pitgs2, which encode for enzymes in prostanoid H2 and E2 biosynthesis, were upregulated in stimulated MC as compared to cMoP, Hpgds, which is involved in prostanoid D synthesis, was increased in cMoP (Fig. 3d). Differences in the expression of inflammatory effector molecules are further signs of discrete immune regulator properties of these cells. In LM-stimulated MC, genes encoding for interleukins like IL-1β and chemokines like Cxcl3, Cxcl2, and Ccl5 (Supplementary Figs. 2g and h) were upregulated. In cMoP, chemokines such as Ccr5, Ccl8, and Ccl12 were potently induced by LM (Supplementary Fig. 2h). Since chemokine and interleukin production are essential for the immune response to mycobacteria25, the contrast...
between cMoP and MC with respect to their transcriptomes might also be connected with their differences in longevity, cell death, and transformation into MGC.

**Cholesterol and fatty acid biosynthesis are essential for MGC formation.** In order to dissect the role of lipid and cholesterol metabolism in the formation of MGC, we performed staining with the cholesterol and lipid dye filipin in MGC from cMoP infected with BCG-RFP. We observed the accumulation of cholesterol/lipids in MGC (Fig. 4a). Filipin staining in flow cytometry revealed that cMoP had a significantly faster accumulation of cholesterol/lipids upon stimulation with fixed BCG compared to MC, i.e., already after 2 days (Fig. 4b).

In addition, we quantified the cholesterol content in cMoP with the fluorometric amplex red assay. We found higher cholesterol concentrations in cMoP infected with BCG as compared to resting cMoP (Supplementary Fig. 3a), highlighting a net upregulation of cholesterol synthesis as a consequence of the transcriptional changes in fatty acid and cholesterol producing metabolic pathways (Figs. 3d and e). Lipid accumulation, however, requires that the cellular export systems are not upregulated in parallel to biosynthesis. The transcriptomes of resting and stimulated cMoP did not unveil downregulation of
**Abcg1** and **Abca1**, genes coding for the two main cholesterol transporters (Fig. 3d). However, this analysis was performed 48 h after induction of the MGC transformation, i.e., before MGC have fully developed (compare Fig. 1d). In order to complement this analysis, mRNA expression levels of **Abca1** and **Abcg1** were established 6 days following differentiation. At this time point these genes were downregulated in cMoP-derived MGC (Fig. 4c). Accordingly, it seemed safe to conclude that the accumulation of cholesterol/lipids originated from dysregulated metabolic pathways and, additionally, a reduction in cholesterol and triglycerides efflux. We tested whether cholesterol accumulation was, besides being a signature trait of cMoP, causally linked to the MGC transformation process. Accordingly, we interfered with the cholesterol metabolism in MGC during the transformation process and analyzed the effect on the phenotype. First, we employed cholesterol oxidase, a flavoenzyme that associates with lipid bilayers, binds cholesterol and desorbs it from the membrane. Coincubation of cMoP with cholesterol oxidase during the transformation process decreased MGC formation by about 50% when stimulated with live or fixed bacteria (Fig. 4d and Supplementary Fig. 3b). In addition, cholesterol was extracted from the cells with methyl-β-cyclodextrin (MBCD), which facilitates the efflux of cholesterol by complexing and solubilizing the lipids. In line with the effects of cholesterol...
Fig. 3 cMoP-derived MΦ keep their potential to form MGC and show a characteristic metabolic profile. a Dot plot of cMoP analyzed immediately after isolation for initial surface marker expression. Surface marker expression of cMoP and MC from Cx3cr1gfp/+ mice analyzed by FACS related to CD45+ cells after 4, 8, 12 or 48 hours in culture with M-CSF. Depicted are mean ± SEM of n = 3-4 (CD117), n = 5-9 (CD11b), n = 3-5 (F4/80) biologically independent samples. Detailed sample numbers (n) are presented in Supplementary Table 4. ns = not significant, *p = 0.0161, **p = 0.0009 (CD117), ***p = 0.0005 (CD11b), ****p < 0.0001 (two-way ANOVA, Sidak’s MCT). b Representative FACS dot plots for F4/80 and CD11b expression of cMoP and MC after 48 h in medium supplemented with M-CSF. c cMoP or MC from Cx3cr1gfp/+ mice were stimulated either directly with LM (4 µg/ml) for 4 days or after 2 days in culture with M-CSF (cond-cMoP, cond-MC). MGC formation was quantified based on Hemacolor stainings (scale bar: 100 µm). Bars show mean ± SEM of n = 4 (cMoP) and n = 3 (MC) independent biological samples. ns = not significant (cMoP — cond-cMoP; p = 0.8162; MC — cond-MC: p = 0.9942), *p = 0.0256 (cMoP — MC), **p = 0.0109 (cond-cMoP — cond-MC, one-way ANOVA, Tukey’s MCT). d Heatmaps of gene expression in cMoP and MC from Cx3cr1gfp/+ mice after LM stimulation (4 µg/ml, 48 hours) with M-CSF (50 ng/ml). M-CSF alone served as control (-). Depicted are standardized log2 differences of significantly up- (red) or downregulated (blue) genes in cMoP-LM compared to MC-LM or cMoP (-) compared to MC (-). The analysis was performed with two biologically independent samples per group (1 = sample 1; 2 = sample 2) by gene-ontology terms (GO-Terms), (GO:0006631 fatty acid metabolic process, GO:0008203 cholesterol metabolic process, GO:0001516 prostaglandin biosynthetic process, GO:0006694 steroid biosynthetic process, GO:0006869 lipid transport). If GO-Terms overlapped, respective genes were only assigned to one group.

