Long-term culture expanded alveolar macrophages restore full epigenetic identity in vivo

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Long term culture expanded alveolar macrophages restore full epigenetic identity in vivo

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Abstract

Alveolar macrophages (AM) are tissue resident macrophages of the lung that can be expanded in culture, but it is unknown to what extent culture affects their in vivo identity. Here we show that long term ex vivo expanded AM (exAM) maintain core AM gene expression but show culture adaptations related to adhesion, metabolism and proliferation. Strikingly, even after several months in culture exAM reacquired full transcriptional and epigenetic identity upon transplantation into the lung and could self-maintain in the natural niche long term. Changes in open chromatin regions (OCR) observed in culture were fully reversible in transplanted exAM (texAM) and resulted in a gene expression profile indistinguishable from resident AM. Our results demonstrate that long term proliferation of AM in culture does not compromise cellular identity in vivo. The demonstrated robustness of exAM identity provides new opportunities for mechanistic analysis and highlights the therapeutic potential of ex vivo expanded macrophages.

Highlights:

- Specific tissue resident macrophage identity can be maintained through long term culture
- Long term proliferation does not compromise differentiated macrophage identity
- Epigenetic and transcriptional culture adaptations are fully reversible in vivo
- Rare example of long term cultured somatic cells restoring full epigenetic identity in natural niche in vivo
- Shuttling cells between ex vivo culture and natural niche in vivo provides a valuable system for genetic and biochemical investigation
- Robustness of macrophage identity through culture highlights potential of macrophage based cellular therapies, including in respiratory disease such as COVID19

Introduction

Resident macrophages can be found in every tissue of the body where they fulfill diverse functions in homeostasis, immunity and repair^{1,2}. Organ specific functions and challenges induce functional specialization of macrophages resulting in highly diverse phenotypes of tissue macrophage populations^{3,4}.

The potential determinants and stability of macrophage identity have been a subject of intense investigation and debate^{5,6}. For example, in the lung, various transplantation protocols have shown adaptations of macrophages from different origin to the alveolar macrophage niche^{4,7,8}, which has led to the suggestion that the niche has a powerful, possibly dominant role⁵. Conversely, macrophages lose part of their tissue specific gene expression signature when placed in culture^{1,9,10,11,12}. This is likely due to the loss of critical environmental signals from the niche in vivo, as well as diverse new chemical and physical stimuli encountered in culture. It has remained unclear, however, whether these adaptations to the cell culture environment are permanent or reversible. In particular it is unknown, whether they involve irreversible changes in chromatin accessibility, or whether core epigenetic identity and the sensitivity to respond to correct environmental cues can be maintained through long periods and multiple rounds of cell division in culture.

Tissue culture models have been tremendously important for the advance of immunology but there are only few primary macrophage culture systems available. The most commonly used protocol is based on the differentiation of bone marrow cells in M-CSF/CSF-1 containing medium. Despite the appeal of being simple and accessible the protocol has major drawbacks. Since it involves differentiation from heterogeneous progenitor cells, it bears the problem of giving rise to a non-uniform population of cells of non-synchronized differentiation states. The inherent danger of this is exemplified by a similar differentiation protocol of bone marrow cells in GM-CSF, which turned out to contain a mixed population of macrophages and dendritic cells that went undetected in many previous studies¹³. Thioglycolate-elicited peritoneal macrophages, another popular culture model, do not reflect a homeostatic cell population and contain an eosinophilic contamination¹⁴. Macrophage differentiation from monocytes, in particular from human donors, suffers from batch to batch variability. Cell culture has been critical for the generation of large numbers of immune cells, in particular T-cells, for cancer cellular therapies, but in contrast to T-cells, the proliferative capacity of macrophages in culture is very limited^{15,16}. All common macrophage culture protocols therefore have the drawback to only generate a limited number of cells and to be usable only short-term.

We have shown previously that alveolar macrophages constitutively express low levels of the anti-proliferative transcription factors MafB and cMaf, which enables them to access a network of self-renewal genes that are repressed by MafB and cMaf in other macrophage populations^{17,18}. As a consequence, alveolar macrophages are unique among tissue resident

macrophages in that they can expand in culture to large numbers and proliferate for extended periods of time^{18 19}. It is therefore of high interest for experimental science and potential cellular therapy applications, whether macrophage identity can be maintained through long term proliferation in culture.

Here we have addressed these questions by comparing transcriptional and epigenetic identity of alveolar macrophages in long term culture before and after re-transplantation into the natural niche environment in the lung. We observed that adaptations of alveolar macrophages to the culture environment were transient and did not compromise functional long-term integration into the natural alveolar niche of the lung. Even after long term proliferation in culture, ex vivo expanded alveolar macrophages showed a transcriptional and epigenetic signature after transplantation that was indistinguishable from resident AM that had never transitioned through culture. This indicated that alveolar macrophages sustained sensitivity to critical environmental cues encountered in vivo through long periods of culture. Together our findings establish a unique macrophage culture system of high fidelity for shuttling between ex vivo experimental manipulation and in vivo validation. It also demonstrates that macrophage expansion ex vivo can provide large scale preparations for cellular therapy applications with maintained normal macrophage identity in vivo.

Results

Alveolar macrophages show a massive expansion potential in long term culture

We have shown previously that alveolar macrophages obtained from broncho-alveolar lavage (BAL) can be cultured¹⁹ and expanded in GM-CSF containing medium¹⁸. Here we have further characterized such cultures and named them “exAM” for expanded AM (Fig.1A). We observed that these cells could be kept in continuous culture for at least 10 months, resulting in 33 theoretical population doublings and an amplification factor of 10^{10} (Fig.1B). This correlated with a three- to four-fold higher percentage of cells in S-phase of the cell cycle in exAM compared to fresh BAL (Fig.1C). Furthermore, the exAM cultures could also be taken through freeze/thaw cycles without compromising growth capacity (not shown). Together these observations indicated an enormous, potentially unlimited expansion potential of exAM.

