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## Trained immunity: a program of innate immune memory in health and disease

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### Abstract

The general view that only adaptive immunity can build immunological memory has recently been challenged. In organisms lacking adaptive immunity as well as in mammals, the innate immune system can mount resistance to reinfection, a phenomenon termed *trained immunity* or *innate immune memory*. Trained immunity is orchestrated by epigenetic reprogramming, broadly defined as sustained changes in gene expression and cell physiology that do not involve permanent genetic changes such as mutations and recombination, which are essential for adaptive immunity. The discovery of trained immunity may open the door for novel vaccine approaches, for new therapeutic strategies for the treatment of immune deficiency states, and for modulation of exaggerated inflammation in autoinflammatory diseases.

### Introduction

Host immune responses are classically divided into innate immune responses, which react rapidly and nonspecifically upon encountering a pathogen, and adaptive immune responses, which are slower to develop but are specific (due to antigen receptor gene rearrangements) and result in classical immunological memory. This schematic distinction has been challenged by the discovery of pattern recognition receptors that confer some specificity to the recognition of microorganisms by innate immune cells (1), and by a growing body of

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literature showing that the innate immune system can adapt its function after previous insults (2, 3). Protection against reinfection has been reported not only in plants and invertebrates that do not have adaptive immunity (4), but also in mammals, with old and new studies demonstrating cross-protection between infections with different pathogens (5). These studies have led to the hypothesis that innate immunity can be influenced by previous encounters with pathogens or their products, and this property has been termed *trained immunity* or *innate immune memory*.

Compared to classical immunological memory, trained immunity has a number of defining characteristics. Firstly, it involves a set of cells (myeloid cells, natural killer (NK) cells, innate lymphoid cells (ILCs)) and germline encoded recognition and effector molecules (e.g. pattern recognition receptors, cytokines) different from those involved in classical immunological memory. Secondly, and in contrast to classical immunological memory that depends of gene rearrangement and proliferation of antigen-specific lymphocyte clones, the increased responsiveness to secondary stimuli during trained immunity is not specific for a particular pathogen and it is mediated through signals impinging on transcription factors and epigenetic reprogramming. These are broadly defined as sustained changes in transcription programs through epigenetic rewiring, leading to changes in cell physiology that do not involve permanent genetic changes such as mutations and recombination. Finally, trained immunity relies on an altered functional state of innate immune cells that persists for weeks-to-months, rather than years, after the elimination of the initial stimulus.

In this context, it important to note that some innate immune cells such as NK-cells display both trained immunity characteristics as defined above, as well as antigen-dependent (or even antigen-specific) immunity that is related to the classical immunological memory mediated by T- and B-lymphocytes (see below for detailed description). In addition, it is important to clearly discriminate between trained immunity and other immunological processes such as immune cell activation and immune cell differentiation. During immune cell activation transcription of genes takes place at the time of stimulation in response to a ligand directly acting on the cell. In contrast, during trained immunity innate immune cells display gene- or locus-specific changes in their chromatin profiles induced by a previous stimulation. These changes, however, allow increasing response to restimulation of the cells through both the same and different PRRs.. The discrimination between trained immunity and immune cell differentiation is more difficult, and to a certain degree is even semantic: one could argue that macrophage differentiation could also be considered an example of trained immunity. However, immune cell differentiation can (and does) occur also during homeostatic conditions, while trained immunity is defined as a reaction to a foreign insult. In addition, while the term “circulating differentiated monocyte” could also be used instead of “trained monocyte”, we believe that this may be confusing, as monocyte differentiation is generally considered equivalent to the process through which blood monocytes differentiate into macrophages in the tissues. Moreover, differentiated cells such as macrophages can be trained as well (e.g. after infection or vaccination), and thus their capacity to display increased function should be defined differently than cell differentiation.

Defining the properties of trained immunity will critically integrate our understanding of host defense, and in this review we will describe the concept as well as discuss recent data

that support its important role in health and disease. We will not review classical immunological memory, as this is the subject of many excellent reviews.