Fig. 4 Cholesterol metabolism is an essential prerequisite for MGC formation. a Filipin (blue) and wheat germ agglutinin (WGA, green) staining of cMoP-derived MGC induced with heat-fixed (hf) BCG (106/ml) and additionally infected with BCG-RFP (MOI 1, red). Scale bar: 20 µm. Representative staining out of three independent experiments shown. Channels were adjusted individually with respect to contrast and brightness. b Filipin staining, depicted is the mean fluorescence intensity (MFI) of cMoP (blue) and MC (green) from Cx3cr1gfp/+ mice stimulated with hf BCG (106/ml) (treatment) normalized to M-CSF control at day 2. Shown are mean ± SEM of n = 8 biologically independent samples examined over three experiments. ***p < 0.0001 (student’s unpaired t-test, two-tailed). c Abcg1 and Abca1 mRNA expression levels relative to Gapdh mRNA of cMoP stimulated with heat-fixed M.tb (50 µg/ml) for 6 days determined by qRT-PCR. Bars show mean ± SEM of n = 5 biologically independent samples. **p = 0.0099 (Abcg1), ***p = 0.0095 (Abca1), (student’s unpaired t-test, two-tailed). d cMoP from Cx3cr1gfp/+ mice were stimulated with BCG (MOI 20), heat-fixed BCG (106/ml) or heat-fixed M.tb (10 µg/ml) and treated with cholesterol oxidase (2 µM/ml, beige) or PBS (vehicle, blue) for 6 days. MGC quantification by Hemacolor staining. Bars show mean ± SEM of n = 17 (-), n = 9 (BCG), n = 15 (MC) independent biological samples examined over 11 experiments. ns = not significant (p = 0.9504), *p = 0.0251, **p = 0.0052, ***p = 0.0004 (two-way ANOVA, Sidak’s MCT). e cMoP from Cx3cr1gfp/+ mice stimulated with heat-fixed BCG (105/ml) or heat-fixed M.tb (10 µg/ml) and treated with methyl-β-cyclodextrin (MBCD, 1 mmol, beige) or dH2O (vehicle, blue) for 6 days. MGC quantification by Hemacolor staining. Bars show mean ± SEM of n = 20 (-), n = 8 (BCG), n = 7 (M.tb) biologically independent samples. **p = 0.0038, ***p = 0.0001 (two-way ANOVA, Sidak’s MCT). f cMoP from Cx3cr1gfp/+ mice were stimulated with heat-fixed M.tb (10 µg/ml) and treated with methyl-β-cyclodextrin (MBCD, 1 mmol), LDL (50 µg/ml) or PMA (1 µg/ml) for 6 days. Vehicle control for MBCD was dH2O. Bars show mean ± SEM of n = 15 biologically independent samples in seven experiments (M-CSF control) and n = 7 biologically independent samples in three experiments (M.tb). *p < 0.05, **p = 0.0004, ***p < 0.0001 (one-way ANOVA, Tukey’s MCT).
Fig. 5 Altering fatty acid metabolism by orlistat affects MGC formation. a cMoP from Cx3cr1<sup>−/−</sup> mice were stimulated with BCG (MOI 20), heat-fixed BCG (10<sup>6</sup>/ml), heat-fixed M.tb (50 µg/ml) and treated with orlistat (100 µmol, beige) or vehicle control (ethanol, blue) for 6 days. MGC were quantified by Hemacolor staining (scale bar: 100 µm). Gamma-correction was adjusted to 0.45 in both images. Bars depict mean ± SEM of 3 biologically independent samples. b Hoechst staining of cMoP from Cx3cr1<sup>−/−</sup> mice stimulated with BCG (MOI 20) and treated with orlistat (100 µmol) or ethanol (control) for 6 days. MGC were quantified as polyploid cells (N > 4) from histogram peaks. Bars show mean ± SEM of n = 3 biologically independent samples. c Cell density determined by FACS counting particles of cMoP from Cx3cr1<sup>−/−</sup> mice treated as indicated in Fig. 5b. Bars show mean ± SEM of n = 5 biologically independent samples. d TNFα production of cMoP from Cx3cr1<sup>−/−</sup> mice stimulated with heat-fixed BCG (10<sup>6</sup>/ml, squares) for 6 days compared to M-CSF (50 ng/ml, circles) control (−). Shown are cholesterol, desmosterol, and 7-dehydrocholesterol (7-DHC) content normalized to cell number. Depicted are mean ± SEM of n = 4 biologically independent samples. e Sterol analysis of cMoP (blue) and MC (green) from Cx3cr1<sup>−/−</sup> mice stimulated with heat-fixed BCG (10<sup>6</sup>/ml, squares) for 6 days compared to M-CSF (50 ng/ml, circles) control (−). Shown are cholesterol, desmosterol, and 7-dehydrocholesterol (7-DHC) content normalized to cell number. Depicted are mean ± SEM of n = 5 biologically independent samples. f-Sqle, Dhc24, Hmgcr and Fasn mRNA expression levels of cMoP and MC stimulated with heat-fixed BCG (10<sup>6</sup>/ml, diamonds, hatched bars) for 6 days relative to Gapdh compared to M-CSF (50 ng/ml) controls (circles, gray bars). Bars show mean ± SEM of n = 5 biologically independent samples in two experiments. | p = 0.0328 (Fasn), *p = 0.0155 (Hmgcr, cMoP-control = cMoP-BCG), *p = 0.0313 (Hmgcr, MC-control = cMoP-BCG), *p = 0.0057 (Sqle), **p = 0.0013 (Dhc24), **p = 0.001 (Hmgcr), ***p = 0.0016 (Fasn), ****p < 0.0001 (two-way ANOVA, Tukey’s MCT). Moreover, orlistat prevented the foamy, lipid-rich cytoplasmic appearance and conserved relatively small MΦ with several pseudopodia (Fig. 5a). Since interfering with lipid metabolism is potentially impacting on cell viability, we carefully assessed any toxic effects of cholesterol oxidase, MBCD and orlistat. We found that cMoP incubated with BCG and orlistat proliferated more than cMoP treated with BCG alone and were similar to untreated control cells in this respect (Fig. 5c). In full agreement, orlistat treated cells did not differ from untreated cells with respect to TNFα formation in response to LPS (Fig. 5d). Upon addition of cholesterol oxidase, cell density and TNFα production after LPS stimulation were not affected compared to vehicle controls (Supplementary Figs. 3e and f). When measuring cell density for MBCD stimulated cells, we found a reduction, when LDL was oxidase, MBCD substantially inhibited MGC formation (Fig. 4e and Supplementary Fig. 3c). We then employed the reverse approach by adding human low-density lipoproteins (LDL) to the culture. In order to facilitate the uptake of LDL, PMA was added<sup>27</sup>. Using this approach, we found orlistat to support of the model that lipid provision is rate limiting for the MGC formation process by cMoP, we found orlistat to inhibit MGC formation in response to fixed and live mycobacteria (Fig. 5a). Orlistat reduced the total number of nuclei per MGC and the percentage of polyploid cells (N > 4) (Fig. 5b).
added additionally, which was in line with the high amount of MGC formed under these conditions (Supplementary Fig. 3g). The reduction in cell number upon addition of MBCD alone to \textit{M. tb} stimulated cMoP (Supplementary Fig. 3g) was likely due to the reduced availability of cholesterol for membrane formation and proliferation. The efficacy of MBCD to extract cholesterol from the cells was examined by filipin staining (Supplementary Fig. 3h). Similar to cholesterol oxidase and orlistat, MBCD did not impact on the TNFa response to LPS (Supplementary Fig. 3i).

Together, these findings suggested that blocking of the fatty acid synthase and depriving the cells of cholesterol inhibit the MGC transformation process driven by mycobacteria, and conserves the mononuclear MΦ state. Next, we dissected the differences in lipid composition of BCG-stimulated cMoP and MC by liquid chromatography-mass spectrometry-based sterol analyses. After 6 days of BCG stimulation, we found cholesterol accumulation in both cMoP and MC. However, the accumulation of cholesterol precursors significantly differed in these cell types. BCG-stimulated cMoP showed an increase in desmosterol content, in contrast to MC, which rather contained 7-dehydrocholesterol (7-DHC) (Fig. 5e and Supplementary Fig. 3j). Both sterols are precursors of cholesterol. Desmosterol is formed via the Bloch pathway, and 7-DHC via the Kandutsch-Russell pathway.

