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Opinion

Teaching Old Dogs New Tricks? The Plasticity of Lung Alveolar Macrophage Subsets

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Alveolar macrophages (AMs) are highly abundant lung cells with important roles in homeostasis and immunity. Their function influences the outcome of lung infections, lung cancer, and chronic inflammatory disease. Recent findings reveal functional heterogeneity of AMs. Following lung insult, resident AMs can either remain unchanged, acquire new functionality, or be replaced by monocyte-derived AMs. Evidence from mouse models correlates AM function with their embryonic or monocyte origin. We hypothesize that resident AMs are terminally differentiated cells with low responsiveness and limited plasticity, while recruited, monocyte-derived AMs are initially highly immunoreactive but more plastic, able to change their function in response to environmental cues. Understanding cell-intrinsic and -extrinsic mechanisms determining AM function may provide opportunities for intervention in lung disease.

Tidy Past and Messy Present

Classically, immunologists thought of mononuclear phagocytes, including tissue macrophages and circulating monocytes, as bone marrow (BM)-derived cells that act as sentinels against pathogens and damage [1]. **Myeloid cells** (see Glossary) were considered relatively short lived [2] and therefore unable to maintain over a long period of time any functional changes that arose as a consequence of prior stimulus. With advances in lineage tracing and cell-type-specific depletion methods in recent years, these views were challenged on several levels: First, steady-state **tissue-resident macrophages** are often long-lived, self-replenishing cell populations of embryonic origin rather than offspring from BM-derived cells [3–5]. Second, tissue-resident macrophages are heavily involved in tissue maintenance and repair processes, sometimes at the expense of their immune function [6–9]. Third, when the steady state is perturbed – for instance, by infection – recruitment of additional monocyte-derived cells is crucial to accelerate the immune response [10,11]. At the end of the perturbation, recruited cells may remain in the tissue to contribute long term to the pool of macrophages that live permanently in that organ [12]. Finally, a range of long-term changes in the reactivity of innate immune cells following a stimulus have been described under the notion of **trained immunity** [13].

These novel concepts and observations highlight the notion that mammalian tissue-resident and **recruited macrophages** are not only of different origins (embryonic versus BM) but can also be functionally different. Besides their origin, important determinants of macrophage responsiveness are the tissue where they reside [14], the time spent in that tissue, and how inflamed or quiescent this environment is. What role does each of these factors play and how do they combine to ultimately fine-tune macrophage function at a given moment in time? What are the underlying mechanisms? Recent studies on murine **AMs** have started to shed light on some of these questions, through the identification of subsets and their role in health and disease. We propose here an overarching model in which most of the functional alterations to lung immunity derive from recruitment or changes in monocyte-derived AMs, while resident AMs are terminally differentiated and largely unable to modify their function: the old dogs that do not learn new tricks.

Highlights

In mice at steady state, embryonically derived tissue-resident alveolar macrophages (AMs) self-sustain over a lifetime and fulfill their homeostatic function of surfactant removal. Following various lung insults, including influenza A virus infection and bleomycin-induced lung fibrosis, these cells show only minimal transcriptional and functional changes, suggesting that resident AMs are terminally differentiated, highly specialized, and not very plastic.

In naïve mice, the contribution of monocyte-derived cells to AMs is negligible, but they become major components of the AM population post-lung insult. The functionality of recruited AMs can determine increased antibacterial protection, reduced asthma, and elevated lung fibrosis, depending on the specific initial insult studied.

When the newly recruited population of monocyte-derived AMs persist in the mouse lung, they start to resemble tissue-resident AMs, suggesting that the lung environment sends cues to shape their relatively non-inflammatory, immunosedated phenotype.

The high reactivity of recently recruited AMs might be best explained as a transcriptional and epigenetic legacy from their prior monocyte identity. Alternatively, training, acting through epigenetic and transcriptional reprogramming on hematopoietic stem cells or monocytes in the blood or in the lung, may imprint the high reactivity of recruited AMs.

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The Complexity of AM Subsets

After years of debate, a clear picture of murine tissue macrophage origin is now emerging, with macrophage populations in organs such as the brain (microglia), liver (Kupffer cells), and lung (AMs) being seeded early in ontogeny from an embryonic precursor [15]. These organs are considered 'closed', as there is almost no steady-state recruitment of monocyte-derived macrophages [3,5,15]. Other tissues are thought to be 'open' for replacement with blood monocytes at either slow (i.e., heart) or fast (i.e., dermis and gut) rates [5,15,16]. Lineage-tracing experiments and BM-chimeric mice (Table 1) have shown that tissue-resident AMs self-maintain without further input from monocytes in the steady-state lung [3–5], whereas in situations of lung insult, BM-derived cells can contribute to the AM pool [10,12]. Embryonic precursors and blood monocytes are considered highly 'plastic' as they are capable of differentiating into many myeloid cell

Table 1. Examples of Commonly Used Techniques to Distinguish Tissue-Resident and Monocyte-Derived AMs

Species	Model	Explanation	Refs
Mouse	*Ccr2* knockout (KO)	CCR2 is crucial for monocyte egress from the BM and recruitment from the blood into inflamed tissue. Therefore, in *Ccr2* KO mice, changes in AMs can be attributed to the resident AMs, as monocyte recruitment is severely impaired.	[40,75]
	Busulfan chimeras using *Ccr2* KO host	Busulfan is used to ablate BM cells, which are reconstituted with allogeneically marked BM cells (e.g., using CD45.1 or CD45.2 alloantigens). Donor-BM-derived monocytes are recruited into the CCR2-deficient host lung.	[10]
	Irradiation chimeras	In host mice, BM cells can be depleted through irradiation while preserving immune cells in the lung if the thorax is shielded. Reconstitution with marked BM cells allows tracing of BM-derived AMs.	[11,12,76]
	Parabiotic chimeras	Conjoined mice sharing blood flow develop a high level of chimerism of circulating monocytes. AMs that are marked with the alloantigen from the conjoined mouse must be monocyte derived.	[40,55]
	Fate mapping using Cre-loxP system	Granulocyte-monocyte progenitors express high amounts of *Ms4a3*. Monocytes express high amounts of *Mx1*, *S100a4*, and *Flt3* as well as high and intermediate amounts of *Cx3cr1*. Resident AMs do not express these genes. Therefore, the promoter of the above genes is used to drive the expression of Cre recombinase that enables permanent induction of a fluorescent marker in monocyte-derived cells.	[3,4,16]
	Labeling with fluorescent antibody/dye	Intravenous injection of fluorescent antibody (e.g., against CD45) or dye (PKH26) labels monocytes and can be traced to monocyte-derived AMs. Dye (PKH26) administration into the lung labels phagocytic cells including resident AMs, so if it is administered before lung insult, newly incoming monocytes and monocyte-derived AMs will not be labeled. These are time-restricted labeling techniques.	[42,76]
Human	Lung transplant HLA mismatch	After lung transplant, donor resident AMs (bearing donor HLA) can be distinguished from host monocyte-derived AMs using mismatched HLA antigens.	[18]
	Lung transplant sex mismatch	In female recipients of a male-donor lung or vice versa, donor-lung-resident AMs can be distinguished from recipient-monocyte-derived cells using sex-specific transcripts [X-inactive specific transcript (*XIST*) for females and ribosomal protein S4 Y-linked 1 (*RPS4Y1*) for males] or fluorescence *in situ* hybridization (FISH) with X/Y probes.	[19,20]
	scRNA-seq	In scRNA-seq, AMs can cluster based on their expression of classical AM genes (potentially resident AMs) or peripheral monocyte-like genes (potentially monocyte-derived AMs). This method is correlative.	[77]