## Immunological memory in plants and invertebrate animals

A first line of evidence that innate immune system has the capacity to build memory to previous insults comes from a plethora of immunological studies in plants. Collectively, they provide compelling evidence of the capacity to respond more efficiently to reinfection, a phenomenon termed *Systemic Acquired Resistance* (SAR) (6). The molecular mechanisms and biochemical mediators of SAR are largely known (6), with epigenetic-based rewiring of host defense playing a central role (7). In addition, there is increasing evidence to suggest that innate immunity displays memory traits, not only in plants, but also in invertebrate animals (4). For example the microbiota has been shown to induce innate immune memory to protect mosquitoes against *Plasmodium* (8); the social insect *Bombus terrestris* displays innate immune memory against three different pathogens (9), and the tapeworm *Schistocephalus solidus* induces memory in the copepod crustacean (10): in these models the organism is protected against re-encounter with the pathogen by an improved clearance of the infection. It is therefore reasonable to conclude that immunological memory is found not only in vertebrates, but also in plants and lower animals (3, 4).

Several mechanisms have been proposed to account for innate immune memory in invertebrates, including the sustained up-regulation of immune regulatory pathways such as the Toll and Imd receptors on the haematocytes (11) or of the bacterial peptidoglycan recognition molecules and lectins (12), and quantitative and phenotypic changes in immune cell populations (8). Alternatively, memory may be due to the presence of diversity-generating mechanisms in insects, such as generation of variation in fibrinogen-related proteins (likely acting as pathogen sensors) with high rates of diversification at the genomic level through point mutations and recombinatorial processes (13). The Toll-like receptors (TLRs), that are the animal counterpart of Toll in *Drosophila*, also show great diversity in the sea urchin, which have an estimated 222 receptors (14).

## Innate immune memory in vertebrates

The presence of memory characteristics in innate host defense of different plant and animal lineages suggests that innate immune memory may also be present in vertebrates. Important clues that vertebrate innate immunity also has adaptive characteristics came from experimental studies in mice showing that priming (or training) of mice with microbial ligands of pattern recognition receptors can protect against a subsequent lethal infection. For example, trained immunity induced by  $\beta$ -glucan (a polysaccharide component of mainly fungal cell walls) induces protection against infection with *Staphylococcus aureus* (15, 16). Similarly, the peptidoglycan component muramyl dipeptide induces protection against *Toxoplasma* (17), and prophylactic treatment with TLR9 agonists such as oligodeoxynucleotides containing unmethylated CpG dinucleotides three days before the infection protects against sepsis and *Escherichia coli* meningitis (18). Furthermore, flagellin can induce protection against *S. pneumonia* (19) and rotavirus (20), the latter being independent of adaptive immunity and induced by dendritic cell-derived interleukin (IL)-18,

which in turn drives production of IL-22 by epithelial cells. In addition to microbial ligands, there is evidence that certain proinflammatory cytokines may induce trained immunity: injection of mice with one dose of recombinant IL-1 three days before infection with *Pseudomonas aeruginosa* protected the mice against mortality (21). The nonspecific character of the trained immunity argues against a classical immunological memory effect and suggests instead the activation of nonspecific innate immune mechanisms.

Compelling evidence that trained immunity is induced in vertebrates and mediates at least some of the protective effects of vaccination came from studies showing that immunization of mice with bacillus Calmette Guerin (BCG, the tuberculosis vaccine that is also the most commonly used vaccine worldwide), induces T cell-independent protection against secondary infections with *Candida albicans* or *Schistosoma mansoni* (22, 23). The hypothesis that trained immunity can be elicited in vertebrates is further supported by studies investigating the mechanism of protection against disseminated candidiasis conferred by attenuated strains of *C. albicans*. For example, when an attenuated PCA-2 strain of *C. albicans* that is incapable of germination is injected in mice, protection is induced against the virulent strain CA-6 (24). Importantly, this protection was also induced in athymic mice and *Rag1*-deficient animals (that cannot rearrange their antigen receptors), demonstrating a lymphocyte-independent mechanism (25, 26). The protection against reinfection was instead dependent on macrophages (24) and proinflammatory cytokine production (27), both prototypical innate immune components.

In addition to BCG and *C. albicans*, some viral and parasitic organisms can exert protective effects through mechanisms independent of adaptive immunity. Herpes virus latency increases resistance to the bacterial pathogens *Listeria monocytogenes* and *Yersinia pestis* (28), with protection achieved through enhanced production of the cytokine interferon (IFN) $\gamma$  and systemic activation of macrophages. Similarly, infection with the helminth parasite *Nippostrongylus brasiliensis* induces a long-term macrophage phenotype that on the one hand damages the parasite and on the other induces T and B lymphocyte-independent protection from reinfection (29).