Previously, the accumulation of desmosterol was reported to be characteristic for foam cell transformation in atherosclerosis-associated MΦ. Furthermore, an immunomodulatory function was described for specific cholesterol precursor derivatives. Accordingly, the accumulation of different cholesterol precursors in BCG-stimulated cMoP and MC indicated that cholesterol metabolism was differentially regulated in these subsets. In order to explore, whether cMoP keep cholesterol biosynthesis upregulated during the entire transformation process into MGC, we analyzed the expression of key enzymes of the cholesterol and fatty acid biosynthesis. We found upregulation of \textit{Sdc1}, \textit{Dhc24}, \textit{Hmgcr}, \textit{Fasn}, \textit{Acat2}, and \textit{Cyp51} in BCG-stimulated cMoP in contrast to MC (Fig. 5f and Supplementary Fig. 3k). However, the expression of \textit{Acat1} involved in β-oxidation and the degradation of fatty acids was downregulated in stimulated cells (Supplementary Fig. 3k). In addition, the upregulation of cholesterol biosynthesis in cMoP-derived MGC in contrast to monocytes was in line with the transcriptome analysis for LM-stimulated cMoP (Fig. 3f).

In order to further dissect the intrinsic metabolic signature in MGC, we analyzed the glycolytic phenotype in cMoP-derived MGC using an extracellular flux analyzer. MGC showed a higher extracellular acidification rate in glycolysis stress tests than control cells (Supplementary Fig. 4a), as well as a significant upregulation of glycolysis. This indicated that in addition to fatty acid and cholesterol metabolism, glycolysis plays an important role in MGC transformation, which hints at high ATP consumption requirements. These observations are in full accordance with a decrease in MGC formation in the presence of 2-deoxy-D-glucose (2-DG), a competitive inhibitor of glycolysis (Supplementary Fig. 4b). Since the target of rapamycin (mTOR) has previously been implicated in the switch from oxidative phosphorylation to glycolysis in DC, we tested the impact of rapamycin on MGC formation and found a decrease of MGC transformation in cMoP (Supplementary Figs. 4c and d). Together, these findings emphasize the close interaction between the immune response to mycobacteria/TLR2 ligands and metabolic changes in cMoP.

\textbf{iMoP — a circulating monocyte progenitor in M. \textit{tb} infection.}

After establishing the high potential of cMoP to form MGC, we set out to define their role in vivo. We employed two different mouse models for pulmonary tuberculosis. Aerogenic exposure to a low-dose inoculum of virulent \textit{M. tb} results in progressive disease, with a peak of infection after 21 days, in both the resistant mouse strain C57BL/6 and in the susceptible strain 129S2. Whereas C57BL/6 mice survive and develop a chronic infection, 129S2 mice succumb to infection within 5 weeks and mimic active tuberculosis in humans. In both mouse strains, we found increased frequencies of cMoP in the bone marrow at day 21 post-infection. In contrast, cMoP were hardly detectable in the blood during tuberculosis progression (Fig. 6a). We postulated that cMoP undergo early changes with respect to their cell surface marker expression, similar to our in vitro observations (Fig. 3a).

In line with this assumption, we identified a circulating cell population that differed from cMoP only with respect to CD117 expression (Fig. 6b). This cell type, which we denominated induced monocyte progenitors (iMoP), fully correlated with the differentiation phenotype of cMoP in vitro, where 70% of cells lost CD117 expression within 4 hours. Similar to cMoP, we found iMoP to be present in the bone marrow of uninfected mice, and an increase of iMoP frequency at day 21 in infected mice. In contrast to cMoP, which almost exclusively reside in bone marrow and spleen, iMoP were detectable at a low frequency in peripheral blood already in steady state. During infection, iMoP markedly increased up to 1% of CD45-positive blood cells at day 14 and 21, especially in the susceptible 129S2 mice (Fig. 6c).

Hemacolor staining of freshly sorted progenitors showed iMoP with decreased nucleus to cytoplasm ratio compared to MDP (Fig. 6e). In contrast to MDP, which also showed an intermediate potential to form MGC, iMoP more specifically formed multinucleated giant cells (Supplementary Fig. 5a). Furthermore, we extracted cMoP, iMoP, and MC from the bone marrow of \textit{Ccr2\textsuperscript{Ldp\textsuperscript{-/-}}} mice. iMoP showed an intermediate potential to produce MGC upon exposure to both live and fixed bacteria (Fig. 6e). In contrast to MDP, which also showed an intermediate potential to form MGC, iMoP more specifically formed multinucleated giant cells (Supplementary Fig. 5a). Furthermore, iMoP, CMoP, and MC produced TNFa within 5 h after stimulation with fixed \textit{M. bovis} BCG, whereas more immature progenitors like \textit{Lin\textsuperscript{-} c-kit\textsuperscript{+}} cells and MDPl did not show this ability (Fig. 6f).

To further analyze the recruitment of iMoP into the blood stream, we infected 129S2 mice with a low dose of \textit{M. tb} and neutralized CCR2-positive cells with the application of a CCR2 antibody (MC-21) starting day 14 p.i.. Mice injected with the CCR2-specific antibody showed a significant decrease of iMoP frequency in the BM and completely failed to mobilize iMoP into the blood (Fig. 6g and Supplementary Fig. 5b). We also observed a significant reduction of cMoP frequency in the BM after antibody treatment (Fig. 6g). These results were in line with our flow cytometry analysis of CCR2 expression on the different bone marrow progenitor subsets, where we observed increased binding of the MC-21 antibody depending on the maturity state of the progenitor subsets (Supplementary Fig. 5c). Injection of the CCR2-specific antibody did not influence bacterial burdens in the lung or spleen (Supplementary Fig. 5d). Consequently, circulating iMoP were not essential to control \textit{M. tb} and neutralized CCR2-positive cells with the application of a CCR2 antibody (MC-21) starting day 14 p.i.. Mice injected with the CCR2-specific antibody did not inactivate bacterial burdens in the lung or spleen (Supplementary Fig. 5d). Accordingly, circulating iMoP were not essential to control \textit{M. tb} in these mouse models, where MGC can hardly be found at the investigated time points (Supplementary Figs. 5e, f and g). However, the spontaneous formation of neutralizing antibodies against MC-21 antibodies in treated mice precludes longer depletion experiments. Thus, in order to further explore the role of CCR2 in MGC formation at later stages of infection, we analyzed \textit{M. tb} infected \textit{Ccr2\textsuperscript{Ldp\textsuperscript{-/-}}} mice from day 60 to day 300 p.i.. We found an increase in the total area of inflammation in lungs from \textit{Ccr2\textsuperscript{Ldp\textsuperscript{-/-}}} mice with time, which was not visible in wild-type infected mice (Supplementary Figs. 5e, f and g). This is compatible with the notion that circulating monocyte progenitors and/or mature monocytes contribute...
to inflammation control in pulmonary Tb infection. In contrast, the relative immune cell composition was largely similar in both mouse strains, including MGC with high lipid content and foam cell formation after 100 and 300 days of infection (Supplementary Fig. 5f). Of note, monocytes (and probably their precursors) are reduced by 80–90% in Ccr2−/− mice. Thus, the remaining monocyte progenitors in the circulation of Ccr2−/− mice might fill the MGC progenitor niche during the mycobacterial infection, in particular since chronic lung inflammation in M.tb infection is a slow process with relatively low cell turnover. Notably, isolated progenitors from wild-type and Ccr2−/− mice treated with heat-fixed BCG exhibited a largely similar potential to form MGC in vitro, with only a modestly reduced capacity of CCR2-deficient cMoP (Supplementary Figs. 5h and i).