Glossary

Alternatively activated macrophages: induced by IL-4, IL-10, or IL-13 to perform mainly tissue-repair functions.
Alveolar epithelial type II cells: cells in alveoli, secreting lipoproteins called surfactants.
Alveolar macrophages (AMs): specialized lung macrophage population present mainly in alveoli, whose main function in lung maintenance is the removal of surfactant and whose immunoreactivity is comparably low.
Bacillus Calmette–Guérin (BCG): live attenuated *Mycobacterium* strain used for tuberculosis vaccination.
Bleomycin: drug commonly administered to mice to study post-injury pulmonary fibrosis.
Clodronate liposomes: lipid vesicles encapsulating clodronate, used to specifically deplete macrophages and other phagocytic cells by apoptosis without causing much inflammation.
Immunosedation: a term we have coined to describe the effect of the non-inflamed, steady-state lung environment to induce low reactivity in immune cells present in the lung.
Inflammaging: chronic low-level, age-associated inflammation.
Inflammatory macrophages: generated on IFN-γ and LPS stimuli; they rely on glycolysis to perform host-defense functions, including secretion of IL-6, IL-1, IL-12, IL-23, TNFα, and other proinflammatory cytokines.
Lymphopenia: condition of reduced numbers of lymphocytes in the blood.
Monocyte legacy: epigenetic and transcriptional features in monocyte-derived macrophages, which may be remnants from their prior monocyte identity.
Myeloid cells: monocytes, macrophages, dendritic cells, granulocytes, mast cells, megakaryocytes, and erythrocytes; arise from a common myeloid progenitor in hematopoiesis.
Niche model: according to this model, local macrophage development is determined by niche accessibility (is there a barrier?), niche availability (is it unoccupied?), and precursor plasticity (is there a more-suited precursor?).
Plasticity: capacity to respond to various environmental cues by developing different functional profiles.
Polarization: functional profile of macrophages developed in response to environmental stimuli. The extremes of

subtypes; by contrast, terminally differentiated tissue macrophages are committed to the phenotype imposed by their specific organ of residence and do not fully acquire the characteristics of AMs when transplanted into the lung [14,17].

Until recently, in neither the mouse nor human datasets was it possible to determine AM origin (Table 1). The absence of reliable markers of origin limits our understanding of AM pool composition in healthy humans, but studies in lung transplant recipients use human leukocyte antigen (HLA) or sex mismatch between donor and recipient as markers for the distinction between AMs that were present in the lung prior to the transplantation (resident) or that migrated in later (recruited). These studies have shown that lung macrophages comprise both resident (donor) cells and newly recruited BM-derived (recipient) cells [18–20]. In humans, being surrounded by pollutants and undergoing infections or other lung insults multiple times during our lifetime, the AM pool will comprise a mix of macrophages, some of embryonic origin and others of BM origin, with differing arrival times and duration of residence in the lung. Laboratory mice living under **specific-pathogen-free (SPF)** conditions will not have experienced major lung insults, so the predominance of embryonically derived AMs has not been lost and, as a consequence, naïve laboratory mice contain mostly, if not only, AMs of embryonic origin [3–5].

RNA-seq of bulk cell populations in mice have shown a unique tissue-specific transcriptional profile for macrophages, which is distinct from blood monocytes [14]. Recent single-cell transcriptional analyses have indicated a vast variety of lung macrophage clusters in lung cancer patients with their biography of varied lung insults [21,22], but also in mice living under SPF conditions [22]. While there are similarities between mouse and human macrophage populations, these cannot be unequivocally mapped onto each other [22]. As these studies have not determined the embryonic or BM origin of macrophage subsets, future single-cell analysis should be combined with models employing fate-mapping tracing techniques [16] to broaden our understanding of the impact of origin or differing sojourns in the lung on AM functionality.

Increased AM Reactivity Post-Influenza Virus Infection (IVI) May Emerge from Monocyte-Derived Cells

Recent results have shed new light on the link between origin and AM functionality. Using chimeric mice that provide markers to distinguish between adult BM and embryonic origin, our group showed that, following recovery from IVI, the AM pool comprised both embryonically derived 'resident' AMs and CCR2-dependent, BM-derived 'recruited' AMs, most likely of monocyte origin [10]. The two AM subsets were indistinguishable by surface phenotype and morphology but showed significant transcriptional, epigenetic, and functional differences. While IVI had no impact on the transcriptional profile or functional phenotype of resident AMs, recruited AMs maintained open chromatin at loci controlling the expression of inflammatory genes, including *Il6*, which made these cells significantly more responsive to stimulation with bacterial **Toll-like receptor (TLR) agonists** [10] (Figure 1A). It should be noted that resident AMs can be important producers of type I interferons (IFNs) and cytokines post-viral infection [23–26], but this response is most prominent on day 1 and is short-lived. On days 3–9 post-infection, resident AMs are often found to be depleted, and monocytes are recruited into the inflamed tissue [10,26–29]. In the case of severe IVI, monocytes destined to become recruited AMs arrive in the lung between day 3 and 7 of infection [10]. Even after a 1-month residence in the lung, these BM-derived AMs have not acquired the relatively unresponsive profile of resident AMs as determined by transcriptional and functional analysis [10]. These results highlight a differential responsiveness between resident and recruited AMs and reconcile functional changes at the population level with a lack of functional changes in resident AMs; the new functionality comes in with the newly recruited cells [10]. Similar observations with regard to BM-derived AMs have been made in other lung-insult models, showing

polarization defined *in vitro* are inflammatory and alternatively activated macrophages, but *in vivo*, intermediate and independent profiles exist.