Other studies have shown that natural killer (NK) cells also display immune memory. This was first demonstrated in mice that showed hapten-induced contact hypersensitivity dependent on NK-cells that persisted for at least 4 weeks (30). Consistent with this notion, several subsequent studies reported that infection with murine cytomegalovirus (mCMV) induces immunological memory independent of T- and B-cells (31-34). The protection in these models is mediated by NK-cells, which proliferate and persist in lymphoid and non-lymphoid organs. Upon reinfection, these “memory” NK cells undergo a secondary expansion, rapidly degranulating and releasing cytokines, thus inducing a protective immune response (31). Interestingly, it has also been shown that NK cells can prime monocytes in the bone marrow during infection, and this may also induce long-term effects on innate immune responses (35).

In addition to experimental studies showing induction of innate immune memory in mice, emerging data suggest that similar trained immunity effects can be generated in humans. Firstly, a large number of epidemiological studies have shown nonspecific beneficial effects

of live vaccines such as BCG, measles vaccines, and oral polio vaccine against infections other than the target diseases (36). The identification of these nonspecific (or heterologous) effects suggests that these vaccines induce trained immunity that protects against unrelated pathogens. This hypothesis was proposed in proof-of-principle trials with BCG vaccine in healthy adult volunteers (37), and thereafter validated in clinical trials in newborn children vaccinated with BCG (38) or exposed *in utero* to hepatitis B vaccine (39). Secondly, certain infections such as malaria can also induce a state of hyper-responsiveness that is functionally equivalent to the induction of trained immunity (40, 41). Finally, non-specific protective effects through innate immunity-dependent mechanisms are provided by the use of BCG for treatment of malignancies such as bladder cancer (42), melanoma (43), leukemia (44), and lymphoma (45): although direct inflammatory effects are likely important, long-term innate immune memory persisting between the BCG treatments is also likely involved. In this respect, it has been recently suggested that these anti-cancer effects of BCG are directly dependent on the capacity to mount trained immunity, as individuals unable to mount trained immunity due to autophagy defects show a diminished recurrence-free survival after BCG treatment in bladder carcinoma (46).

Taken together, these complementary murine and human studies suggest that innate immune responses have the capacity to be “primed” or “trained,” and thereby exert a new type of immunological memory upon re-infection, for which the term *trained immunity* has been proposed (Figure 1).

## Mechanisms responsible for mediating trained immunity

### Innate immune cells that build innate immune memory

Innate immune memory properties have been described in several cell populations including monocytes/macrophages and NK cells, while preliminary observations suggest that similar characteristics may also be present in other cell types such as innate lymphoid cells (ILCs) or polymorphonuclear leukocytes. Unlike lymphocytes, innate immune cells do not express rearranging antigen receptor genes, but they do express pattern recognition receptors (PRRs) and other receptors that allow them to recognize and respond to pathogen-derived structures (pathogen-associated molecular patterns, PAMPs) and endogenous danger signals (damage-associated molecular patterns, DAMPs) (47, 48). Although these responses are not specific to the degree conferred by antigen receptors, there is evidence that expression of distinct members of pattern recognition receptor families (e.g., Toll-like receptors, NOD-like receptors, C-type lectin receptors, RIG-I-like receptors, or combinations thereof) on macrophages and dendritic cells does indeed trigger different signaling pathways that lead to innate immune responses that are tailored on the type of pathogen encountered (49).

Among the various cell types implicated in innate immune memory, the major focus has been on monocytes/macrophages and NK cells. We note that this attention does not necessarily mean that these cells are more amenable to training than other innate immune cells (or conceivably other somatic cell types). Instead, this focus may merely reflect historical connection of these cells with lipopolysaccharide (LPS)-induced tolerance (LPS is a component of Gram-negative bacterial cell walls). Indeed, some of the first evidence that macrophages may exhibit memory-like features came from investigations of LPS-induced

tolerance at the molecular level (50). In one such study, gene-specific chromatin modifications were associated with silencing of genes coding for inflammatory molecules, while priming other genes coding for antimicrobial molecules (50). These findings suggested that macrophages could be primed by LPS to become more or less responsive to subsequent activation signals. This observation was expanded by studies that demonstrated that exposure of monocytes/macrophages to *C. albicans* or  $\beta$ -glucan enhanced their subsequent response to stimulation with unrelated pathogens or PAMPs, process termed as trained immunity (26). Training was demonstrated to be accompanied by significant reprogramming of chromatin marks (26, 51) (50), as detailed further below. Besides bacterial and fungal pathogens, monocytes/macrophages can also mount trained immunity responses following infection with parasitic (29) and viral (28) pathogens.