In summary, we identified a circulating monocyte progenitor subset termed iMoP, which has a high capacity to form MGC and circulates at increased frequency in mycobacterial infection.

**iMoP — a progenitor cell between cMoP and monocytes.** The surface marker expression (CX3CR1+ CD117− CD115+ Ly6C+ CD11b−) of iMoP differed from cMoP with respect to CD117 expression and from MC with respect to the lack of CD11b expression. CD115 upregulation classified iMoP as part of the monocyte/macrophage lineage. Since, in contrast to cMoP, iMoP
were capable of circulating in the blood, we aimed to further characterize this so far undescribed bone marrow subset and to distinguish these cells from other progenitors. We therefore performed a transcriptomic analysis of iMoP and cMoP, sorted directly from murine bone marrow and found 391 genes to be significantly upregulated, and 260 genes to be significantly downregulated in iMoP as compared to cMoP (Fig. 7a). The analysis revealed that cell cycle and cell differentiation were differentially regulated in iMoP compared to cMoP, which corresponded to their different progenitor status. Kit oncogene (CD117) was clearly downregulated in iMoP, in line with our previous flow cytometry analysis. In contrast to this, the gene expression of hematopoietic cell kinase (Hck), important for regulation of immune response in myeloid cells and reported to be involved in cell recruitment to the lung and cytokine production, was increased in iMoP (Fig. 7b). Other genes, e.g., Tlr13, C3, Cd180, and Il1rn, which are involved in innate immune response and cytokine-mediated signaling, were upregulated in iMoP. The upregulation of Trem2, which is expressed in iMoP, is reported to be connected with immune response to mycobacterial infections. This spurs the hypothesis that iMoP are involved in mycobacterial immunity. In addition, we found differences between iMoP and cMoP with respect to cell adhesion and cell migration, which might explain their various potential to circulate in the blood (Fig. 7b). Gene ontology enrichment analysis of genes overexpressed in iMoP compared to cMoP yielded terms such as positive regulation of monocyte chemotaxis, regulation of inflammatory response and regulation of phagocytosis, which further supported iMoP to be part of the monocyte/macrophage lineage (Fig. 7c). Although the transcriptomic analysis indicated an increased expression of *Igdam* (CD11b) in iMoP as compared to cMoP (Fig. 7b), quantitative analysis (qRT-PCR) analysis of sorted lineages revealed that compared to MC, iMoP were low for *Igdam*, as well as *Adgre1* (F4/80). For *Mpo*, *Tlr13* and *Msrl*, iMoP showed intermediate expression levels compared to cMoP and MC (Fig. 7c). These results are in line with our hypothesis that iMoP represent a circulating monocyte progenitor subset, which originates in the bone marrow. In contrast to cMoP, iMoP did not form colonies in MethoCult assay. Instead, they rather formed groups of 2–3 cells, indicating that they lost their stem cell potential and only undergo a limited number of divisions (Fig. 7e). We further analyzed the capacity of the different subsets to kill intracellular bacteria and found more live BCG in MC as compared to cMoP and iMoP 4 days post infection (Supplementary Fig. 5j), although all cell types failed to eliminate mycobacteria. It has been reported before that cMoP show a high expression of *Ms4a3*. Thus we examined *Ms4a3* expression levels in the progenitor subsets and found cMoP to have the highest expression. MC from the bone marrow, defined by their CD11b expression, did not express *Ms4a3*, whereas iMoP and MDP showed distinctly lower levels compared to cMoP (Fig. 7f). To confirm that iMoP can give rise to CD11b positive monocytes in vivo, we adoptively transferred iMoP, which had been isolated from bone marrow of β-actin gfp+/− mice, i.e., (Fig. 7g). After 14 h all transferred iMoP still expressed Ly6C. In the blood, almost all GFP+ cells had upregulated CD11b. In the spleen and bone marrow, around 70% showed CD11b expression, whereas F4/80 expression was still rather low in all compartments (Figs. 7g and h). These observations confirmed that iMoP isolated from the bone marrow could indeed give rise to inflammatory monocytes. Together these data indicate, that iMoP are a bone marrow precursor subset between cMoP and CD11b-positive monocytes, which can give rise to circulating monocytes.

**Transferred bone marrow precursors migrate to spleen and liver granulomas in *M. bovis* BCG infected mice.** Next we addressed whether immature bone marrow cells localize to the site of mycobacterial infections and become part of the granuloma. Therefore, we made use of an established model, where *M. bovis* BCG i.v. infection leads to granuloma formation in liver and spleen (Fig. 8a). BCG infection resulted in substantial splenomegaly at day 18 and to a lesser extent at day 30 post infection (Figs. 8a and b), with CD68-positive, BCG containing granulomas in the spleen (Fig. 8c) and liver. In accordance with our data on pulmonary *M. tb* infection, CD11b−, Ly6C−, CD115− cells (including cMoP and iMoP and excluding MC) were increased in spleen and bone marrow. With respect to the kinetics, these cells were most frequent at day 18, at the peak of infection, but they stayed high in number until day 30, especially in the bone marrow (Fig. 8d and Supplementary Fig. 6a). In spleen and blood, Ly6C+ monocytes were transiently increased at day 18 post infection, but they decreased to steady state levels until day 30. Interestingly, the monocyte compartment in the bone marrow was not significantly affected in BCG infection, in contrast to the precursor subsets (Supplementary Fig. 6b). In addition, granulocytes increased during the infection, whereas B-cells slightly decreased at day 18 post infection referred to all CD45-positive cells. The proportions of the T-cell subsets were not altered during the time course of infection (Supplementary Fig. 6c). Next, we made use of the BCG infection model to further study the role of precursor cells in Ccr2−/− mice during infection. As outlined above, the recruitment of monocyte progenitors is CCR2-dependent (Fig. 6g). In contrast to the application of the CCR2 antibody MC-21, which depletes all CCR2-expressing cells...
including those in the bone marrow, we found an accumulation of bone marrow progenitors both in wild-type and CCR2-deficient mice at day 18 post infection (Fig. 8e). In line with the fact that recruitment of monocytes and their progenitors is reduced in Ccr2−/− mice, we observed only a minimal increase in CD115+ CD11b− Ly6C+ cells in the spleens of Ccr2−/− compared to wild-type mice. In BCG infected Ccr2−/− mice, Ly6Chigh monocytes were reduced by over 80% in the bloodstream and the spleen as compared to wild-type mice. In contrast, bone marrow Ly6Chigh monocytes were present at similar frequencies in Ccr2−/− and control mice (Fig. 8f and Supplementary Fig. 6b). In order to explore, whether monocyte progenitors, in
Fig. 7 iMoP - a precursor cell between cMoP and monocytes. a Volcano plot of Clariom S gene array analysis of cMoP and iMoP. Depicted are −log10 (p-value) on the y-axis versus log2(fold change) on the x-axis. Shown are significantly upregulated (red) and downregulated (green) genes in iMoP compared to cMoP. b Heatmaps of gene expression in cMoP and iMoP from C3H/HeN mice. Depicted are standardized log2 differences of significantly up- (red) or downregulated (blue) genes. The analysis was performed by GO-Terms (GO:0045087 innate immune response, GO:0030154 cell differentiation, GO:0007075 cell adhesion, GO:0006629 lipid metabolic process, GO:0016477 cell migration, GO:0019221 cytokine-mediated signaling pathway, GO:0007049 cell cycle). If GO-Terms overlapped, respective genes were only assigned to one group. Affymetrix Clariom S gene array was performed with two biologically independent samples per group (1 = sample 1; 2 = sample 2). c Compilation of over-represented gene-ontology-processes with respect to the significantly upregulated genes in iMoP compared to cMoP. d qRT-PCR analysis of Il13 (Cd11b), Adgre1 (F4/80), Mpo, Tlr13, Mar1 and Kit oncogene mRNA relative to Gapdh mRNA in cMoP, iMoP (red) and MC. Bars show mean ± SEM with n = 3 biologically independent samples. *p = 0.0101 (Tlr13), **p = 0.0142 (Mpo), ***p = 0.0038 (Tlr13), ****p = 0.0098 (Mpo), ****p = 0.0022 (Il13, Msr1 cMoP - MC), ***p = 0.0004 (Il13, Msr1 iMoP - MC), ****p < 0.0001 (ordinary one-way ANOVA, Tukey's MCT). e GFP and iMoP (red) were cultured in M3434 Methocult medium. Colonies consisting of at least 10 cells were counted at day 10. Depicted are mean ± SEM of n = 4 biologically independent samples examined over two experiments. f qRT-PCR analysis of Mfsd3a mRNA relative to Gapdh in MDP, cMoP, iMoP and MC. Bars show mean ± SEM of n = 4 biologically independent samples examined over at least two experiments. g iMoP isolated from β-actin-gfp/+ mice were transferred intravenously into WT mice. Blood, spleen and bone marrow were analyzed for GFP+ cells (green) 14 h post transfer by FACS for Ly6C, CD11b and F4/80 expression. Representative FACS plots are shown. h Quantification of FACS analysis described in Fig. 7g. Bars show mean ± SEM of n = 5 (blood) and n = 4 (bone marrow, spleen) mice examined over at least three experiments.