Expanded alveolar macrophages maintain characteristic phenotype and function in long term culture

We further analyzed whether exAM cultures maintained typical macrophage phenotypic and functional characteristics in culture. We observed that long term exAM cultures had a typical AM phenotypic appearance and Diff-Quik dye staining properties (Fig.2A). Furthermore, the exAM cultures showed macrophage typical acidified lysosomal structures as indicated by acridine orange staining. They also showed labeling with Magic Red dye, a sensor of enzymatic

activity of the lysosomal protease Cathepsin B (Fig.2B). The cells could also take up fluorescently labelled latex beads, indicating that they are capable of phagocytosis (Fig.2C). In order to quantify this and demonstrate active phagocytosis, we took advantage of zymosan labelled with the pH sensitive dye pHRhodo that only fluoresces upon acidification of the phagocytosed particles in the lysosomes. FACS quantification of this dye showed the same strong signal in exAM cultures as in freshly harvested BAL, that was present only at 37°C but not at 4°C (Fig.2D). This indicated that exAM maintain full ability of AM to phagocytose pathogen associated material in an active metabolic process that involves the uptake into acidic and enzymatically active lysosomal structures. In order to analyze whether exAM could also mount a macrophage typical immune response, we stimulated exAM cultures with E.coli LPS as a TLR4 stimulating PAMP and mimetic of infection with a gram-negative bacterial pathogen. As shown in Fig.2E, LPS stimulation resulted in production of reactive oxygen species (ROS) in exAM cultures similar to fresh BAL. Together our results indicated that exAM cultures showed typical macrophage functional characteristics.

We further analyzed whether exAM not only maintained general macrophage functions but also a specific AM identity in long term culture. Flow cytometric analysis demonstrated that exAM maintained typical macrophage surface marker staining including the characteristic AM markers SiglecF and CD11c (Fig.2F), which were also maintained after passage through a freeze/thaw cycle (Fig.S1). Furthermore, RNAseq analysis revealed that exAM showed high general expression levels of AM specific core macrophage genes³ compared to other tissue resident macrophage populations such as peritoneal macrophages (Fig.2G). This was reflected in high expression levels of AM specific macrophage transcription factors genes³, including *Pparg*, *Car4*, *C/ebpb* and *Bhlhe41* (Fig.2H), which have been shown to be important regulators of AM identity and self-renewal^{4 20 21 22 23}. Together these results showed that exAM not only maintained macrophage functional characteristics but also AM specific gene expression through long term culture.

The exAM transcriptome shows adaptations to the culture environment

The microenvironment has emerged as a potent determinant of macrophage gene expression^{2 5}. For example, the transfer of mouse⁹ or human¹⁰ microglia from the brain environment to ex vivo culture results in major changes in gene expression and AM in culture show significant changes in cytokine responsiveness and glucose metabolism compared to in vivo conditions¹¹. We therefore wondered whether the culture environment also imposed changes in gene expression on exAM, despite the observed overall conservation of macrophage function and phenotype (Fig.2).

Indeed, a general comparison of exAM to AM in vivo by RNAseq analysis revealed a substantial number of significant transcriptional changes (FDR 0.05) with 1370 genes up-regulated more

than 2-fold and 376 genes upregulated more than 10-fold, whereas 2317 genes were down-regulated more than 2-fold and 857 genes were down-regulated more than 10-fold (Fig.3A).

We hypothesized that some of these changes in gene expression might be related to the expansion capacity in culture. Indeed, consistent with the observations in Fig.1B we found enrichment of GO term cell cycle gene sets and increased expression of cell cycle regulators (Fig.3B). We also found enrichment and overexpression of TGFbeta signaling related genes, consistent with reports that AM self-renewal depends on an autocrine TGFbeta signaling loop²⁴ (Fig.3C).

Although exAM maintained overall similar high expression levels of core AM specific genes³ as AM in vivo, compared to other resident tissue macrophage populations (Fig.2G), there appeared to be reductions in some genes. To investigate this in more detail we analyzed the individual expression levels of AM core genes and observed that whereas the majority showed no or low changes in gene expression, several genes were substantially downregulated. These genes included surfactant proteins, lipid metabolism genes and Epcam, a homotypic adhesion molecules of the CAM family (Fig.3D ,Fig.S2). This is consistent with the loss of the natural alveolar niche environment in culture, where these genes might be obsolete or lack appropriate induction cues.

To analyze the nature of these changes in more detail we performed k-means clustering. Whereas the majority of expressed genes showed no significance difference between culture and in vivo conditions, we identified two smaller clusters of up- and down-regulated genes, respectively (Fig.3E).

Pathway analysis of the up-regulated cluster revealed cytoskeletal, adhesion and migration pathways, consistent with adaptation to the different surface properties of the culture environment (Fig.3F,G). Furthermore, oxygen response, amino acid and glucose metabolism pathways (Fig.3F,G and Fig.S2) were upregulated, indicative of the altered oxygen pressure, the availability of nutrients in culture and the upregulation of glycolytic pathways in culture that are restricted in vivo¹¹.

Pathway analysis of the down-regulated cluster revealed predominantly immune system pathways, including bacterial and viral response, inflammatory and antigen presentation terms. This is consistent with the fact that the alveolar macrophages were taken from a barrier tissue constantly exposed to environmental microbes to a sterile tissue culture environment (Fig.3F,G).

In conclusion, these data indicated that despite the conservation of core AM identity through long term culture, a significant number of genes are sensitive to lost or gained cues in culture that regulate the interaction of AM with their microenvironment.

Transplanted exAM restores full transcriptional AM identity in vivo

Based on the observed transcriptional adaptations to the culture environment we wanted to determine whether these changes were stable or reversible. We therefore employed an intra-tracheal transplantation protocol to establish whether full transcriptional identity of AM could be restored, once the microenvironmental cues of the natural niche *in vivo* were provided again. Several protocols have been previously used to transfer different macrophage populations into the alveolar space of the lung. These protocols typically involve transplantation into an empty niche, from which the resident AM have been depleted genetically, chemically or by irradiation^{7 21 25 26}. Since these procedures all involve strong disturbance of tissue environment and homeostasis, we considered them not ideal to investigate the effects of the natural niche environment on exAM identity *in vivo*. Here we therefore established a transplantation protocol into an unmodified niche of wt mice. To control for the effects of transplantation itself we compared transplanted fresh AM from BAL (tAM) and transplanted *ex vivo* amplified exAM (texAM) to the resident host AM populations (Fig.4A). Using this protocol, we could demonstrate stable long-term contribution of transplanted cells to the AM pool for at least 4 months with highly similar contribution rates of 5-15% for both tAM and texAM (Fig.4B). Furthermore, FACS analysis demonstrated undistinguishable expression levels between all three populations for the general myeloid and macrophage markers CD11b and CD64 as well as for the AM specific markers CD11c and SiglecF (Fig.4C). Together this showed that despite extended passage time in culture, exAM maintained the capacity of long-term contribution to the AM pool with normal stable phenotype and self-renewal *in vivo*.