An important aspect to be considered regarding trained immunity in monocytes is the lifespan of these cells. Monocytes are cells with a short half-life in circulation, with recent studies suggesting it to be up to one day (52). The observation that trained monocytes have been identified in the circulation of BCG-vaccinated individuals for at least three months after vaccination (37) suggests that reprogramming must take place at the level of progenitor cells in the bone marrow as well. Indeed, recent evidence has emerged that innate immune memory can be transferred via hematopoietic stem and progenitor cells. Macrophages derived from hematopoietic stem and progenitor cells rendered tolerant by TLR2 ligand exposure and transferred to irradiated mice retain a tolerant phenotype and produce lower amounts of inflammatory cytokines and reactive oxygen species in response to inflammatory stimulation (53). Furthermore, exposure of the skin of mice to UV radiation induces immunosuppression that was originally attributed to defective T-cell priming by dendritic cells (54), but was subsequently shown to involve epigenetic reprogramming and a long-lasting effect on dendritic cell progenitors in the bone marrow that altered the function of their differentiated progeny (55). In addition, recent studies have suggested that microbiota can induce long-term functional reprogramming of bone marrow progenitors, and subsequently dendritic cells, to induce protection against *Entamoeba histolytica* (56). Whether vaccines know to induce trained immunity, such as BCG, can also confer or induce similar effects at the level of progenitor cells remains to be established.

Emerging evidence suggests that NK cells also respond more vigorously after a previous challenge. NK cell memory has been documented following exposure to cytokine combinations (e.g., IL-12, IL-15, and IL-18) (32) or hapten sensitization, which induced long-lived NK cells that mediate contact hypersensitivity and long-lived antigen-specific recall responses independently of B and T cells (30). In addition, NK cells undergo expansion during virus infections such as those with murine CMV (31), influenza A (57), or vaccinia virus (58). Studies of CMV infection have shown that NK cell activation may provide T cell-independent protection against reinfection by rapidly degranulating and producing cytokines (31). Furthermore, adoptive transfer experiments have demonstrated that activated NK cells can proliferate *in vivo* and protect naïve recipient mice against virus infection, suggesting that they could confer protective immunological memory. The nonspecific protective effects of BCG infection have also been linked with activation of NK cells. NK cells from BCG-vaccinated individuals have enhanced pro-inflammatory cytokine production in response to mycobacteria and other unrelated pathogens, and studies in mice

have shown that BCG confers nonspecific protection against *C. albicans* at least partially through NK-cells (59).

A number of mechanisms have been put forward that may mediate the memory properties of NK cells: some of them are responsible for induction of innate immune memory, and others for the survival of the NK memory cells. The former include enhanced responsiveness of the IL-12/IFN $\gamma$  axis (32) or the activation of the co-stimulatory molecule DNAM-1 (DNAX accessory molecule-1, CD226) on the membrane of the cells (60), while survival of the memory NK cells during the contraction phase after murine CMV infection necessitates mitophagy through an *Atg3*-dependent mechanism (61). The issue of specificity of the NK memory immune responses is complex. Evidence that NK memory is specific was provided by the demonstration that in the mouse mCMV-induced NK cells protected against mCMV but not Epstein-Barr virus, another herpesvirus (62). Interestingly, mCMV impaired heterologous immunity against influenza and *L. monocytogenes* (57, 63). Memory responses of NK cells towards other stimuli such as haptens and viruses also induced antigen-specific immune memory (41). Another important aspect concerns the mechanisms responsible for the persistence of NK memory cells. NK cell memory of haptens and viruses depends on CXCR6, a chemokine receptor on hepatic NK cells that is required for the persistence of memory NK cells but not for antigen recognition (41). In addition, recent studies revealed evidence of NK memory in primates: splenic and hepatic NK cells from Adenovirus 26-vaccinated macaques efficiently lysed antigen-matched but not antigen-mismatched targets five years after vaccination. These data demonstrate that robust, durable, antigen-specific NK cell memory can be induced in primates after both infection and vaccination. This finding has important implications for the development of vaccines against HIV-1 and other pathogens (64).

In addition to studies showing antigen-specific mechanisms of NK cell immune memory, other recent studies have suggested that memory in NK cells is also mediated by epigenetic changes. In a study in patients recovering from CMV virus infection, the DNA methylation patterns of NK-cells and cytotoxic T-cells were similar, and very different from those of canonical NK cells. Subsequently, the capacity of these adaptive NK cells to secrete cytokines was modulated and this was dependent on the transcription factor promyelocytic leukemia zinc finger (PLZF) (34, 65). Similarly, another study showed that these “memory-like NK cells” are defective in the Syk-dependent stimulation pathway, which is correlated with epigenetic changes at the level of the gene promoter (60).