Discussion

Adaptation of the host to its microbiota requires tight local immune control, which balances containment of microorganisms and avoidance of collateral tissue damage with subsequent organ failure. In other words, a very targeted, yet overall restrained host response is the basis for efficient commensalism and thus ultimately for the benefit of the microorganism. It is an intriguing hypothesis that MGC are involved in mycobacterial latency, a particular form of host pathogen coexistence. They are reduced or missing in situations of mycobacterial dissemination, such as primary immunodeficiency syndromes or anti-TNFα treatment. Yet, the specific contribution of MGC to mycobacterial immunity is still uncertain. MGC may inhibit mycobacterial cell-to-cell spread, but may just as well promote tissue destruction due to their high expression of extracellular matrix-degrading enzymes. The findings presented here suggest that the single-cell adaptation to becoming an MGC requires progenitor traits, especially proliferative activity, and commitment to the monocyte lineage. However, the progression from a dedicated monocyte progenitor to the fully differentiated MGC involves stepwise cellular reprogramming in a fashion distinct from that leading to the formation of mature mononuclear MΦ.

Diverse, and in part controversial, evidence for the role of differentiated monocytes in mycobacterial pathogenesis has been previously provided. In humans with active tuberculosis monocytes exhibit a regulatory program that predisposes them to differentiation into alternatively activated MΦ, which are relatively permissive for mycobacteria, Ccr2−/− mice, which are deficient in circulating monocytes, develop smaller lung granulomas and release less abundant IFNγ into the draining lymph nodes after embolization with beads coupled to M. bovis components. This may be due to impaired trafficking of antigen-presenting cells, since monocytes acquire DC characteristics while delivering M.tb to lymph nodes. Moreover, type 1 IFN leads to a CCR2-dependent accumulation of permissive lung MΦ and neutrophils in mice, which promotes tuberculosis exacerbation. These data are generally in line with our finding that CCR2-expressing monocyte progenitors contribute to the formation of the granuloma characteristic MGC in mycobacterial infection. Yet, CCR2

granulomas (Fig. 9e1). In addition, we identified transferred cells with atypical nuclear structures, including very large nuclei (Fig. 9e3) and GFP+ cells with 3 nuclei (Figs. 9e4 and 9e5) in line with our definition of MGC. Thus, individual bone marrow progenitors have the potential to acquire signature MGC properties in M.tb infection in vivo.
expression appeared to not be an absolute prerequisite for MGC formation, both in vitro and in vivo. It is tempting to speculate that interference of monocyte egress from the bone marrow in chronic infection triggers complex compensatory programs at the very heterogeneous host microbe-interface in the tissue.