In order to further determine whether the global transcriptome could be restored to the *in vivo* state upon transplantation of exAM from the culture environment to the natural niche *in vivo*, we investigated the similarity of the different AM populations by RNAseq. Spearman's correlation analysis revealed that the differences of cultured ex AM to *in vivo* samples disappeared upon transplantation. TexAM showed a high degree of correlation to the other two *in vivo* samples, both tAM and host AM populations (Fig.4D). This was further confirmed by comparison to published data sets from other tissue resident macrophage populations using PCA analysis. As shown in Fig.4E, texAM were indistinguishable from tAM or resident host AM but different to all other resident macrophage populations. As an indication of the high degree of similarity, our samples were closer to each other than to published AM data sets from another lab generated with a different protocol (Fig.4E).

In a more precise analysis of changes in gene expression between tAM and texAM, we only detected 56 genes that were significantly expressed with more than twofold change (FDR: 0.05). This contrasted with 3547 differentially expressed genes between exAM in culture and after transplantation (texAM) (Fig.4F,G). A similarly high number of 3687 differentially

expressed genes were detected when comparing exAM to host AM, which were reduced to only 217 differentially expressed genes after transplantation. When analyzing whether any of the genes that differed between texAM and tAM or host AM, only very few genes overlapped (Fig.2H), indicating that likely none of the other differences were due to the retention of culture specific genes expression *in vivo*, but to experimental noise or transplantation specific effects, as similar differences as for texAM versus host AM were also detected between tAM and host AM (Fig.S3)

Finally, the analysis of the core AM signature genes showed that the few genes related to lipid metabolism, surfactant and adhesion molecules that were lost in the culture environment (Fig.3D) were fully restored after transplantation of exAM into their natural niche (texAM) with nearly no detectable differences to the other *in vivo* AM samples (Fig.4I). Similar observations were made for the few AM specific genes that had been upregulated in culture (Fig.4I).

Together this showed that the transcriptomic adaptations of long-term cultured exAM to the culture environment were fully reversible and could be fully restored to the characteristic signature of resident AM upon exposure to the cues of the natural niche environment *in vivo*.

Epigenetic changes of exAM in culture are fully restored in vivo

The full restoration of transcriptomic identity upon transplantation of exAM into the natural alveolar space and the complete loss of the transcriptional adaptations to the culture environment opens the question as to the underlying epigenetic regulatory mechanisms. We therefore performed ATACseq analysis to determine the status of chromatin accessibility during these transcriptional changes. As one potential explanation of the complete reversibility of transcriptional changes we considered the possibility of unchanged chromatin accessibility between *in vivo* and culture conditions. The comparison of open chromatin regions (OCR) by ATACseq in cultured exAM and BAL AM *in vivo*, however, revealed a substantial number of differentially closed (723) or accessible (1504) OCR between the two conditions (Fig.5A), and thus excluded this explanation.

GO term analysis of the genes associated with these OCR alterations yielded similar terms of adaptation to the cell culture environment as the transcriptomic analysis. Whereas terms for metabolism, cellular proliferation, cell adhesion and migration were enriched, immune response terms were depleted in culture (Fig.5B). As individual examples of this, peaks not present in fresh BAL AM were induced in culture in the regulatory regions of cyclin D1 and mdm2, two cell cycle genes, of Sphk1 and Pkp2, two genes important for cell adhesion, and of HK1 and HK2, critical regulators of glycolysis. Similarly, OCR of several genes important in lipid metabolism (Acaa1b and Cidec) and in the immune response, such as several MHCII genes (H2-Aa, H2-Ab1, H2-Eb1) disappeared in exAM culture (Fig.5C).

Environmental cues can induce long- lasting epigenetic changes despite full reversibility of the transcriptional alterations induced by such signals. For example, we have shown previously that LPS stimulated hematopoietic stem cells (HSC) show transcriptional changes that quickly come back to normal but conserve a long-term epigenetic memory of the stimulus²⁷. We therefore wondered whether culture-induced epigenetic changes in exAM were conserved upon re-transfer into the natural environment in vivo. In order to address this question, we used the same transplantation protocol as before (Fig.4A). Strikingly, culture specific alterations in chromatin accessibility were fully restored upon transplantation of exAM into the natural alveolar niche. This was indicated by nearly identical OCR in transplanted cells (texAM) compared to resident AM of the recipient (host AM), whereas the comparison of cells in culture (exAM) showed the same difference to host AM as to freshly isolated BAL (Fig.5A). From the over 2500 differential OCR detected between AM in culture and in vivo (exAM versus host AM), only 15 were conserved after re-transplantation of exAM into the natural niche (texAM versus host AM, Fig.5D). Consistent with this, the comparison of exAM to texAM showed similar differences, whereas BAL and host AM showed a nearly identical OCR profile (Fig.S4). The similarity of all in vivo samples among each other, including transplanted expanded AM (texAM) and the similar strong differences of all in vivo samples to exAM in culture was also indicated by a heat map of unsupervised cluster analysis (Fig.S5). Importantly, analysis of individual OCR showed that the culture-induced peaks in regulatory regions of cell cycle, adhesion or glycolysis genes were lost again, whereas peaks in immune response genes that were lost in culture were re-established, when cultured cells (exAM) were transplanted into the natural in vivo environment (texAM) (Fig.5C).

Analysis of transcription factor binding motifs associated with differential OCR between culture and in vivo samples revealed that besides ATF3 and KLF motifs, binding sites for the core myeloid PU.1, C/EBP and Runx transcription factors were associated with both the differential open and closed OCR (Fig.S6), suggesting that the changes in chromatin accessibility might occur on pre-existing myeloid enhancer platforms.

Finally, analysis of regulatory regions for core AM specific transcription factors and surface markers identified AM specific OCR that were not detected in other myeloid cells or other cell types of the immune system, identifying them as markers of epigenetic AM identity (Fig.5E). For example, AM specific peaks of the transcription factor PPAR γ ¹⁸, a key regulator of AM identity, are not detected in other macrophage populations or immune cells, whereas the surface marker CD11c (*Itgax*) that is expressed in AM and other cell types shows AM specific and general OCRs. Importantly, these key regulatory regions of AM identity did not change across samples and were equally present in AM in vivo, after expansion in culture (exAM) and upon re-transplantation in vivo (texAM), indicating that epigenetic AM identity was not lost in culture.

Together the analysis of epigenetic chromatin accessibility indicates that AM identity was conserved through culture expansion and that adaptations to the culture environment were transient, reversible and fully restored to the *in vivo* status upon transplantation into the alveolar niche.