Taken together, the published data suggest that NK immune memory is complex and may display aspects of both antigen-dependent (and in certain circumstances antigen-specific) memory, as well as epigenetic reprogramming as seen in trained immunity.

### **The molecular basis of trained immunity: transcriptional and epigenetic reprogramming**

A distinguishing feature of the trained innate immune cell is its ability to mount a qualitatively different – and to some extent quantitatively stronger – transcriptional response compared to untrained cells when challenged with pathogen or danger signals. The molecular bases of such enhanced activation of a subset of inflammatory genes are only partially defined, but evidence supports the convergence of multiple regulatory layers,

including changes in chromatin organization and the persistence of microRNAs (miRNAs) induced by the primary stimulus.

In myeloid cells, many loci encoding inflammatory genes are in a repressed configuration (66-68), as inferred by their limited accessibility to nucleases (used as tools to probe chromatin structure), the low acetylation of the nucleosomal histones, and the very low amount of RNA polymerase II loaded onto both the coding body of the genes and the genomic regulatory elements (enhancers and promoters) that control their expression (69). Upon primary stimulation, the changes observed at these loci, in terms of gain in chromatin accessibility, increased histone acetylation and RNA polymerase II recruitment, are massive and of magnitudes that are uncommonly observed in other responses to micro-environmental changes. These significant alterations, which in some cases result in the activation of gene expression hundreds of times higher than baseline in a short window of time, are driven by the recruitment of stimulation-responsive transcription factors (e.g., NF- $\kappa$ B, AP-1, and STAT family members) to enhancers and gene promoters, which are usually pre-marked by lineage-determining transcription factors such as PU.1 (70-73). In turn, transcription factors control the recruitment of coactivators (including histone acetyltransferases and chromatin remodelers) (67, 68) that locally modify chromatin to make it more accessible to transcriptional machinery.

Maintenance of such enhanced accessibility may underlie the more efficient induction of genes primed by the initial stimulation (50). Moreover, since histone modifications are specifically bound by recognition domains contained in various proteins implicated in transcriptional control (as in the case of the bromodomain-acetyl lysine interaction) (74), the persistence of histone modifications deposited at promoters or enhancers after the initial stimulus may itself impact the secondary response (26). The possible contribution of chromatin modifications to trained immunity must be examined taking into account the different stability of individual covalent chromatin modifications, with more stable modifications (e.g., histone methylation) being potentially more suitable to perpetuate a functional change than those with a typically short half-life (e.g., histone acetylation). Therefore, the observed long-term persistence of some histone modifications in myeloid cells after removal of the initial activation stimulus may reflect either their stability or, alternatively, the sustained activation of the upstream signaling pathways and transcription factors that control their deposition.

One interesting paradigm is provided by latent or *de novo* enhancers (75, 76), which are genomic regulatory elements that are epigenetically unmarked or marked at low levels in unstimulated cells but gain histone modifications characteristic of enhancers (such as monomethylation of histone H3 at K4, H3K4me1) only in response to specific stimuli. *In vitro*, upon removal of the stimulus that triggered their functionalization, a fraction of latent enhancers retain their modified histones and can undergo a stronger activation in response to restimulation (Figure 2). This observation is reminiscent of the fact that *in vivo* macrophages acquire repertoires of active enhancers that are largely instructed by the micro-environmental signals specific to a given tissue, and are thus to a large extent different depending on the organ in which a macrophage is located (77, 78). In turn, such signals act by specifically inducing regulation by distinct combinations of transcription factors eventually responsible



for the activation of different sets of genes mediated by epigenetic modifying enzymes. Transferring macrophages from one tissue to another results in an extensive reprogramming of the enhancer repertoire (78). Therefore, a complex equilibrium exists between mechanisms that promote the persistence of the modified epigenome instructed by the previously encountered stimuli, and mechanisms that reprogram it in response to a changing environment. The very same dynamic equilibrium likely underlies the persistence of chromatin states that are relevant to enhanced transcriptional responses in trained immunity.