Previously, it was described that monocytes and MΦ can be renewed independently of DCs from a committed clonogenic progenitor present in the bone marrow and spleen denominated cMoP. cMoP can be found, albeit in much lower frequency than in the bone marrow, in the spleen, as an important site of extramedullary hematopoiesis in mice. In the spleen, cMoP-derived monocytes have the ability to differentiate into spleen-resident MΦ after depletion of MΦ or upon inflammation. In addition, the precursor reservoir in the spleen was not empty during BCG infection of Ccr2−/− mice. This may be due to the reduced, but not abolished, progenitor recruitment in CCR2-deficient mice. On the other hand, it may point towards a bone marrow independent reservoir in the spleen as an alternative source of progenitor cells in Ccr2−/− mice to cope with the infection.
Importantly, a human cMoP analogue has been recently identified as a CLEC12A^{high}CD64^{high} subset in umbilical cord blood and in bone marrow. Using established gating strategies, this subset is included in the conventional GMP fraction^{15}. Accordingly, cMoP have become established as the most specific monocyte progenitor in bone marrow and spleen. Under homeostatic conditions, cMoP differentiate into Ly6C^{high} monocytes in the bone marrow, egress and, in part, give rise to Ly6C^{low} monocytes and Mo^{50}. However, it had not previously been clarified how monocyte and Mo transformation is achieved under conditions, when these cells are most needed for immune defense, i.e., when microorganisms infect the tissue. Here, we introduce a circulating Mo progenitor, which, according to our in vitro and in vivo differentiation analysis, is interposed between cMoP and inflammatory monocytes. This cell type, which we denominate iMoP, is best characterized by the loss of CD117. iMoP remain negative for the myeloid lineage marker CD11b, but are positive for CD115, which is why we attribute them as a monocyte/Mo progenitor. Chong et al. established a new classification of mature bone marrow monocytes with respect to expression of CXCR4^{51}. As the CXCR4^{+}Ly6C^{+} cells, they described, are partly CD11b^{−}, our iMoP subset might be a further subpopulation of the CXCR4^{+}CD11b^{−} bone marrow subset. In vitro, the iMoP phenotype is, similar to the cMoP phenotype, very transient and lasts only a few hours. In accordance with this, iMoP differentiated in vivo within 14 h into CD11b^{+}Ly6C^{+} monocytes. The specificdedication of cMoP to its progenitor trait was based in part on strongly reduced Ccr2 mRNA expression compared to monocytes of the bone marrow, which was in line with the notion that cMoP hardly egress from the bone marrow, and on their inability to take up particles as an essential functional phagocyte feature^{15}. Here, we substantially extend the cellular potential of cMoP and its descendants. First, we found cMoP to express CCR2 on the protein level, and their number to be reduced in the bone marrow of CCR2-mAb treated mice. This indicates their potential to circulate and home to target tissues under certain conditions. Yet, this may occur at a very low frequency and thus evade conventional analysis. Second, we found that cMoP exhibit sensors and effector molecules involved in mycobacterial immunity. They express TLR2, a cognate receptor for mycobacterial glycolipids and lipoproteins and produce abundant TNFα in response to TLR2 ligation. In addition, a unique regulation of lipid metabolism in cMoP appears to bridge progenitor and immune cell functions. Conversion of Mo into MGC is associated with foamy cellular transformation, as indicated by the accumulation of cholesterol and lipids in the cytosol. Our data strongly suggest that reprogramming of fatty acid and cholesterol metabolism and efflux are critical prerequisites for MGC formation. The metabolic MGC imprint likely involves high availability of free fatty acids, either through de novo fatty acid synthesis or degradation of triacylglycerols, since both are inhibited by the general lipase inhibitor tetrahydrolipstatin (orlistat). Moreover, and probably more important in the context of our study, orlistat tightly binds and irreversibly inhibits the fatty acid synthase^{28} and triacylglycerols degradation^{29}. However, the cellular processes leading to the accumulation of lipids and the transformation into a foamy cell remain highly cell specific. As an example, whereas orlistat has potent activity against the accumulation of fatty acids in MGC, it increases lipid droplets in hepatic stellate cells in number and size. Under these conditions, orlistat inhibits the breakdown of cholesterol esters^{29}. Accumulation of fatty acids is a clear indication for changes in the lipid handling by MGC, yet the biological role of this phenomenon is less clear. The accumulation of cholesterol and lipids in MGC may represent an enlarged reservoir, since less membrane material is needed in one slowly enlarging cell as compared to macrophages undergoing cellular fission and daughter cell formation. The inhibition of MGC formation by orlistat highlights the role of fatty acid de novo synthesis and metabolism for cellular energy production. In addition, *M.tuberculosis uses host lipids to produce energy largely through β-oxidation, to generate biosynthetic precursors needed for pathogenesis and to incorporate them into membrane phospholipids to maintain cytoplasmic membrane integrity*^{52}. Overall, host cholesterol appears to be required for proper growth and persistence of *M. tuberculosis in vivo*^{53–55}. In addition, a high cholesterol content in the cell membrane supports the uptake of mycobacteria^{56}. In line with this, interfering with cholesterol synthesis has been shown to reduce mycobacterial numbers both in Mo in vitro and in experimental mouse models^{57,58} and has been used as an adjunct therapy in patients with tuberculosis^{59}.

Furthermore, cholesterol import is a prerequisite for mycobacterial persistence in the lungs of chronically infected animals, where mycobacteria localize to Mo^{60}. It is well appreciated that *M. tuberculosis infection induces a foamy phenotype in mononuclear Mo, which may provide a permissive growth environment for the bacteria. Our data on increased mycobacterial loads in monocyte progenitor as compared to MC is in line with this notion, although in vitro killing assays have to be taken with some caution, given the complexity of a mixed bacterial-host cell culture (e.g., competitive metabolism, cell death etc.). Moreover, our data clearly indicate that the dependence on cholesterol for MGC transformation is independent of mycobacterial metabolism, since MBCD inhibited MGC in response to both pure TLR2 ligands and metabolically inactive mycobacteria. With regard to...
the findings of Tornack et al. who detected \( M. tb \) DNA in peripheral blood and hematopoietic stem cells of humans during latent Tb infection, the accumulation of lipids in progenitor cells might also provide a suitable environment for mycobacteria. Moreover, it is conceivable that monocyte progenitors may serve as Trojan horses for mycobacteria contributing to Tb reactivation.

Spann et al. reported a link between the process of foam cell transformation and inflammatory response via accumulation of desmosterol. Our sterol analysis of cMoP-derived MGC also revealed an accumulation of the cholesterol precursor desmosterol. Nonetheless, TLR2-induced cMoP-derived MGC differ in the expression of e.g., \( Dhcr24 \) and \( Abca1 \) from the atherosclerosis-associated foam cells Spann et al. described, indicating further regulatory mechanisms to play a role during the transformation process into a MGC.

The role of MGC has been a matter of considerable debate. The most accepted hypothesis, which incorporates findings in osteoclasts, states that MGC are superior to mononuclear phagocytes.
in the uptake and degradation of large particles due to their size and increased functional properties of the endosomes. However, this hypothesis has been disputed recently since MGC have been found to be a less hostile environment to mycobacteria than other macrophage-like cells and rather allow for cellular persistence. Our finding that dedicated monocyte progenitors are programmed by the infecting mycobacteria to progress towards cell subsets facilitating replication and persistence extends existing models of host-mycobacteria adaptation. Mycobacteria piggy-back the evolutionarily conserved antibacterial nitric oxide response to create a permissive cellular environment. At the same time, MGC locally contain the infection rather than allowing bacterial spread. MGC are relatively immobile and exhibit heightened phagocytic properties, which allow them to engulf apoptotic infected cells in the immediate vicinity. Thus, mycobacteria-driven genesis of MGC encompasses reprogramming of dedicated monocyte progenitors and represents an intriguing example of host-microbe coevolution offering mutual benefits. The hypothesis that mycobacteria have the potential to reprogram progenitor cells is supported by the observations that BCG and M. tb differentially impact on the transcriptional program in stem cells in vivo. Adoptive transfer of CD115+ CD11b− immature bone marrow cells yielded that these cells can migrate to M. bovis BCG-induced liver granulomas and acquire the morphology of granuloma-associated macrophages. As most Tb mouse models do not show typical granuloma formation in M. tb infection, we further analyzed the role of progenitor cells in M. tb infected IL-13−/− mice which are capable of forming MGC. After intratracheal transfer of CD115+ Ly6C+ CD11b− bone marrow cells, we observed cells with 3 nuclei and iNOS expression as markers for MGC formation. At this stage the contribution of a T helper 2 inflammatory environment remains to be established.

In summary, we hereafter introduce both cMoP and their circulating progeny iMoP as myeloid cell hybrids, which comprise both progenitor potential and immune cell functions in mycobacterial infections. The identification and characterization of dedicated, and potentially circulating MGC progenitors, opens perspectives in defining, and eventually modulating, the role of MGC in mycobacterial granulomas and beyond.

Methods
Mice. All knock-out mice were on C57BL/6 genetic background and used at 6 to 10 weeks of age. Female and male mice were included in the experiments. C57BL/6 and C57BL/6N mice were purchased from Jackson Laboratories (USA) or Charles River Laboratories (Germany). Cx3ctgfl/fl mice were obtained by breeding Cx3ctgfl/fl mice (B6.129S2-Cx3ctgfl/fl/J, Jackson Laboratories) with Flq−/− mice. Mice were lethally anesthetized with ketamine/xylazine i.p. and blood was readily collected from the inferior vena cava in syringes containing anti-coagulant (EDTA). Bone marrow was collected by flushing femoral bones with RPMI1640 media. Cells were resuspended in FACS buffer (1% FBS, 2 mM EDTA), blocked for unspecific binding with rat-IgG and anti-FcR antibodies and stained in the dark for 30 min at 4 °C. Details on antibodies used are presented in the section: Surface and intracellular staining. Finally, samples were fixed in 4% PFA for 30 min at room temperature (RT). Staining of blood cells was performed in whole blood. Following blocking and antibody incubation, it was transferred into fix/lavage solution (ebiscience) for 30 min at RT. Cells were collected on the CytoFLEX S (Beckman Coulter) flow cytometer and analyzed with the FlowJo software (Treestar).