Poly I:C: synthetic analog of double-stranded RNA used to mimic RNA-virus infections.

Pulmonary alveolar proteinosis (PAP): pulmonary disease due to impaired clearance of surfactants by AMs, leading to surfactant accumulation in alveoli.

Recruited macrophages: develop from circulating monocytes that originated in the BM and were recruited into a peripheral organ. They can develop self-renewal capacity, similar to tissue-resident macrophages. The terms 'recruited', 'monocyte-derived', and 'BM-derived' macrophages are used interchangeably in this opinion article.

Resident, or tissue-resident, macrophages: population of sessile macrophages that self-renew *in situ* by proliferation. They originate from fetal precursors and colonize organs during development. 'Embryonically derived macrophages' is used synonymously.

Specific pathogen free (SPF): term applied to laboratory animals that are proven to be free of particular pathogens that might cause interference with experimentation.

Surfactant: complex of lipids and proteins lowering the surface tension in alveoli.

Toll-like receptor (TLR) agonist: substance that stimulates immune responses by TLR activation.

Trained immunity: changes in the immunoreactivity of innate immune cells resulting from previous exposure to a stimulus. Here, we use it to discuss medium- to long-term changes in monocyte/macrophage reactivity due to stimulus-induced epigenetic reprogramming.

Type 2 immune response: triggered by helminths, allergens, and other stimuli; characterized by cytokine production, including IL-4, IL-5, and IL-13.

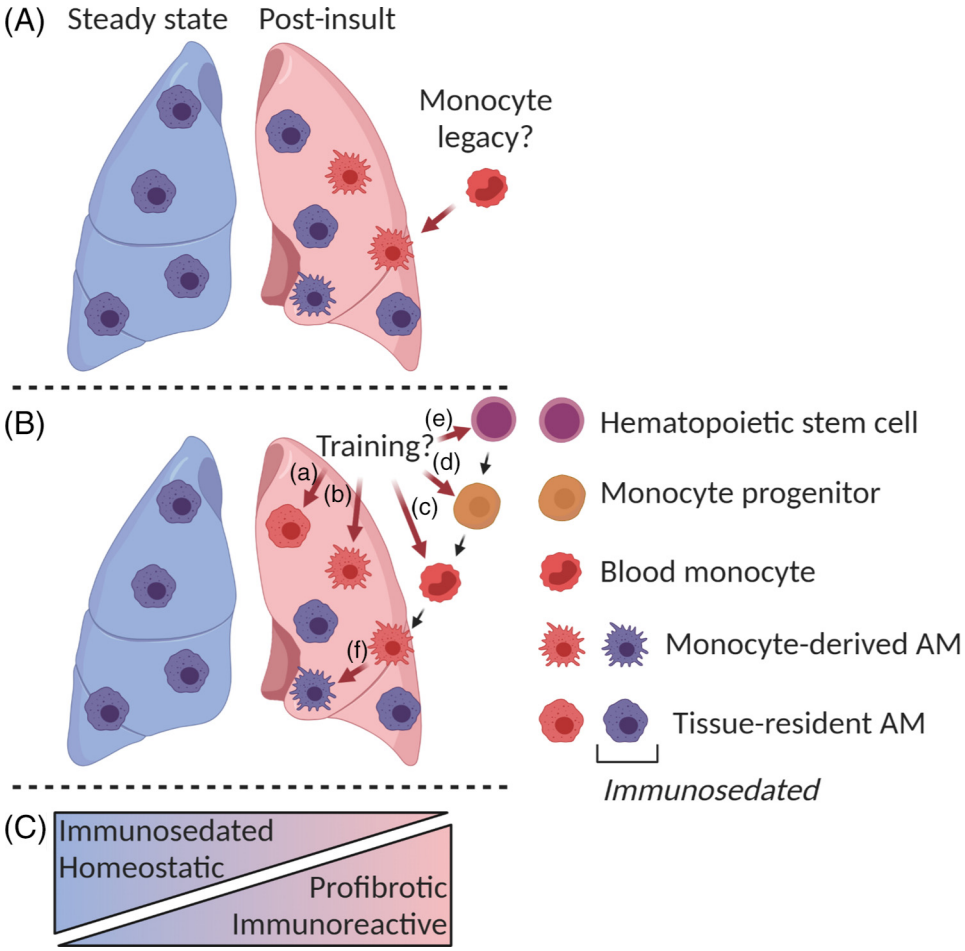

Figure 1. Model of Changes in Alveolar Macrophage (AM) Phenotype in the Post-Insult Lung. AMs found in the post-insult lung (A,B) (e.g., in mice) can be profibrotic or immunoreactive, compared with the steady-state, immunosedated resident AMs that are responsible for homeostatic functions (C). This may be explained by (A) monocyte-derived AMs retaining functionality similar to that of monocytes [10] due to their recent recruitment; or by (B) innate training. (a) Resident AMs can be trained to develop a more immunoreactive phenotype [39,40]. Recruited AMs can change functionally as a result of innate training at various stages, including (b) as AMs in the lung, (c) as monocytes in the blood [43], and (d) as monocyte progenitors or (e) hematopoietic stem cells in the BM [44–46]. (f) Monocyte-derived AMs can lose reactivity and become immunosedated [10] by anti-inflammatory signals from the uninflamed lung environment. This figure was created using BioRender (https://biorender.com/).

that BM-derived AMs provide protection against murine house dust mite (HDM)-induced asthma [11] or can be a driving force for lung fibrosis following mouse lung injury [12,30,31]. Also, similar to IVI, tissue-resident AMs have been reported to remain mostly unchanged following **bleomycin** treatment [12], consistent with the notion that resident AMs of embryonic origin can play important roles in tissue homeostasis and exhibit reduced functional **plasticity** [17,32].

Why Is Proliferation of Resident AMs Not Sufficient to Replenish the AM Pool?