Recent studies have investigated the changes in epigenomic programs in innate immune cells during induction of trained immunity. One early study proposed that changes in epigenetic status underlie the repression of inflammatory genes during LPS tolerance, however genes mainly involved in antimicrobial responses were either normally produced or even displayed an increased production capacity (50). The repression of inflammatory mediators production and the potentiation of antimicrobial proteins synthesis were accompanied by histone repressive or activating marks, respectively. Similarly, exposure of monocytes/macrophages to *C. albicans* or  $\beta$ -glucan modulated their subsequent response to stimulation with unrelated pathogens or PAMPs, and the changed functional landscape of the trained monocytes was accompanied by epigenetic reprogramming (26, 51). Pathway analysis identified important immunological (cAMP-PKA activation) and metabolic (aerobic glycolysis) pathways that play crucial roles in induction of trained immunity (51, 79). In addition, a recent study showed that both LPS and  $\beta$ -glucan induce trained immunity through a MAPK-dependent pathway that phosphorylates the transcription factor ATF7, subsequently reducing the repressive histone mark H3K9me2 (80). Moreover, the immunological networks activated in trained monocytes depend on STAT1 activation (80), and the importance of STAT1 for the induction of trained immunity is supported by the defects in trained immunity reported in patients with chronic mucocutaneous candidiasis due to *STAT1* mutations (81).

BCG vaccination has also been shown to result in an increase in inflammatory mediators produced by monocytes from healthy volunteers, which correlated with parallel changes in a histone modification associated with gene activation (37). Similarly to monocytes and macrophages, the induction of CMV-induced NK cell memory at least partially relies on epigenetic reprogramming, which is linked to reduced expression of PLZF (34) and the tyrosine kinase SYK (65). Finally, human CMV also drives epigenetic priming of the *IFNG* locus in NK cells, which 'tags' the gene and leads to consistent IFN $\gamma$  production in a subset of NK cells, providing a molecular basis for the adaptive feature of these cells (82). Finally, we note that the epigenetic machinery of the immune system may also be hijacked by certain bacterial pathogens such as *L. monocytogenes* (83), and this may represent a more general escape mechanism from host defense (84, 85).

miRNAs may also contribute to trained immunity (86), mainly because of the reportedly long half-life of these molecules (87) that, combined with the limited proliferative ability of myeloid cells, would result in their persistence after removal of the primary stimulus. Among miRNAs, miR-155 may have particular relevance because its up-regulation in response to inflammatory signals such as microbial components is associated with the hyperactivation of myeloid cells, possibly due to the derepression of phosphatases that negatively regulate transducers of several signaling pathways (88). It can be predicted that

myeloid cells expressing miR-155 in a sustained manner would remain in a primed, hyper-sensitive state: upon exposure to a secondary stimulus of identical strength, they would be able to respond in an enhanced manner compared to the primary stimulation.

While the discussion above addresses the role of epigenetic programming as a mechanism for mediating innate immune memory, one crucial aspect remains open: what cellular processes induce and maintain these epigenetic changes? There is increasing evidence to suggest that rewiring of cellular metabolism is involved, with a role for metabolites as cofactors for enzymes involved in epigenetic modulation of gene transcription.

### **Immunometabolic circuits: the role of cellular metabolites for shaping the epigenetic program of trained innate immune cells**

Recent work revealed extensive rewiring of metabolic pathways in different immune cells upon activation (89). The best example concerns macrophages, where the M1 phenotype (i.e., macrophages activated with LPS and IFN $\gamma$ , producing mainly inflammatory cytokines) and M2 phenotype (macrophages activated by IL-4-related cytokines and expressing genes involved in tissue repair) use distinct metabolic pathways (90, 91). M1 macrophages are largely glycolytic, with impairment of oxidative phosphorylation and disruption of the Krebs cycle at two steps, after citrate and after succinate. Citrate is withdrawn for fatty acid biosynthesis (which enables the increased production of inflammatory prostaglandins), whereas succinate activates the transcription factor HIF1 $\alpha$ , which regulates a wide range of genes, including the one encoding the inflammatory mediator IL-1 $\beta$  (90, 91). In M2 macrophages, the Krebs cycle is intact; a key feature is the synthesis of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) from glucose and glutamine, which is needed for the extensive glycosylation occurring in receptors such as mannose-binding lectin, which are hallmarks of the M2 phenotype (91).