CCR2-antibody injection in vivo. For analysis of CCR2-dependent recruitment of iMoP, 1292S2 mice were injected with a low dose of Mtb (H37Rv) as indicated. Starting on day 14 post infection (p.i.), mice were intraperitoneally (i.p.) injected with 20 µg of a CCR2 specific antibody (MC-21) or an isotype control (MC-67) daily. On day 14 and 19 p.i., five mice per group were euthanized and lungs and spleen plated for CFU assessment. Cells from blood and bone marrow were analyzed for frequency of cMoP and iMoP at day 19.

M. bovis BCG infection model. C57BL/6 or C3H/HeN mice were injected with 1×10^7 CFU M. bovis BCG intravenously. Eighteen and thirty days after infection mice were sacrificed and spleen size and weight were measured. Spleen...
blood, and bone marrow were analyzed by flow cytometry for frequency of progenitors and other immune cells. Spleens were analyzed for granuloma formation by confocal microscopy (Carl Zeiss). Mice, mice were euthanized and sacrificed by 

Adoptive transfer model of M. bovis BCG-infected mice. C57BL/6 mice were infected with 1–5 × 10⁶ CFU M. bovis BCG intravenously. At day 20 post infection CD115⁺ CD11b⁺ GFP⁺ bone marrow cells were intravenously injected. These cells were isolated from β-actin gfp⁺/− mice, continuously expressing GFP on their bone marrow cells except erythrocytes. The Dynabeads™ (Thermo Fisher Scientific) purification of bone marrow cells for cell sort is described in the section: Isolation of bone marrow progenitor cells. The lung was washed through a 100 µm strainer, followed by a RBC lysis (eBioscience) and stained in the dark for 30 min at 4 °C, Details on antibodies used are presented in the section: Surface and intracellular staining. Cells were analyzed at a 3-laser flow cytometer (Galilos™, Beckman Coulter) and processed with the Kaluza software (v1.5, Beckman Coulter).

Histopathological analysis of M. bovis infected CCR2-deficient mice. Ccr2⁻/⁻ and C57BL/6 control mice were infected with a low dose (100 CFU) of M. bovis (bacterIALIZED M. tuberculosis H37Rv). Infection was achieved by aspiration of the resuspended progenitors. In short, mice were anesthetized with isoflurane, progenitor cells resuspended in 30 µl PBS and injected in the oropharynx of the infected mice. Control mice only resipated PBS. Recipients were analyzed 7 days post transfer. Lungs were paraffin-embedded for histology and microscopic slices of 6-µm thickness cut on a microtome. After air-drying slides were deparaffinized and antigen-retrieval performed in citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) for 20 min at 90 °C. Blocking and immunofluorescence staining for NOS2 (Thermo Fisher Scientific), anti-GFP DyLight 488 (Rockland Immunocoulants), and Hoechst 33342 were performed simultaneously in the same section. Confocal microscopy was performed on a LSM 880 with a ×20 objective (numerical aperture 1.2) and analysis followed with ZEN 2012. Brightness and contrast were adjusted for ideal evaluation conditions.

Cell culture. After isolation, cells were cultured at 37 °C in Opti-MEM® I Reduced Serum Medium, GlutaMAX® Supplement with 10% FBS (Gibco, Thermo Fisher Scientific) and Ciprofloxacin (5 mg/ml, Frenesi) in 48- or 96-well plates (Cell-Bind Surface, Corning). Cells were cultured with 50 ng/ml recombinant murine M. tuberculosis (Thermo Fisher Scientific) in a Glas-Col inhalation exposure system. Lungs were collected from mice, con

For intracellular staining, cells were fixed and permeabilized with the Fixation/Permeabilization Solution Kit (BD Bioscience) according to the manufacturer’s instructions. Samples were analyzed at a 3-laser flow cytometer (Galilos™, Beckman Coulter) and processed with the Kaluza software (v1.5, Beckman Coulter). Hoechst, TNFα and IFN-γ staining were recorded with a BD LSR Fortessa flow cytometer (Becton Dickinson). Details on FACS antibodies are provided in Supplementary Table 1.
Isolation of blood monocytes. Mice were anesthetized with Ketamin/Xylazine i.p. and blood was drawn from the retro-orbital plexus. Erythrocytes were lysed in RBC Lysis buffer solution (eBioscience® 1X RBC Lysis Buffer). The remaining cells were washed in FACS buffer and stained for CD45, CD11b, and Ly6C. Progenitor populations were separated using a MoFlo® Astrios® cell sorter (Beckman Coulter).

Hemoccolor staining. Hemacolor® rapid staining for blood smears (Merck Millipore) was used to fix and stain cells. Briefly, cells were fixed in staining solution 1 for 5 seconds (Methanol), followed by staining with solutions 2 and 3 for 5 s each. Ly6Chigh and Ly6Clow blood monocytes were isolated with a MoFlo® Astrios® cell sorter (Beckman Coulter).

CFSE staining. 5-(and-6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from eBioscience (eBioscience® CFSE, Thermo Fisher). Bone marrow progenitors were stained in a 5 µmol CFSE staining solution immediately with appropriate stimulants and together with GolgiStopTM (BD, Germany). After scraping and washing, cells were fixed by the Fixation/Permeabilization Solution Kit (BD Bioscience) according to the manufacturer’s instructions. For intracellular staining, TNFα-APC-antibody from BD Pharmingen was added and staining performed at room temperature for 20 min. After washing, samples were analyzed at a BD LSR Fortessa flow cytometer (Becton Dickinson). Unstimulated cells served as control.

Microarray. Sample preparation for microarray hybridization was carried out as described in the Affymetrix GeneChip WT Pico Reagent Kit User Guide (Affymetrix, Inc., Santa Clara, CA, USA).

1. Affymetrix Mouse Gene 2.1 ST (compare Fig. 3 and Supplementary Fig. 2) In brief, 5 ng of total RNA was used for reverse transcription to synthesize single-stranded (ss) cDNA with a T7 promoter sequence at the 5′ end. Next a 3′ adaptor was added and the ss cDNA was converted to double-stranded cDNA, which acted as a template for a pre-in vitro transcription (IVT) amplification by a low-cycle PCR. Afterwards antisense RNA (complementary DNA or cRNA) was synthesized and linearized by in vitro transcription (IVT) of the double-stranded cDNA template. 20 µg of cRNA was purified and reverse transcribed into sense-strand (ss) cDNA, whereas unnatural dUTP residues were incorporated at a fixed ratio relative to dTTP. Purified ss cDNA was fragmented using a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) at the dUTP residues followed by terminal labeling with biotin. 3.8 µg of fragmented and labeled ss cDNA were hybridized to Affymetrix Mouse Gene 2.1 ST Array Plates. For hybridization, washing, staining, and scanning an Affymetrix GeneTitan system, controlled by the Affymetrix GeneChip Command Console software v4.2, was used.