Resident AMs are known to be able to proliferate and contribute to the repopulation of the lung, but some of the AMs found after IVI and other insults are monocyte derived [10–12]. Why do resident AMs not fully repopulate the AM pool during recovery from IVI in mice? Why is the contribution of a monocyte-derived AM population needed? The **niche model** [33] may provide a

framework to explain AM dynamics. At the steady state, the lung epithelium can prevent access by monocytes to engraft in this niche. Moreover, the AM niche is unavailable, already occupied with embryonically derived resident cells. Injury such as IVI would lead to AM depletion and render the niche temporarily available. The empty niche may instruct resident AMs to proliferate locally or, if necessary, allow recruited monocytes to engraft [3,10,17]. *In vivo* studies in mice have highlighted the capacity of resident AMs to self-sustain via proliferation at the steady state and expand after targeted depletion [3]. For instance, accumulation of pleural macrophages was demonstrated in a **type 2 immune response** induced by infection with the nematode *Litomosoides sigmodontis*. Here, local proliferation of **alternatively activated macrophages** was driven via IL-4 signaling, given that it was absent in IL-4-deficient mice, with minimal contribution from recruited macrophages [34,35]. It should be noted that monocyte-derived macrophages could also be driven to proliferate by IL-4 activation, as shown by BrdU incorporation by recruited peritoneal macrophages after IL-4 treatment [34]. The infection-site-restricted IL-4-dependent proliferation of resident macrophages was independent of the M-CSF receptor, in contrast to M-CSF-dependent proliferation at the steady state [35]. In the mouse lung, IL-4-dependent AM proliferation is enhanced by **surfactant** protein A (SP-A), potentially produced by **alveolar epithelial type II cells** [36]. By contrast, repopulation of lung macrophages after genotoxic depletion is dependent on both M-CSF and GM-CSF, but independent of IL-4 [3]. Together, these data suggest that proliferative capacity may not be restricted to macrophage precursors, but resident macrophages may be capable of expansion *in situ* for maintenance in type 2 responses or when restoring tissue homeostasis in situations where the tissue-damage potential of inflammatory cell recruitment is to be avoided.

In contrast to our findings describing how depleted AMs are repopulated with both resident AMs and highly immunoreactive recruited AMs following IVI [10], another IVI study in mice found that the reduction of AMs was recovered by local repopulation with resident AMs, independent of monocyte contribution [3]. Similar patterns of AM depletion and proliferation *in situ* were observed with **poly I:C** and **clodronate liposome** administration [3], confirming the ability of resident macrophages to expand in inflammatory settings. Most likely, the contribution of embryonically derived versus monocyte-derived cells to the AM pool post-IVI may depend on the degree of AM depletion during infection, which in turn may depend on the severity of inflammation. This might also contribute to explaining why, in another IVI study, AMs were desensitized for TLR signaling 1–2 months post-recovery [37], reminiscent of the lipopolysaccharide (LPS) tolerance previously described [38].

Is There Trained Immunity of Resident AMs?

Trained immunity in monocytes and macrophages has been described as a stimulus-induced epigenetic alteration leading to long-term functional changes. Where does trained immunity come into play and where is macrophage origin sufficient to explain functional differences? The above results [10–12] demonstrate that resident AMs do not change during certain lung insults, in line with their status as terminally differentiated, tissue-adapted cells. However, in other experimental settings, long-lived resident AMs can also show long-term functional alterations (Figure 1Ba). For instance, the *Pseudomonas aeruginosa* vaccine induces expansion of local AMs independent of monocyte recruitment, providing protection against bacterial challenge in a mouse model of chemotherapy-induced depletion of BM-derived immune cells [39]. Similarly, resident trained AMs provide prolonged antibacterial protection after adenoviral infection independent of monocytes and BM progenitors, as shown in BM chimeras and parabiotic mice ([40]; Table 1). Here, the induction of trained AMs required priming by $CD8^+$ T cells via IFN-γ production [40]. In these studies, increased lung macrophage reactivity did not require newly recruited monocyte-derived cells as it was independent of the monocyte chemoattractant CCL2; instead, increased reactivity was deemed to be

dependent on resident cells having changed their functionality [39,40]. It is currently unclear whether this effect is restricted to certain challenge models; also, it is unclear why other viral infections or bleomycin-induced lung insult might instead lead to the strong presence of monocyte-derived AMs with changed functionality. In addition, not all lung insults will lead to strong $CD8^+$ T cell-derived IFN-γ, which appears to be a key cytokine in the imprinting of increased macrophage reactivity in the abovementioned mouse model of adenovirus infection [40]. Given the variety of results obtained in different systems, the jury is still out on the circumstances under which embryonically derived resident AMs adopt long-term functional changes (Table 2). It is possible that, depending on the type of insult (e.g., whether it is more local or systemic), different processes may lead to changed reactivity of macrophages, some relying on long-term changes in resident populations, others on recruitment of new cells of differing reactivity.

Table 2. Recent Studies of Phenotypic Changes in Alveolar Macrophages (AMs) and Their Progenitors after Challenges

Cell	Species	Challenge	Phenotype	Duration	Refs
BM-derived AMs	Mouse	Murid herpesvirus (MuHV-4) infection	Decreased IL-4, IL-5, IL-13, and IL-6 production Reduced allergic response to HDM	28–30 days	[11]
	Mouse	Bleomycin-induced fibrosis	Increased expression of *Adam8*, *Arg1*, *Apoe*, *Itga6*, *Mfge8*, *Mmp12*, *Mmp13*, *Mmp14*, and *Pdgfa* Drive lung fibrosis	14–19 days	[12]
			Increased expression of *Ccl2*, *Ccl12*, *Ccl24* Drive lung fibrosis	7–14 days	[30]
			Increased expression of ApoE Drive lung fibrosis resolution	8 weeks (fibrosis resolution stage)	[78]
	Mouse	Asbestos-induced fibrosis	Self-sustaining via M-CSF/M-CSFR signaling Increased expression of *Pdgfa* Drive lung fibrosis	14 days	[31]
	Mouse	Influenza A virus infection	Increased IL-6 production Increased protection against *Streptococcus pneumoniae*	28 days (phenotype lost at 2 months)	[10]
Tissue-resident AMs	Mouse	*Pseudomonas aeruginosa* vaccine	Increased protection against *P. aeruginosa* pneumonia in chemotherapy settings T cell dependent	4 weeks	[39]
	Mouse	Adenovirus infection	Increased production of neutrophil chemokines (MIP-2 and KC) Increased glycolysis $CD8^+$ T cell-dependent priming via IFN-γ Increased protection against *S. pneumoniae* and *Escherichia coli*	4 weeks (up to 16 weeks for *S. pneumoniae* protection)	[40]
	Mouse	*E. coli*	Reduction of phagocytosis of extracellular bacteria	7–14 days	[42]
Blood monocytes	Human	BCG vaccine	Increased IL-1β production Reduced yellow fever viremia	1 month	[43]
			Increased production of IFNs, IL10, IL-1β, IL-6, IL-1RA, TNF	14–90 days	[44]
		Severe trauma or sepsis	Reduction of phagocytosis of extracellular bacteria Reduced amounts of SIRPα, CD206, CD14, and CD16	Up to 4 weeks post-sepsis Up to 6 months post-trauma	[42]
BM cells	Human	BCG vaccine	Enhanced myelopoiesis Increased expression of *IFNG*, *TNF*, and *IL1B* (BMDMs) Increased protection against *Mycobacterium tuberculosis*	4 weeks (BMDMs up to 5 months)	[46]
			Upregulation of myeloid and granulocytic lineage-associated transcripts Upregulation of TFs *HNF1A* and *HNF1B*	90 days	[44]
	Mouse	β-Glucan	Increased G-CSF and IL-1β production Increased glycolysis Enhanced myelopoiesis Increased protection against LPS challenge	24 h 24 h and 7 days 7–28 days 28 days	[45]