Expanded alveolar macrophages show full long-term reconstitution of alveolar macrophage niche *in vivo*

Given the full restoration of transcriptional and epigenetic identity of exAM *in vivo*, we wondered whether they were also functionally capable of reconstituting an empty alveolar macrophage niche. Since transplantation into the full niche of wt mice only allows a small contribution of donor cells (Fig.4A,B), we took advantage of GM-CSFRbeta KO (*csf2rb*^{-/-}) mice, which are deficient for alveolar macrophages and can be transplanted with different sources of monocytes or macrophages^{7 8 25 28 29}. We therefore transplanted CD45.1 exAM long term cultures into the empty niche of neonatal CD45.2 *csf2rb*^{-/-} mice, which allows the distinction of donor and recipient cells. Interestingly, 8 months after transplantation we detected nearly 100% of CD45.1 donor cells with a SiglecF⁺/CD11c⁺ AM phenotype in transplanted *csf2rb*^{-/-} mice, the same proportion as in CD45.1 WT mice of the same age, whereas untransplanted *csf2rb*^{-/-} mice showed no CD45.1 cells or SiglecF⁺/CD11c⁺ AM (Fig.6B,E,F). Transplanted and WT control cells showed the same morphology by brightfield microscopy and staining with Kwik-diff dye of cytopsin samples (Fig.6C). We also obtained the same absolute number of cells from the BAL fluid of transplanted *csf2rb*^{-/-} mice as from CD45.1 WT control mice (Fig.6G). This demonstrated that long term cultured exAM were capable of full long term reconstitution of an empty AM niche *in vivo*. A similar full reconstitution was also already seen 3 months after transplantation (Fig.6E-H, Fig.S7), demonstrating a similar potency of seeding the niche *in vivo* as fetal monocytes, the most prominent source of populating the lung in normal development^{7 30}.

Due to the complete absence of AM, *csf2rb*^{-/-} mice show a pulmonary alveolar proteinosis (PAP) pathology that is characterized by accumulation of mucus and debris in the alveolar lung fluid. Microscopic inspection (Fig.6C) or FACS analysis (Fig.6D,H) demonstrated a strong reduction of debris in the transplanted lungs (Fig.6D,H) that in some cases showed complete clearance of debris similar to WT control mice (Fig.6 C,D,H). Thus, in culture expanded exAM maintained the key homeostatic function of AM to clear alveolar mucus.

Together these results showed the capacity of expanded AM in culture (exAM) to functionally repopulate an empty alveolar niche *in vivo* and to self-maintain homeostatic numbers long term.

Discussion

Here we have shown that alveolar macrophages can be maintained through long term culture and can be expanded massively without losing tissue resident macrophage identity. Although significant epigenetic and transcriptional culture adaptations occur, they are fully reversible upon transplantation into the natural alveolar niche in vivo (Fig.7). This is conceptually important as it indicates a robust cell endogenous epigenetic setup that is stable through many cell divisions and provides the flexibility to adapt to different environmental cues without losing identity. In practical terms, expanded alveolar macrophages provide a nearly unlimited source of genetically unmodified and untransformed normal macrophages and a new cell culture system that provides the convenience of a cell line but the ability to quantitatively repopulate the natural niche in vivo with full restoration of epigenetic and transcriptomic identity. The potential to shuttle cells between ex vivo culture and natural niche in vivo provides a unique experimental system for screening and in vivo validation approaches or combined large-scale biochemical and genetic investigation in an untransformed cellular system with direct in vivo relevance.

Cell culture has had enormous impact on the progress of biological research ranging from cancer to stem cell research³¹. In immunology it has boosted vaccine development, the generation of monoclonal antibodies and cellular therapy and has been essential for many fundamental discoveries. Despite this general usefulness it has been put into question how close cells in culture are to their in vivo equivalents due to the loss of essential in vivo cues and new culture stimuli not encountered in vivo. For example, mouse and human microglia cells in culture undergo major changes in gene expression and lose in vivo functionality^{9 10 12}. Similarly, AM show significant changes in cytokine responsiveness and metabolism when put in culture and it has been suggested that cultured macrophages do not reflect macrophage identity in vivo¹¹. Indeed, here we also observed significant changes in gene expression related to the adaptation to the culture environment, including for genes involved in cellular adhesion/migration, proliferation and metabolism. This was in particular the case for genes related to glycolysis, consistent with the high availability of glucose in the culture medium compared to the low levels in the alveolar niche and the shift to glycolytic energy metabolism of AM in culture¹¹. We also observed changes in adhesion molecules and cytoskeletal genes, which is consistent with the different surfaces and general physical cues that AM encounter in the culture dish compared to the natural alveolar niche. Furthermore, immune response related gene expression was lost in culture, which might be explained by the loss of the stimulation from the microbiome of the lung mucosal surface in the sterile culture environment. Importantly, however, both positive and negative changes in gene expression were reversible and fully restored upon transplantation into the natural alveolar niche in vivo, confirming a potent role of the microenvironment as determinant of macrophage gene expression².

The interesting question emerging from these observations is how AM conserve the ability to respond to these different environmental cues. We observed that the changes in gene expression were also accompanied by changes in chromatin accessibility. Differentially open and closed OCR in culture were similarly associated with adhesion, proliferation, metabolism and immune response terms, respectively (Figs.5,7). Interestingly, both differentially opened and closed OCR were enriched for binding sites of PU.1, C/EBP and Runx (Fig.S6), transcription factors that determine macrophage identity³². It has been suggested that these factors establish a core macrophage enhancer platform on which additional signal induced transcription factors converge^{33 34}. Consistent with this, culture induced OCR were also enriched for ATF3 and KLF transcription factor binding sites, which are typical signal induced factors. Together this suggests that a stable enhancer architecture maintains cellular identity through culture, on which accessory transcription factors can activate environment specific gene expression. Consistent with this, the expression of core macrophage and alveolar macrophage specific transcription factors like *Runx2*, *Car4*, *Pparg*, *C/ebpb* and *Bhlhe41* (Fig.2H) and downstream AM specific genes (Fig.2G,4I) as well as AM specific surface markers (Fig.2E) were maintained through culture. Furthermore, core AM specific transcription factors and surface markers showed maintained OCR in their regulatory regions (Fig.5E). This could explain how highly stable AM specific identity is maintained through culture, while allowing a spectrum of responsiveness to environmental cues in vivo and in culture with the corresponding repertoire of adapted gene expression. In more general terms this might also explain the high degree of plasticity of macrophages in responding to a diverse set of stimuli³⁵.