2. Clariom S Array (compare Fig. 7) In brief, 5 ng of total RNA was used for reverse transcription to synthesize single-stranded (ss) cDNA with a T7 promoter sequence at the 5′ end. Next a 3′ adaptor was added and the ss cDNA was converted to double-stranded cDNA, which acted as a template for a pre-in vitro transcription (IVT) amplification by a 6 cycle PCR. Afterwards antisense RNA (complementary DNA or cRNA) was synthesized and linearly amplified by an in vitro transcription (IVT) of the double-stranded cDNA template. Twenty micrometers of cRNA was purified and reverse transcribed into double-stranded (ds) cDNA, whereas unnatural dUTP residues were incorporated at a fixed ratio relative to dTTP. Purified ds cDNA was fragmented using a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) at the dUTP residues followed by terminal labeling with biotin. 5.5 µg fragmented and labeled ds cDNA were hybridized to Affymetrix Clariom S mouse arrays for 16 h at 45°C in a GeneChip hybridization oven 640. Hybridized arrays were washed and stained in an Affymetrix Fluidics Station FS450, and the fluorescent signals were measured with an Affymetrix GeneChip Scanner 3000 7G. Fluorics and scan functions were controlled by the Affymetrix GeneChip Command Console v4.1.3 software.

Sample processing was performed at an Affymetrix Service Provider and Core Facility, KFB - Center of Excellence for Fluorescent Bioanalytics (Regensburg, Germany: www.kfb-regensburg.de). Summarized probe set signals in log2 scale were calculated by using the RMA72 algorithm for Affymetrix Gene Array 2.1 ST or the GCCN-SST-RMA algorithm for Clariom S array with the Affymetrix GeneChip Command Console software. After exporting into Microsoft Excel, average signal values, comparison fold changes and significance p-values were calculated. Probe sets with a fold change above 2.0 and a student’s t test p value lower than 0.05 were considered as significantly regulated.

The principal component analysis was performed using the prcomp function in R (Version 3.2, R Core Team (2016), R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) and visualized with the ‘factoextra’ package.

Heatmaps show z-scores, generated with the software GEDEE. (https://software.broadinstitute.org/GDEE) Significance was calculated by an ANOVA test, used to create a volcano plot. The frequency of Gene-Ontology processes and the pathway analysis (PANTHER pathways) were determined with the Panther Classification System.

Rapamycin and 2-DG assays. Sorted bone marrow progenitors from C57BL10® mice were cultured as described above. 2-Deoxy-D-glucose (2-DG) (10, 20 or
Confocal microscopy of cMoP as described above using (Thermo Fisher Scientific) was performed with absolute qPCR SYBR Green Mix (Thermo Fisher Scientific) or iScript cDNA Synthesis Kit (Bio-Rad) or by Superscript IV (Invitrogen). RT-qPCR was mixed with MethoCult™ Medium M3434 (Stem Cell Technologies) to a cell density of 10^4/ml. 1.1 ml of the cell suspension for 10 min, then Filipin lipin stained cells was added on live cells for 10 min, then Filipin was added on live cells for 10 min, then Filipin staining was performed (abcam chromatography assay kit) according to manufacturer’s instructions. Analysis was performed via 740 nm two-photon-microscopy using a ZEISS LSM 880.

RNA extraction and RT-qPCR. Cells were lysed in RLT lysis buffer (Qiagen) with 1% β-mercaptoethanol. RNA was extracted with RNeasy MicroKit (Qiagen) according to the manufacturer’s instructions. RNA was transcribed into cDNA by iScript cDNA Synthesis Kit (Bio-Rad) or by Superscript IV (Invitrogen). RT-qPCR was performed with absolute qPCR SYBR Green Mix (Thermo Fisher Scientific) in HBSS was added on live cells for 10 min, then Filipin was added on live cells for 10 min, then Filipin staining was performed (abcam chromatin assay kit) according to manufacturer’s instructions. Analysis was performed via 740 nm two-photon-microscopy using a ZEISS LSM 880.

Methyl-β-cyclodextrin (MBCD) was purchased from Sigma Aldrich, c) in HBSS was added on live cells for 10 min, then Filipin staining was performed. Cells were cultured for 6 days at 37 °C and MGC were quantified based on Hemacolor staining. 2-DG was bought from Sigma (vehicle, equivalent amount for 1 mmol MBCD) were added immediately to the samples or mice as described in detail in the respective figures. Experimental repeats in the case of representative data were performed as follows. Phalloidin stained cells were performed as a cell formation of at least 10 cells. For statistical analysis was used to compare the mean of the two groups. One- or two-way ANOVA was applied for multiple comparisons, followed by a multiple comparison analysis. The statistical analysis was based on the number of biological samples or mice as described in detail in the respective figure legends. Experimental repeats in the case of representative data were performed as follows. Phalloidin and Hoechst stainings of cMoP were performed in at least three independent experiments (Fig. 1a). Hemacolor stainings, as presented in Fig. 1d, were conducted in independent experiments. The figure legends in Fig. 1a depicts one representative out of three independent experiments. Hemacolor stainings in Supplementary Fig. 3b were performed of n = 15 and in Supplementary Fig. 3c of n = 6 biologically independent samples. Cytosins of bone marrow precursors were performed in three independent experiments (Fig. 6d). Granuloma stainings shown in Fig. 5a were conducted with 4 × n = 5 mice. Immunofluorescence stainings of live cells after transfer were performed for n = 5 mice (Fig. 7a and Supplementary Fig. 6d). Individual 3D reconstructions of GFP transfected cells were performed for five images (Fig. 9c).

**Reporting summary** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** All microarray data are deposited at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database. The accession numbers for the 'Genome-wide expression study of cMoP and monocytic from murine bone marrow compared to their differentiated descendants’ (Fig. 3 and Supplementary Fig. 2) is GSE117456 and for the ‘Genome-wide expression study of cMoP and iMoP from murine bone marrow’ (Fig. 7) is GSE117460. Other data supporting the results can be provided by the authors upon reasonable request.
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Author contributions

P.H. and A.K.L. designed and analyzed the experiments and wrote the manuscript. A.K.L. performed the majority of the experiments with help of K.G. and F.L. M.tb in vivo experiments (Figs. 6a, c, g and Supplementary Figs. 5b, d) were performed and analyzed by L.S. and A.D. (M.tb infection and tissue processing for cell analysis). Individual experiments were performed by F.L. (confocal microscopy of filipin staining, CCR2 expression on myeloid progenitors), J.N. (confocal microscopy of spleen and liver), C.K. (Killing assay), I.K. (PCA analysis, microscopy analysis), and A.F. (Extracellular flux analysis). X.L.G. and Y.Y.P. performed the liquid chromatography mass spectrometry. M.M. provided the CCR2-antibody and isotype control. A.T. performed initial experiments. M.L. performed the histopathological analysis of Ccr2−/− mice. M.D.D. and S.A.K. performed M.tb infections of Ccr2−/− mice and provided lung paraffin sections. A.H., C.H., and A.K.L. performed the progenitor transfers in IL-13Fl mice. All co-authors edited the manuscript.

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