Why Are Recruited AMs More Immunoreactive Than Resident AMs?

We argue that the simplest explanation for the increased reactivity of monocyte-derived AMs is that this represents a 'legacy' of their former monocyte profile. Recruited monocytes seeded the mouse lung niches early post IVI to contribute to the AM pool [10,28,29]. Nevertheless, when recruited AMs were analyzed 28 days post-infection, they showed great similarities to blood monocytes from naïve animals, transcriptionally and in terms of global chromatin accessibility [10]. A prominent example is the regulation of *Il6* gene expression: more IL-6 protein is produced on a bacterial stimulus in recruited AMs than in resident AMs, and this is reflected in open chromatin regions found upstream of the *Il6* gene in recruited AMs. The open regions are similar to those in blood monocytes, and absent in resident AMs [10]. In addition, another study in mice found enrichment of genes belonging to the IL-6 signaling pathway after stimulation of inflammatory monocytes and interstitial macrophages, but not of AMs [32]. As interstitial macrophages are located in an open niche, they resemble more blood monocytes, in contrast to the closed niche of AMs, which sets them apart [5,15]. This suggests that the heterogeneous AM population post-IVI in mice [10] might not require major transcriptional, epigenetic, or functional changes of any of the constituent cell subsets. Resident AMs may be programmed to be hyporesponsive; hence, the low IL-6 production. By contrast, recruited AMs, due to their monocyte legacy, might still transcriptionally resemble the blood monocytes they once were, with key proinflammatory genes prone for expression (Figure 1A,C).

However, these findings in IVI do not exclude the possibility that in other situations, greater reactivity of recruited AMs is imprinted. Where could this happen? One possibility is that inflammatory conditions in the lung during the monocyte-to-macrophage transition imprint enhanced reactivity onto these cells (Figure 1Bb), similar to what has been described *in vitro* when human blood monocytes were kept in culture for time periods of several days [41]. Alternatively, prior to arrival in the lung, monocytes might receive a cytokine-mediated signal in the blood imprinting their functionality (Figure 1Bc), or monocyte precursors might be imprinted even earlier in the BM (Figure 1Bd,e). The latter hypotheses are testable as they predict that, post-lung injury, the function of monocytes or monocyte-derived macrophages might be changed in peripheral organs in addition to the lung, because presumably, blood monocytes or their precursors would be trained. Inversely, how systemic insults such as sepsis and trauma modify monocyte and macrophage function locally [42] is an important area of research that we do not cover here.

Bacillus Calmette–Guérin (BCG)-induced long-term epigenetic changes were demonstrated in humans, with consequent enhanced innate immune responses to yellow fever vaccination or restimulation with *Candida albicans* in blood monocytes [43,44] concomitant with changes in the hematopoietic progenitor compartment [44]. The hypothesis that these alterations are due to upstream changes induced in BM precursors is also supported by mouse data showing changes in hematopoiesis and the possibility of adoptive transfer of hematopoietic precursors that transfer reactivity [45,46]. In addition, earlier studies have shown an IFN- or IL-12-mediated instructive signal to BM cells during peripheral infection that set up antiviral or regulatory programs, respectively, in nascent monocytes [47,48]. While the underlying mechanisms are yet to be fully understood at the transcriptional and epigenetic levels, it seems clear that murine myeloid progenitors can be trained with BCG or β-glucan exposure, although the potential persistence of these stimuli must be taken into account [44–46]. Whether different types of lung insult can cause reprogramming in the BM to generate trained recruited AMs remains to be determined; if so, whether these are cells trained differently depending on the specific challenge is also unknown. In conclusion, increased immunoreactivity of recruited AMs may sometimes be the result of imprinting along the developmental path from BM precursors to recruited AMs (via blood monocytes). We propose that, in many instances, a simpler explanation is that the specific functionality of recruited

AMs is a remnant of the chromatin landscape and transcriptional profile of the former monocytic identity of these cells.

Maintaining or Losing Monocyte Immunoreactivity in Recruited AMs

If **monocyte legacy** determines the high reactivity of recruited AMs, why, then, do other, less inflammatory interventions to recruit AMs into the lung, such as clodronate liposomes or transfer into GM-CSFR-deficient mice, yield less-reactive recruited AMs [10,11,17,49]? The monocyte legacy model would hypothetically explain this as follows. There is no proinflammatory signal required to imprint high reactivity in monocyte-derived cells: monocytes themselves are highly reactive cells, so this profile simply needs to be maintained. However, the lung will start to exert environmental imprinting on newly recruited cells, and the degree of lung inflammation may make the difference. For example, exposure to tumor necrosis factor (TNF) is known to suppress the LPS-triggered expression of *IL6* and other genes encoding proinflammatory cytokines in human monocyte-derived macrophages [50], but this effect can be abolished by type I IFNs present during TNF priming [51]. IFNs can render cells refractory to a wide range of immunosuppressive signals, such as glucocorticoids, IL-4, and IL-10 [52]. Moreover, IFN-γ and IL-4 can inhibit each other's **polarization** programs [53]. Therefore, while recovering from IVI, the lung may not send the 'immunosedative' signals required to turn off inflammatory programs in recruited cells, or such calming signals may be counterbalanced by activating signals coming from the still-inflamed lung. This might result in a prolonged state of monocyte-like high reactivity in recruited AMs (Figure 2). By contrast, a largely uninflamed lung environment, as found in sterile AM depletion models, might result in faster and more efficient establishment of an 'immunosedated' state in incoming monocytes, relative to those recruited into a highly inflamed lung. At 1 month post-IVI, recruited murine AMs retain a significant epigenetic and transcriptional resemblance to monocytes; after longer periods of lung residence post-IVI, recruited AMs become transcriptionally more similar to resident AMs, suggesting that, given sufficient time, the recovered lung does impose an immunosedated state in the recruited cells [10]. To settle these questions conclusively, BM progenitors, monocytes, and recruited macrophages will have to be compared under different recruitment regimens in careful kinetic studies, paying special attention to the signals given by the lung environment.