The observed full restoration of normal chromatin accessibility in vivo, despite the significant epigenetic changes acquired in long term culture, is distinctly different from scenarios of trained immunity. Although in principle both resident³⁶ and monocyte-derived^{21 26} AM also appear to be capable of trained immunity, the underlying mechanism and inducing signals might be different. For example, we have shown previously that LPS stimulation of HSC induced only short-term transcriptional changes but long-term epigenetic memory through a TRIF and C/EBPb signaling axis²⁷. Perhaps, in this case, C/EBPb acts as a pioneering factor establishing new enhancer landing sites for other transcription factors, whereas in the case of AM these enhancer assemblies are already present in vivo and maintained through culture. Beta-Glucan stimulation can also induce trained immunity through epigenetic changes in monocytes in an Akt, mTOR and glycolysis dependent manner ^{37 38 39}. Interestingly, PI3K/Akt and mTOR pathways as well as glycolysis were also induced in exAM (Fig.3F) but changes in chromatin accessibility were completely restored after re-transplantation in vivo. This indicates that these pathways are necessary but not sufficient for the induction of epigenetic memory in myelo-monocytic cells.

Our results are important on three fronts: conceptually, for their therapeutic potential and as experimental model.

Our observations extend our previous findings that demonstrated the compatibility of self-renewal with the differentiated state of mature macrophages^{17 18 40}. Here we show that AM can be expanded extensively in culture for almost a year or more, but still assume full epigenetic identity and functionality upon re-integration in their natural environment *in vivo*. This is conceptually exciting, because it demonstrates that neither culture adaptations nor long term proliferation erase the epigenetic and transcriptional identity of AM. Such behavior is normally only known for stem cells that can be expanded extensively or indefinitely in culture and still functionally integrate into a tissue environment, for example pluripotent stem cells that can integrate into an embryo and give rise to a whole new organism or epithelial stem cells that can provide functional skin tissue for transplantation^{31 41}.

Our results are therefore also highly promising for all potential future applications of macrophage cellular therapy. T-cells have been highly successful in cancer immune-therapy¹⁵. Despite their potential for cancer therapy, the use of macrophages for such applications, has so far been limited by the difficulty to grow large numbers of macrophages in culture¹⁶, as has been possible for T-cells¹⁵. Our results now demonstrate that in principle large scale and long-term expansion of macrophages *ex vivo* is possible without compromising the cells' identity *in vivo*. The robustness of macrophage identity through culture could also be important for macrophage based cellular therapies in respiratory disease such as COVID19, where macrophages and the relative contributions of monocyte-derived and resident alveolar macrophages play important roles in disease development⁴².

Finally, we show that expanded AM are a long-term reliable culture model for resident macrophages that provides similar advantages as a cell line, in terms of providing large quantities of cells and the possibility of starting and stopping cultures by freeze/thawing without being dependent on complex differentiation protocols. In addition, the possibility to shuttle between *in vivo* and culture environment provides a unique experimental system for genetic and biochemical manipulation that cannot be provided by other macrophage culture systems.

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Methods

Animals

C57BL/6 CD45.1+ and CD45.2+ mice were obtained from Charles River and Janvier, respectively. The *csf2rb*^{-/-} mice were kindly provided by Dr. Melanie Greter (University of Zurich) and described previously⁴³. Age of donor and recipient mice were between 6 and 8 weeks old. Mice were housed under specific pathogen-free conditions in individually ventilated cages in a controlled 12-hour reverse light/dark cycle and were provided with food and water *ad libitum*. In vivo procedures were performed following the protocols approved by the ethics committee of n°014, Marseille, France (APAFIS#3292-2015122109359224) and the TU Dresden and the Landesdirektion Sachsen in accordance with institutional, national and European animal welfare legislation (TVV28/2018 AKZ: IC 114-G0160/16 and DD24.1-5131/449/30).

isolation and ex vivo expansion of alveolar macrophages (AM)

Isolation of AM from BAL was done as previously described¹⁹. The expansion and maintenance of exAMs ex vivo was done as described previously¹⁹. Briefly, cells were plated at a density of $1.1 - 1.5 \times 10^6$ per 10 cm petri dish in 10 ml complete medium. The medium was supplemented with murine 2-5% GM-CSF supernatant from J558L cells transfected with murine GM-CSF.

Preparation of lung homogenates for flow cytometry analysis

Mice were euthanized by cervical dislocation, lungs were collected, cut in small pieces and incubated with 1 mg/ml collagenase-2 (Worthington) and 0.15 mg/ml DNaseI (Sigma) at 37°C for 30 min with constant agitation. Cell suspension was filtered through a 70 µm mesh and erythrocytes were removed by RBC lysis (RBC Lysis buffer, Invitrogen).

Flow cytometry

Lung cell suspensions was pre-incubated with Fc receptor blocking antibody (clone 2.4 G2, BD Pharmingen or TruStain FcXTM, Biolegend) and Zombie fixable cell viability dye (NIR, Aqua or UV, Biolegend) or DAPI (Sigma) for 15 - 20 min at 4°C. For antibody staining, cells were incubated with antibody cocktail for 20 min at 4°C. FACS sorting and analysis was done with FACSCanto, LSRII and LSRFortessa systems (BD). The following antibodies were used for staining cells: anti-CD11b (clone M1/70, eBioscience or Biolegend), anti-CD11c (clone N418, eBioscience or B-Ly6, BD), anti-F4/80 (clone BM8, eBioscience), anti-MHCII (clone M5/114.15.2, eBioscience), anti-CD45.1 (clone A20, eBioscience or Biolegend), anti-CD45.2 (clone 104, eBioscience or Biolegend), anti-Ly6C (Clone AL-21, BD), anti-CD64 (clone X54-5/7.1, Biolegend or BD), anti-SiglecF (clone E50-2440, BD). Diva software was used for acquisition of data and Flowjo software V10 for data analysis (TreeStar).

Intra-tracheal (i.t) and intra-nasal transplantation of alveolar macrophages (AM)

Mice were anesthetized by isoflurane inhalation, placed on their back, the tongue was gently pulled out and $0.8 - 1 \times 10^6$ freshAM or exAMs in 80 - 100 μ l PBS were instilled intra-tracheally using 1 ml syringe with a blunt 22G gavage needle. Mice were observed while recovering from anesthesia and then returned to their cages for routine care and handling. Neonatal *Csf2rb*^{-/-} mice (postnatal day 3) were anaesthetized with isoflurane and transplanted with 0.4×10^5 AM resuspended in PBS and in a total volume of 7 μ l.