The importance of cellular metabolism for macrophage programming suggests that similar mechanisms may play a role for the long-term functional changes in monocytes and macrophages during trained immunity. In line with this, an important role for a shift from oxidative phosphorylation towards glycolysis through an Akt/mTOR/HIF-1 $\alpha$ -dependent pathway has been recently reported to be essential for trained immunity induced by  $\beta$ -glucan (51, 79). Whether and how this shift influences epigenetic processes in trained immunity is still under investigation, but important clues have been given by studies linking chromatin regulation to intermediary metabolism (for reviews see (92, 93). In this respect, a critical metabolic intermediate that is increased in trained monocytes, acetyl-CoA, is required for histone acetylation. In addition, the ratio of the TCA cycle metabolites  $\alpha$ -ketoglutarate and succinate is a critical determinant for the activity of two families of enzymes controlling epigenetic modifications, the JMJ (Jumonji domain-containing) family of lysine demethylases and the TET (ten-eleven translocation) family of methyl-cytosine hydroxylases (51, 94). These enzymes require  $\alpha$ -ketoglutarate as a cofactor, whereas succinate limits their activity (Figure 3). An additional possibility for innate immune memory may be that stimulation of macrophages causes an elevation in succinate; this would then inhibit JMJD3, leading to enhanced H3K27 trimethylation of particular genes (e.g., those associated with the M2 phenotype), suppressing their expression (95). This

process would maintain a proinflammatory phenotype of trained macrophages upon restimulation. Important links between altered metabolites and epigenetic changes have been also demonstrated in LPS-induced tolerance, in which NAD<sup>+</sup>-dependent activation of class III histone deacetylases (sirtuins) functions with sirtuin-1 and sirtuin-6 in coordinating a switch from glucose to fatty acid oxidation (96). The challenges to the field are on the one hand to explain how these potentially non-specific functions of metabolites could have locus/gene-specific effects, and on the other hand to provide direct evidence for metabolites altering the activity of enzymes that modify DNA and histones during trained immunity.

## Adaptive and maladaptive programs

As described above, trained immunity most likely evolved as a primitive form of immune memory, aimed to provide improved protection of the host against reinfection, with beneficial effects for survival. It is also likely that trained immunity plays an important role in ontogeny, enabling the maturation of the innate immune system of the newborn (97), a process in which microbiota plays an important role (98). In line with the notion that microbiota might influence the functional program of immune cells, a recent study showed increased H3K4me3 in NK cells from conventionally housed mice compared with germ-free animals (99). However, there may also be situations in which reprogramming of innate immunity and increased inflammatory responses to exogenous or endogenous stimuli may also have deleterious effects.

Several pathological conditions have been described in which innate immune reprogramming may have deleterious effects. During LPS-induced tolerance, reprogramming of innate immune cells likely plays a beneficial role in maintaining a relatively high threshold of cellular activation in organs in which LPS naturally occurs at physiological levels, such as in the gastrointestinal tract (50). In contrast, in the case of systemic activation of innate immune cells during sepsis, LPS-induced tolerance can contribute to immune paralysis, placing the individual at greater risk for opportunistic infections (100). Persistent silencing of important host defense genes, possibly due to epigenetic mechanisms, has been proposed to mediate these effects (101, 102). Hence, maladaptive responses that inappropriately affect cell populations such as systemic monocytes, as opposed to local tissue-resident macrophages, can have detrimental effects for the host.

There are also examples of deleterious systemic consequences of trained immunity. In general, trained immunity is an adaptive response resulting in the long-lasting capacity to respond more strongly to stimuli (36). While this type of high-alert immune state has beneficial effects during host defense, it could also trigger enhanced tissue damage during chronic inflammatory conditions in which trained immunity is induced by endogenous ligands of innate receptors. For example, there is strong epidemiological evidence for an increased susceptibility of atherosclerosis in patients with autoimmunity or chronic inflammatory conditions such as rheumatoid arthritis (103). It is tempting to speculate that the maladaptive state of innate immune cells triggered by the underlying chronic inflammatory condition would change the local immune responsiveness of immune cells in atherosclerotic lesions and that this could contribute to the increased disease risk (104). It is

also possible that Western-type diets, which are known to trigger systemic inflammatory responses, can precipitate maladaptive trained immune responses. A strong argument for this hypothesis is the recent demonstration of trained immunity induced by oxidized LDL in human monocytes *via* epigenetic reprogramming (105). Furthermore, this type of maladaptation of innate immune cells could be a culprit for other common inflammatory diseases prevalent in Western societies such as type 2 diabetes or Alzheimer's disease. In diabetes, a bout of hyperglycemia can result in long-term deleterious effects, a process termed "hyperglycemic memory": this condition is accompanied by sustained NF- $\kappa$ B activation by increased H3K4me1 and decreased H3K9me3 at selected genes (106).