What Signals Are Given by the Lung Environment to Establish AM Functionality in Recruited Cells?

If the above monocyte legacy model is correct, incoming monocytes would receive signals derived from structural cells turning them slowly into less-inflammatory cells, focused towards homeostatic functions and much resembling embryonically derived resident AMs. What might these signals be? As murine adult blood monocytes have the capacity to develop into AMs [17], it may be instructive to consider those signals driving the ontogeny of resident AMs as possible candidates to promote the monocyte-to-AM transition. Important cytokines involved in AM development are transforming growth factor beta (TGF-β) and GM-CSF (Figure 3) [54,55]. The absence of or inability to signal via either of them leads in humans and mice to the development of **pulmonary alveolar proteinosis** (PAP), a disease arising when AMs do not develop to fulfill their key maintenance role of clearing surfactant and lipids from the alveolar space [54–59]. TGF-β acts in an autocrine manner, and signaling via the TGF-β receptor upregulates the master transcription factor (TF) peroxisome proliferator-activated receptor gamma (PPAR-γ) in fetal monocytes and is essential for the development of these embryonic precursors as well as BM-derived cells into AMs in mice [54]. GM-CSF is secreted by lung epithelial cells as well as resident macrophages and plays a vital role in inducing murine fetal monocyte development into macrophages via PPAR-γ in a paracrine manner [58]. Downstream of GM-CSF, the TF PU.1 also appears to be important: GM-CSF induces PU.1, while forced expression of

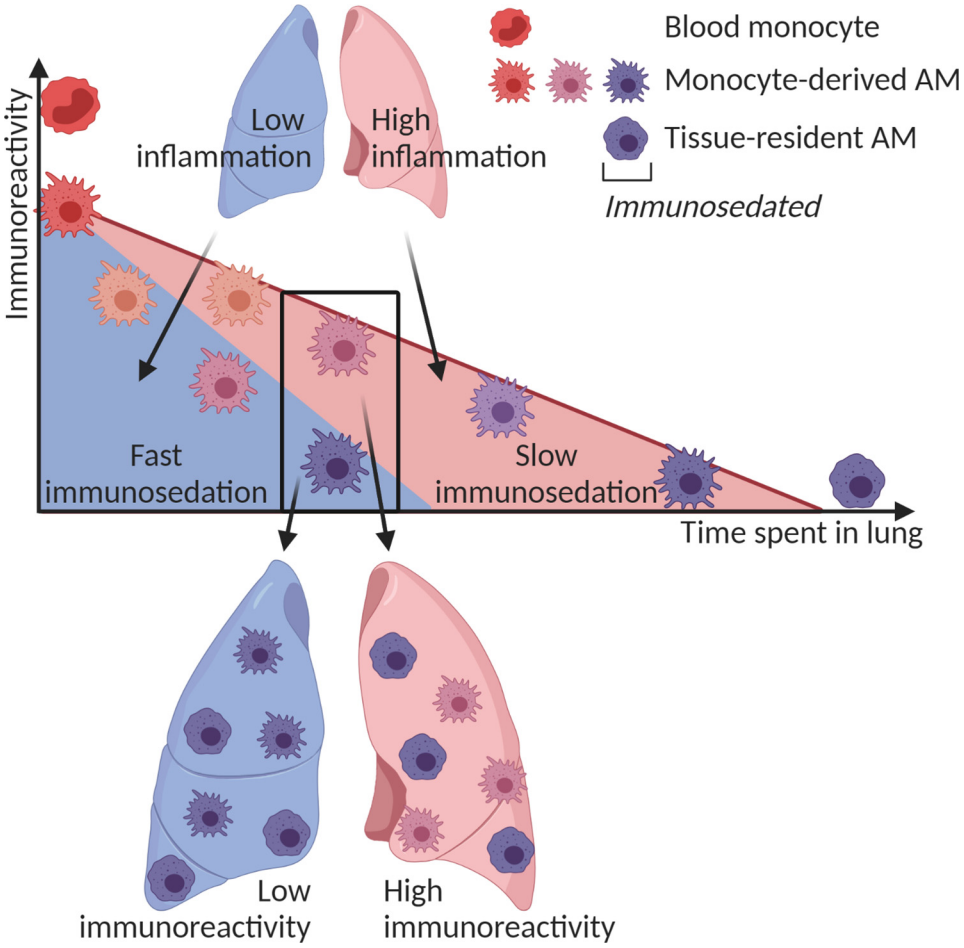

Figure 2. Model of Kinetics of Alveolar Macrophage (AM) Immunosedation in Different Mouse Lung Environments. In this model, the immunoreactivity of monocyte-derived AMs is determined by the lung environment and the duration spent in the lung. After lung insult or depletion of AMs, highly reactive monocytes are recruited into the lung and lose reactivity over time (immunosedation). In a non-inflamed lung (blue), the signals from the environment may be anti-inflammatory and therefore immunosedate these cells fast and efficiently, while activating stimuli are largely absent. In a still-inflamed lung environment (red), both pro- and anti-inflammatory signals may be present, leading to a slower rate of immunosedation. This might explain the comparably low immunoreactivity of monocyte-derived AMs after sterile depletion and the higher immunoreactivity for a longer time period following an inflammatory insult [10–12,49]. This figure was created using BioRender (https://biorender.com/).

PU.1 in GM-CFS-deficient *Csf2*$^{-/-}$ AMs rescues their ability to catabolize surfactants [60]. In the absence of PPAR-γ, murine *Pparg*$^{-/-}$ macrophages exhibit increased proinflammatory and reduced anti-inflammatory phenotypes [61]. PPAR-γ is also highly expressed in adipocytes in mice and regulates a lipid metabolic program in these cells [62]. A similar PPAR-γ-driven metabolic program is likely to promote the key lung maintenance function of AMs by keeping the balance of surfactant via constant removal of these lipid-rich molecules, constitutively produced by lung epithelia.