Ex vivo alveolar macrophage assays

Cytospins and in-well photography

AM were plated at a density of approx. 0.4×10^5 cells/cm² for 4 hours for attachment at either 6-well or 100mm non-treated cell culture dishes. Bright-field images acquired from well center using Zeiss Axio Vert A1 microscope at 10x magnification. Fresh BAL AMs or exAM (2 months culture, detached and counted) were subjected to cytocentrifugation 300 – 450 rpm for 4min using a Cytospin 4 (Thermo Fisher) and performed Diff-Quik staining following manufacturer's protocols (9990700, Thermo Scientific™ Rapid-chrome™ Kwik-diff™ kit). Images were acquired using the 3DHistech slide scanner and images were processed using Qupath v.0.2.0 with built-in Image J image analysis software. For *Csf2rb*^{-/-} transplants, brightfield images were taken with an inverted microscope (Dmi1, Leica) after transfer of BAL cells to a Neubauer counting chamber. Cell suspensions were cytospin at 800 rpm for 3 min using a Cytospin 4 (Thermo Fisher), followed by Kwik-diff staining according to the manufacturer's instructions (9990700, Thermo Scientific™ Rapid-chrome™ Kwik-diff™ kit). Images were taken with a Zeiss AxioLab A1 microscope.

Cell cycle analysis

AM were plated at density of 0.2×10^6 cells/well of non-treated 12-well dishes (Nunc). The cells were directly pulsed with 10 μ M 5-ethynyl-2'-deoxyuridine, EdU (C10634, Thermofischer) for 4 hours at 37°C to label proliferating cells. Cells were then detached, washed and pre-incubated with Fc receptor blocking antibody and cell viability dye followed by antibody staining as described under flow cytometry section. Cells were fixed with 4% PFA for 20 min at 4°C and permeabilized with Click-iT saponin permeabilization and wash buffer for 15 minutes at 4°C. Click-IT reaction was performed using the Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Kit (C10634, Thermofischer) as per manufacturer's instructions. For DNA staining, cells were resuspended in permeabilization buffer containing 1 μ g/ml DAPI for 30 minutes at RT. FACS data acquisition was done in the BD LSR Fortessa and analysis using FlowJo V10.

Phagocytosis assay using fluorescent latex beads

AM were incubated in growth media containing Fluorescent yellow-green, carboxylate-modified polystyrene Latex beads (L4655, Sigma-Aldrich) at 1:400 dilution for 1 hour at 37°C. Supernatant medium containing beads were discarded post-centrifugation, washed three

times (5 min each) in PBS. After a brief cytocentrifugation, the cells were fixed using 4% PFA for 10min and permeabilized with 0.1% Triton-X in PBS for 15 min at RT. Cells were incubated in blocking buffer (5% donkey serum+1%BSA in PBS) for 15 min at RT followed by Actin red staining (2 drops/ml in PBS). All the steps were carried out with adequate washing steps (3 times, 5 min each using PBS). The slides were air-dried and mounted with ProLong™ Gold Antifade mountant (P36931, Thermo Fischer).

Phagocytosis assay by flow cytometry

AMs were plated for 4 hours and replaced with fresh medium containing 2 µg/ml pHRhodo™ Red Zymosan Bioparticles™ Conjugate (P35364, Thermo Fischer) and incubated at 37°C and for controls at 4°C on ice for 2 hours. Subsequently, AMs were detached and processed for flow cytometric analysis.

Detection of lysosome and lysosomal active cathepsin B

AMs were incubated with Acridine orange (1:1000 of stock solution in PBS, 6130 - Bio-Rad) for 20 min at 37°C or with Magic Red solution (1x final concentration, ICT937 - Bio-Rad) for 50min at 37°C. Nuclei stained using Hoechst 33342 solution (1:200, 62249 - Thermo Fischer) for 10min at 37°C. After staining, AMs were mounted on slides and immediately imaged using Zeiss inverse ApoTome microscope and analyzed using FIJI image analysis software.

Detection of LPS induced ROS generation

AMs were stimulated with 2 mg/ml E.coli O55:B5 LPS (L4005, Sigma) overnight. The next day, AMs were detached and incubated with 100 µM CM-H2 DCFDA (1:100, C6827 - Thermo Fischer) for 20min at 37°C. The nuclei were stained using Hoechst 33342 solution (1:200, 62249 - Thermo Fischer) for 10 min at 37°C. After staining, the AMs were mounted on slides and imaged immediately using Zeiss inverse ApoTome microscope and analyzed using FIJI image analysis software.

RNA sequencing.

For expanded AMs (exAM), total RNA was extracted from 3 months culture of exAMs. In vivo AM subsets, were sorted (Live, Singlets, SiglecF+ CD11c+, CD45.2+ or CD45.1+) from total lung homogenates directly into RNA lysis buffer (RLT buffer, Qiagen) for RNA extraction using BD FACSAriaIII machine. Each sample was generated from a pool of 3 mice in duplicates. Amplified cDNA was prepared from 2 ng of total RNA using the Ovation RNA-seq system V2 (NuGEN Technologies, Inc.) following manufacturer's instructions. Briefly, first-strand cDNA was prepared using a combination of random and poly-T DNA/RNA chimeric SPIA (single primer isothermal amplification) primers and reverse transcriptase. Priming sites were used to synthesize second strand cDNA using a DNA polymerase, was purified using Agencourt RNAClean XP beads (Beckman Coulter Inc.). and subjected to SPIA. Amplified cDNA was purified using AMPure XP beads (Beckman Coulter Inc.) and 500 ng was fragmented by sonication using a Covaris E210 instrument. Library preparation was performed using Ovation

Ultralow Library System kit (NuGEN Technologies, Inc.) according to manufacturer's instructions. Briefly, 100 ng of amplified cDNA was blunted, phosphorylated and ligated to indexed adapter dimers. The libraries were then enriched by PCR amplification and surplus PCR primers were removed by purification using AMPure XP beads. DNA libraries were checked for quality using 2100 Bioanalyzer (Agilent) and quantified using Kapa Sybr Fast Light Cyclor 480 qPCR Kit (Kapa Biosystems) following manufacturer's instructions.

ATAC sequencing.

BAL AM pooled from 3 mice were expanded ex vivo for 2 months and i.t transplanted into lungs of 2-3 WT CD45.1 mice. The lungs were collected at 4 months post transplantation and sorted for CD45.2+ exAM or CD45.1+ host AM using BD FACSAriaIII machine. 10×10^3 cells from 3 mice each of BAL AM, exAM, texAM or Host AM were pooled and used for ATAC-seq. Fast ATAC-seq protocol was used as described⁴⁴. Cells were lysed with 1% of digitonin in TD buffer and subjected at the same time to Tn5 transposase (Nextera). The reactions were incubated 30min at 37°C. DNA were purified using Cleanup Minelute kit (28204, Qiagen) according to the manufacturer protocol. Library were prepared using Nextera DNA Library Preparation Kit (FC-121-1030, Illumina) with a 4 cycles PCR according to the manufacturer protocol. DNA was quantified with a quantitative-PCR using Nextera primer and amplified with a second PCR of 12 to 18 cycles. Libraries were cleanup with a ratio of 0,7 of AMPure XP beads (Beckman coulter) and quantified with a Qubit fluorometer (Life Technologies). Library quality was assessed by using 2100 Bioanalyzer (Agilent technologies). Libraries were sequenced with 75 paired-end using NextSeq 500 instrument (Illumina) for an average of 1×10^7 reads per sample (to verify).

Bioinformatic analysis.

RNA-seq analysis

The following analyses were done with R (v3.3). Samples were aligned to mouse genome version mm10 using Bowtie aligner. A table of raw counts was generated using htseq-count and R. All samples were normalized to 1M reads using the cpm function from the edgeR package. In order to check reliability of samples replicates, we conducted PCA analysis on normalized expression data on all expressed genes (excluding lowly expressed genes) and correlation tests on normalized expression data. For Identification of differentially expressed genes, a filtering step was conducted depending on the comparison of interest. Genes having more than 4 cpm in at least one condition in a pairwise comparison were kept for the remaining analysis. For detection of differentially expressed genes, raw data were treated using DESeq package. GO analyses were done on differentially expressed genes after k-means clustering between exAM vs Host with help of ChIPseeker package. R software was used to generate heatmaps.

ATAC-seq analysis

Data were pooled from 3 independent experiments. Fastq files quality control were done by fastqc tool. Adapter trimming was done using Cutadapt. Bowtie2 was used to align and index sequences on the mm10 mouse genome and thus generated the first series of bam files. Then, Peaks detection was made on these bam files by Genrich. Then, from the first series of bam files, a second series was generated as following: after using Bowtie2, mitochondrial DNA was removed with Samtools. Picard was used to remove PCR duplicates. Finally, Samtools allowed to remove non-unique alignments. Bigwig files were generated from bedgraph files thanks to bdg2bw script (<https://gist.github.com/taoliu/2469050>). These bedgraph files were created from the second series of bam files. All peaks detected by Genrich from exAM, texAM, Host, BAL samples were pooled and merged together with bedtools (v 2.26.0). Then a table of counts was generated by bedtools from these peaks. Signal was computed from the second series of bam files. Then peaks with more than 10 counts in at least one condition were kept for the following analysis. Opened and closed peaks were detected with the DESeq package. Peaks with adjusted p-values less than 0.05 were declared as differentially open between two conditions. Comparisons were texAM vs host AM, exAM vs host AM, exAM vs texAM. Then $\log_2(\text{count}+1)$ were represented in scatterplots comparing condition two by two. Peaks with $\log_2\text{FC}$ computed by DESeq2 more than 1.5 (resp. less than 1.5) were highlighted in red (resp. in blue).

For Motif enrichment analysis, 1492 peaks were selected as gained in exAM (as compared to host) and 937 were selected as lost. Then motif analysis was performed on these two groups of peaks using HOMER (<http://homer.salk.edu/homer>). HOMER, inter alia, is a discovery motif analyzer and screens for enrichment of known motifs. HOMER perl script findMotifsGenome.pl was used with the mm10 mouse genome as a background (random genomic sequences samples according to GC content of input sequences). Venn diagrams were plotted using VennDiagram package. GO analysis and Genes association with peaks were done with GREAT tool (v4.0.4) using default options to assign regions and using minimum region-based fold enrichment of 1.2 and term annotation counts (5-1000) were set as global controls for GO term analysis. For peak visualization reference ATAC-seq sample bigwig files were downloaded from the Immgen consortium⁴⁵ (GSE100738). peak visualization on our samples independently or in parallel with the Immgen references using integrative genomics viewer (IGV, version 2.10.2) and were auto scaled by group.

Figure legends

Figure1. Alveolar macrophages show a massive expansion potential in long term culture

- a) Growth curve of AM in liquid culture represented as cumulative population doublings over time. Linear regression of exponential population growth (Orange line) with 95% confidence intervals (Black lines). Data shown are from biological quadruplicates and represented as Mean $\pm$ SD. Inset: Schematic of alveolar macrophages (AM) isolation by broncho-alveolar lavage (BAL) and ex vivo expansion culture (exAM).
- b) Cell cycle analysis on freshly isolated AM or exAM (3.5 months), (Live, singlets, SiglecF+, CD11c+ cells) using EdU (10 μ M, 4 hours) for proliferating cells and DAPI for DNA-content as evaluated by flow cytometry. FACS plots are representative of biological quadruplicates. Data is represented as Mean $\pm$ SD.

Figure2. Expanded alveolar macrophages maintain characteristic phenotype and function in long term culture

- (a-c) Representative images of plated BAL AM or exAM (2 months) ex vivo.
 - a) Brightfield-images (10x magnification) and Kwik-diff histological staining on cytopins. Scale bar represents 20 μ m and 10 μ m for magnified cell examples. Data shown are representative of two independent experiments.
 - b) Representative fluorescence staining for the lysosomal marker acridine orange (AO) and lysosomal cathepsin B activity using magic red (MR).
 - c) Representative fluorescence staining for phagocytosis of green fluorescent latex beads. Cell cytoplasm stained using actin red.
- (b-c) Scale bars represents 5 μ m. Images acquired at 20 x magnification.
- d) Representative FACS histogram and quantification of BAL AM or exAM (2 months) for phagocytosis of pHRhodo Red Zymosan bioparticle conjugates at 4°C and 37°C. Data shown are representative of 2 independent experiments.
- e) Flow cytometric comparison of cell-surface markers F4/80, CD64, CD11c, SiglecF and CD11b expression on BAL AM and exAM. Colored lines indicate specific staining, grey areas indicate staining with isotype control antibody. Data shown are pooled from two independent experiments and representative of four independent experiments.
- f) Representative fluorescence staining of BAL AM or exAM (2 months) for E.coli LPS (O55:B5) induced ROS production indicated by CM-H2 DCFDA staining (green).
- g) RNA-seq gene expression of Core AM UP signature genes ³ in AM or exAM compared to AM and peritoneal macrophages (PM) from Immgen consortium⁴⁶ (GSE122108)
- h) Heatmap of AM-specific transcription factor gene expression on AM or exAM compared to PM and AM from Immgen consortium⁴⁶ (GSE122108).