To summarize, the AM TF signature regulates lipid metabolism and the primary tissue maintenance function of AMs is the degradation of lipid-rich surfactants in humans and mice [57,58,63]. Lung alveoli are not only rich in lipids but also thought to be low in glucose [64],

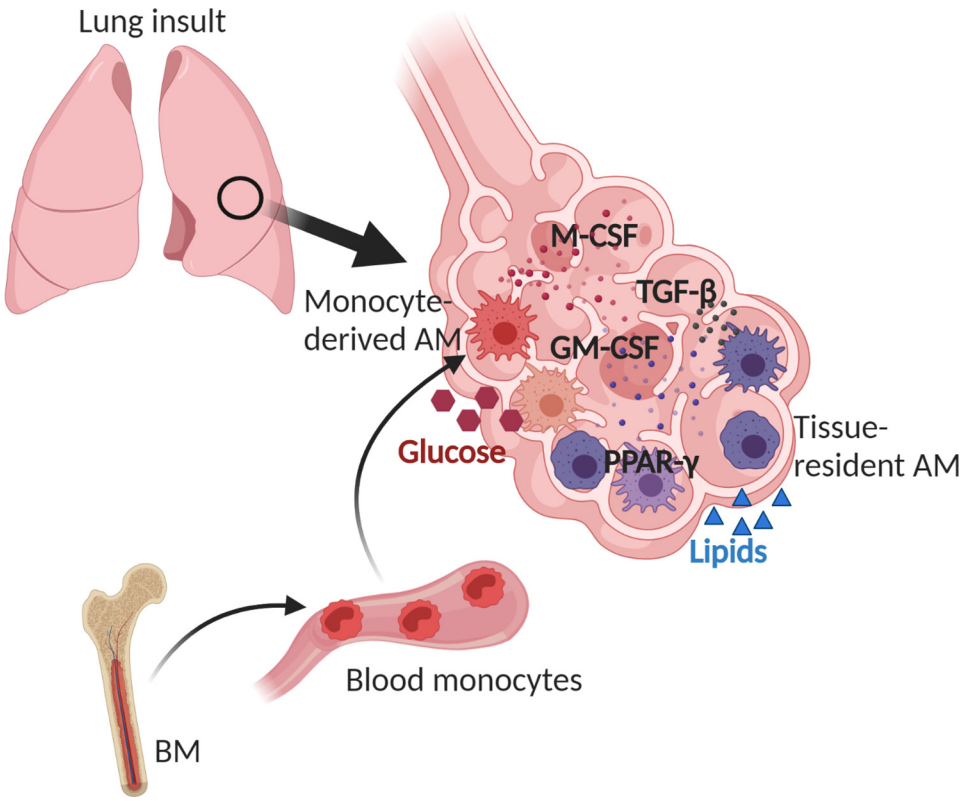

Figure 3. Model of How Signals in the Mouse Lung Shape Newly Recruited Alveolar Macrophages (AMs). The functionality of monocyte-derived AMs can be shaped by various signals in the lung environment, including colony-stimulating factors (CSFs) (GM-CSF and M-CSF) and transforming growth factor beta (TGF-β), acting in a paracrine or autocrine manner. In the lung, monocyte-derived AMs are exposed to different amounts of glucose and fatty acids, which, together with other stimuli, may establish over time the transition from an immunoreactive phenotype initially to the subsequent immunosedation of recruited AMs expressing the signature transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ). This figure was created using BioRender (https://biorender.com/).

suggesting that the low immunoreactivity of AMs may be strongly dictated by the metabolic adaptation to their maintenance role and their environment. Generally, macrophage function is known to be tightly linked to their metabolic profile, with a distinction, largely based on *in vitro* work, between LPS-stimulated **inflammatory macrophages** mostly depending on glycolysis and alternatively activated macrophages employing oxidative phosphorylation to generate energy [65,66]. However, it is becoming increasingly clear that, in addition to increased fatty acid oxidation and oxidative phosphorylation, glycolysis is also essential for the latter phenotype [67]. Glycolysis inhibition studies have highlighted the importance of glucose consumption in determining the alternatively activated phenotype in murine [67,68] and human [69] monocyte-derived macrophages. This fits with the finding that AMs are hyporesponsive to IL-4-induced type 2 inflammation *in vivo* [59]. Of note, removed from their niche and cultured *in vitro*, murine AMs regain responsiveness to IL-4 in a glycolysis-dependent and fatty acid oxidation-independent manner, whereas peritoneal macrophages transferred to the lung become less responsive to IL-4 [59]. Given the now-recognized importance of glycolysis for macrophage functions, combined with the scarcity of glucose in the lung due to active epithelial removal processes [64], the low responsiveness of AMs might be a consequence of their metabolic profile of near-absent glycolysis.

How is metabolism linked with gene expression patterns? A novel link between macrophage transcriptome and metabolism is lactate, a product of glycolysis. The addition of a lactyl group (lactylation) to a lysine residue on histones serves as an epigenetic modification in murine BM-derived macrophages (BMDMs) [70]. The degree of histone lactylation is dependent on the endogenous production rate of lactate via glycolysis, with inflammatory, but not alternatively activated, macrophages having elevated amounts of lactate and increased histone lactylation [70]. Although lowering of lactate concentrations by depletion of lactate dehydrogenase A in BMDMs was shown to be not essential for the inflammatory phenotype, histone lactylation seems to serve as a molecular clock that ensures a late switch from the expression of proinflammatory genes to gene signatures associated with a homeostatic macrophage phenotype [70]. We speculate that similar metabolism-driven epigenetic modifications might contribute to shaping the functional profile of AMs. Presumably, newly recruited monocyte-derived AMs might highly depend on glycolysis, through activation or induced during training [71,72]. Increased glycolysis might set in motion a lactate clock leading to a switch towards a homeostatic phenotype. As lung glucose concentrations are increased in inflammatory conditions and rise transiently during infections [73], recruited cells might initially have access to glucose, which might become less available over the course of recovery. Once settled in the healing lung, where glucose supply is limited [64], recruited AMs would have to downregulate glycolysis, resulting in the cessation of glycolysis-driven histone modifications important for macrophage polarization. Together with a variety of other links between cell metabolism and gene expression (reviewed in [74]), a mechanism as delineated hypothetically here might contribute to the

Box 1. Lessons from the SARS-CoV-2 Infection

Cytokine storm and related immunopathology are hallmarks of severe COVID-19, caused by SARS-CoV-2 infection (reviewed in [79]). Symptoms include profound **lymphopenia** and loss of HLA-DR expression on immune cells [80–83]. Infiltration of inflammatory monocytes and macrophages (IMMs) has been previously reported in fatal cases of SARS [84] and IMMs were shown to be responsible for the increased expression of proinflammatory cytokines, including IL-6 and IL-1β, in the SARS mouse model [85,86]. Single-cell RNA-seq (scRNA-seq) has revealed an altered macrophage composition in the lungs of patients with moderate or severe COVID-19, with a drastically reduced resident AM population and emerging populations expressing monocyte-like markers, but also AMs with a reparative but potentially profibrotic phenotype [77,87]. Also, peripheral blood classical monocytes can express increased amounts of IL-6 and IL-1β [80,88,89]. Therefore, proinflammatory cytokines produced by IMMs might be promising candidate targets for blockade that could hypothetically contribute to the prevention of COVID-19 pathology [80,90,91]. For example, administration of an IL-6-blocking antibody might partially revert immune dysregulation in some COVID-19 patients by increasing circulating lymphocytes, restoring HLA-DR expression on CD14 monocytes [80].

Another potential but complex therapeutic target for SARS-CoV-2 is GM-CSF signaling (reviewed in [92]). On the one hand, increased GM-CSF was reported in the plasma of COVID-19 patients [93], suggesting that IMMs are exposed to proinflammatory GM-CSF produced by peripheral blood T cells and monocytes [94] and might potentially be targeted with GM-CSF inhibitors [79]. On the other hand, GM-CSF can contribute to improvement of epithelial repair in humans [95] and regulate AM differentiation in mice [60]. Therefore, GM-CSF treatment might accelerate the differentiation of monocyte-derived AMs. Because GM-CSF is essential for the repopulation of resident AMs after insults by inducing proliferation [3], one might speculate that this results in the expansion of resident AMs with low immunoreactivity, which in turn might reduce the niche for recruited proinflammatory AMs [94]. Overexpression of GM-CSF post-IVI in an inducible transgenic mouse model resulted in increased survival and reduced expression of proinflammatory genes in AMs [96]. These studies provide a rationale for plans to administer GM-CSF to COVID-19 patients [79,92]. Evidently, robust studies in this regard are still awaited.

In the long term, many patients will have recovered from severe cases of SARS-CoV-2 infection. Within 7 days post-recovery, blood monocytes still express high amounts of inflammatory genes, such as IL-1β, but after 14 days this expression is reduced [88]. However, based on animal studies with various challenges [10,12], we predict that patients who have recovered from COVID-19 might bear a mixed AM population in the lung, which is likely to have a strong component of monocyte-derived AMs recruited during a massive inflammatory episode. In patients with disease, macrophages accumulate in alveolar cavities and are associated with lung lesions [97,98], whereas *in vitro* macrophage co-stimulation with poly I:C and serum from patients with severe COVID-19 induces endothelial barrier disruption [99]. In recovered patients, the role of AMs in short- and long-term lung damage, as well as potentially altered AM phenotypes, should be further investigated and taken into account when treating subsequent diseases.

establishment of the unique immunosedated phenotype of AMs in the long term. This means that homeostatic requirements and **immunosedation** might be intimately linked, opening new potential therapeutic intervention strategies in lung diseases.

Concluding Remarks

To protect its delicate structure and allow gas exchange, the lung is under normal circumstances an immunologically quiet, or immunosedated, site, as not every inhaled particle should trigger inflammation. Non-inflammatory defenses such as the epithelial barrier and the mucus layer are in place for protection. Embryonically derived, long-lived resident AMs are a crucial part of this environment and may be 'terminally sedated', retaining some, but minimal, immunoreactivity. Newly arriving myeloid cells, recruited rapidly on insult, are initially highly immunoreactive due either to their proinflammatory monocyte legacy or to training at various stages of recruitment, as determined in mouse models. Recruited AMs might also become immunosedated over time and develop a functional profile similar to that of embryonically derived AMs, centered around the homeostatic role of lipid catabolism to remove surfactants from the alveoli. This functional change is reflected in alterations to the transcriptional and chromatin landscape. Many questions remain regarding the precise order of events, the signals and TFs at work, and the underlying molecular mechanisms (see Outstanding Questions), but some answers are beginning to emerge. Generally, blood monocytes may be an all-purpose weapon to be deployed in divergent contexts, explaining their unique combination of high immunoreactivity and high plasticity. Monocyte-derived recruited AMs retain this plasticity for some time and therefore may be the macrophages that determine outcomes in circumstances such as cancer, allergy, infections [including IVI and severe acute respiratory syndrome (SARS)-CoV-2 (Box 1)], and chronic lung conditions such as chronic obstructive pulmonary disease (COPD).

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Outstanding Questions

Why do tissue-resident AMs remain unchanged after various lung insults? What is specific to those challenges that do induce changes in resident AMs? Understanding cell-intrinsic or -extrinsic factors and processes that control tissue-resident AM plasticity could allow targeted modulation.

How do short-lived BM-derived monocytes become long-lived, self-sustaining, homeostatic macrophages in the lung? How does the lung environment drive this process? This transition appears to be the reverse of many biological processes and understanding it may be useful when lung homeostasis needs to be re-established.

What are the functions of resident and recruited AMs in resolving a primary lung insult? Are the functions specific, complementary, or overlapping? How are these initial differences reflected during subsequent challenges?

Are the beneficial or detrimental roles of locally recruited AMs merely a reflection of their monocyte origin or does innate training of precursors in BM or blood change macrophage phenotypes systemically? This could have important implications for vaccine strategies and other interventions to reprogram macrophages organism wide.

Under what conditions are monocyte-derived AMs recruited into the human lung? How can we identify resident and monocyte-derived AMs in humans? What is the ratio of resident to recruited AMs in health and disease? Are functional changes specifically in resident or recruited AMs linked to human diseases? Such knowledge may open new avenues for targeted intervention in humans.

Do monocyte-derived AMs accumulate with age across species? If so, are they responsible (or partially responsible) for '**inflammaging**'? If true, this might help to explain why certain diseases disproportionately affect elderly people.

Do the specific metabolic constraints in the lung change AM polarization? Do differently programmed macrophages change their metabolic status? Better

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knowledge might open possibilities for the (re)programming of AM function in disease through metabolic intervention.

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