Figure 3. The exAM transcriptome shows adaptations to the culture environment

- a) Volcano plot showing differential gene expression (DEGs, <0.05 FDR, |FC| >2) between exAM and in vivo AM (Host AM, Fig.4A).
- b) Gene set enrichment analysis (GSEA) enrichment plot for GO biological process (GO: BP) positive regulation of mitotic cell cycle gene set (GO:0045931) in AM vs exAM. Corresponding leading-edge gene expression heatmap shown for AM vs exAM.

- c) Gene set enrichment analysis (GSEA) for Reactome: Signaling by TGF- β family members gene set (R-HAS-9006936) in AM vs exAM. Corresponding leading-edge gene expression heatmap shown for AM vs exAM.
- d) Differentially expressed genes (DEGs) of AM or exAM in core AM signature genes³ in ($|FC| > 4$; < 0.05 FDR). Downregulated genes enriched for GO:BP lipid metabolic processes (GO:0006629, P-value $2.77E-8$) marked by asterix*, AM core genes involved in lipid metabolism⁷ (in bold), potential niche interaction genes in blue.
- e) K-means clustering (K=3) heatmap showing total genes in AM vs exAM.
- f) Gene ontology analysis on K means clusters CI and CII using GO:BP, KEGG, Reactome and Hallmark databases. Selection of representative, meaningful top hits.
- g) Heatmaps of gene expression of selected representative key genes on recurring, complementary GO terms from analysis shown in Figure 3F.

Figure 4. Transplanted exAM restores full transcriptional AM identity in vivo

- a) Experimental setup for intra-tracheal (i.t) transplantation of freshly isolated BAL AM or 2 months expanded exAM (wild type, WT CD45.2) into lungs of WT CD45.1 host mice. Lungs analyzed 4 months post transplantation containing transplanted freshly isolated AM from BAL (tAM) or transplanted ex vivo amplified exAM (texAM) and resident host AM.
- b) Contribution of CD45.2+ tAMs or texAMs and CD45.1 host AM to the total CD11c+SiglecF+ AM population, showing FACS example, individual values (n = 6). and median.
- c) Surface marker expression of CD64, CD11c, SiglecF, CD11b and MHCII on host AM, tAM, and texAM. Data are representative of three independent experiments.
- d) Heatmap of Spearman's correlation matrix with 2 biological replicates.
- e) 3-dimensional principle component analysis (PCA) showing exAM (orange), texAM (red) and host AM (blue) compared to various tissue macrophage subsets⁴.
- f) Overlaid volcano plot showing DEGs in tAM vs texAM in blue compared to exAM versus texAM in red (top), and texAM versus host AM in blue compared to exAM versus host AM in red (bottom) ($|FC| > 2$, < 0.05 FDR).
- g) Venn diagram showing overlap of DEGs up and down for exAM or tAM compared with texAM.
- h) Venn diagrams showing total DEGs on pairwise comparisons on all in vivo AMs.
- i) Heatmap showing core AM UP and DOWN signature gene expression³ from our data compared to published tissue macrophage subsets⁴. Boxed outlines indicate restoration in vivo of gene expression of several Core AM UP genes shown to be altered in culture (Fig. 3D).

Figure 5. Epigenetic changes of expanded alveolar macrophages in culture are fully restored in vivo

- a) Scatter plot analysis of ATAC-seq peaks differentially open or closed. Total peaks were pooled (48,100) from three independent experiments with 2-3 biological replicates. Each point represents the average signal for each comparison.
- b) Gene ontology analysis using GREAT tool⁴⁷ on differential open chromatin regions (OCR) in exAM vs host AM.

- c) Examples of integrative genomics viewer (IGV) tracks for key genes linked to GO terms shown in Figure 5B and Figure 3D,F,G.
- d) Venn diagram showing total differential peaks for exAM or texAM compared with Host AM.
- e) Examples of integrative genomics viewer (IGV) tracks showing open chromatin regions (OCR) for AM specific transcription factors (TF) and cell surface markers and house-keeping genes (HKG) in our samples alongside various cell populations available through the Immgen consortium⁴⁵. ATAC-seq signal pile-up traces were generated by pooling three independent experiment with 2-3 biological replicates each. Peak signals were group auto scaled based on data source.

Figure 6. Expanded alveolar macrophages show functional long-term contribution to alveolar macrophages in vivo

- a) Schematic of intranasal transplant of exAM obtained from CD45.1 mice into empty niche of Csf2rb^{-/-} CD45.2 neonates at post-natal day 1 and subsequent analysis at 3 months weeks or 8 months post transplantation.
 - b) Flow cytometric analysis of exAM contribution to Csf2rb^{-/-} lungs at 8 months post exAM transplantation. FACS plots showing CD45.1 contribution of engrafted texAM (top panel) and SiglecF and CD11c AM surface markers (bottom panel).
 - c) Representative brightfield and Kwik-diff stained images of BAL at 8 months transplantation. WT (CD45.1) and Csf2rb^{-/-} (CD45.2) BAL as controls.
 - d) Flow cytometric analysis of reduction of debris in BAL of Csf2rb^{-/-} (CD45.2) mice post exAM transplantation. FACS plots showing debris clearance in exAM transplanted mice (top panel) and the % debris (bottom panel) quantified in Figure 6H.
 - e) Quantification of CD45.1+ exAM engraftment shown in Figure 6B and Fig.S7.
 - f) %SiglecF+ CD11c+ AM in BAL corresponding to Figure 6B and Fig.S7.
 - g) Cell numbers counted in BAL corresponding to Fig 6C and Fig.S7.
 - h) Percent Debris and dead cells corresponding to Figure 6D and Fig.S7.
- Data in (E-H) shown as Mean $\pm$ SEM.

Figure 7. Schematic of epigenetic changes of exAM in culture and restoration in vivo

- a) The scheme shows symbolic representation of AM identity OCR maintained across conditions, as well as culture adaptation OCR gained or natural environment induced OCR lost in culture and restored in the natural niche in vivo.

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Figure 1

Figure 2

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Supplementary Files

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