The data presented above argue that the adaptive ability of innate immune cells to tune their responses to changing environments appears to be an important feature that evolved to prepare innate immune cells for unpredictable events, such as invading pathogens. However, the epigenetic mechanisms that control the memory of the environmental trigger may also lead to persistence of disease-associated phenotypes. Hence, altering the changed epigenetic landscape by pharmacologic means or behavioral changes could be a promising strategy to restore homeostatic healthy gene expression patterns.

### **Trained immunity: a modified steady-state of innate immunity after infection**

In this review, we reappraised the various arguments pointing to the presence of innate immune memory in plants, lower animals as well as in vertebrates. We defined trained immunity as a non-specific immunological memory resulting from rewiring the epigenetic program and the functional state of the innate immune system, eventually resulting in protection against secondary infections. We also compared data assessing the mechanisms of tolerance and trained immunity. However, one important question remains: are tolerance and training two fundamentally opposing functional programs, or do they represent different facets of the same phenomenon?

When one considers the traditional appraisal of the effects of tolerance as a hypo-inflammatory state, and trained immunity resulting in an increased production of proinflammatory cytokines, these two programs may seem to be functional opposites. However, one needs to consider the evidence carefully: whole-genome transcriptional and epigenetic analysis has clearly demonstrated that while in the process of LPS-induced tolerance many proinflammatory genes are downregulated, others are not modified or even upregulated (50). Similarly, the assessment of the trained immunity program induced by  $\beta$ -glucans also shows that it contains both up- and down-regulated genes (51). It thus becomes evident that both tolerance and training represent manifestations of long-term epigenetic reprogramming of the innate immune system after encountering an infection or a microbial ligand.

A crucial aspect of trained immunity that needs further investigation is its duration. *In vitro* studies have demonstrated long-term memory effects in monocytes and macrophages lasting days (26, 75), while experimental studies reported effects that extended to weeks (26, 107). Epidemiological studies on the non-specific effects of vaccines such as BCG or measles have suggested positive effects on susceptibility to infections lasting for months and even

years (36), although it is highly unlikely for this protection to be as long-lived as classical immunological memory. These data are supported by proof-of-principle studies demonstrating the presence of trained immunity effects on circulating monocytes of volunteers for three months and even one year after vaccination with BCG (108). This would imply effects of vaccination on bone marrow progenitors as well, as pointed out earlier. More studies are warranted to describe in more detail the duration of trained immunity effects after infection and vaccination.

## Conclusions and future directions for research

The arguments presented above suggest that trained immunity is a fundamental property of host defense in mammalian immune response. Whereas classical immunological memory mediated by T and B lymphocytes is specific and antigen-dependent, with antigen specificity being mediated by gene rearrangement in specific lymphocyte clones that undergo expansion and contraction, trained immunity (innate immune memory) is non-specific and mediated through epigenetic reprogramming in myeloid cells or NK-cells. An important difference between classical immunological memory and trained immunity also concerns the persistence of the effects: memory within trained immunity has a shorter duration than classical adaptive immune memory.

Much remains to be learned in this exciting new field of immunology in the coming years. Firstly, the molecular mechanisms that mediate trained immunity should be elucidated at the level of the cell types involved, and the immunological, metabolic and epigenetic processes mediating it need to be unraveled further. It will be also important to delineate the duration of innate immune memory and the impact on the innate immune cell precursors in the bone marrow and tissue macrophage populations. Secondly, the fast progress of cutting edge technologies such as single cell transcriptomics and epigenomics, in particular DNA methylation, will permit the identification of the potential novel subpopulations of cells that are prone to display innate immune memory characteristics. This will enhance our understanding of immunological processes and open up possibilities for new therapeutics that target specific cell subpopulations. Thirdly, future research should explore the impact of trained immunity on disease: on the one hand, its role in diseases with impaired host defense such as post-sepsis immune paralysis or cancers, and on the other hand, its role in autoinflammatory and autoimmune diseases in which maladaptive programs may be in place.

Finally, the concept of innate immune memory has considerable potential in helping the design of novel therapeutic approaches, with at least three potential lines of investigation: (1) the design of new generation vaccines that combine adaptive and innate immune memory, as recently proposed with a novel *Bordetella pertussis* vaccine (109); (2) the use of inducers of trained immunity for the treatment of immune paralysis, such as the muramyl dipeptide preparation mufamurtide for osteosarcoma (110) or beta-glucan in various cancer types (111); and (3) the modulation of the potentially deleterious consequences of trained immunity in autoinflammatory diseases (e.g. the potential use of the recently described iBET inhibitors). Only when this is accomplished will the full potential of the discovery of trained immunity be achieved.

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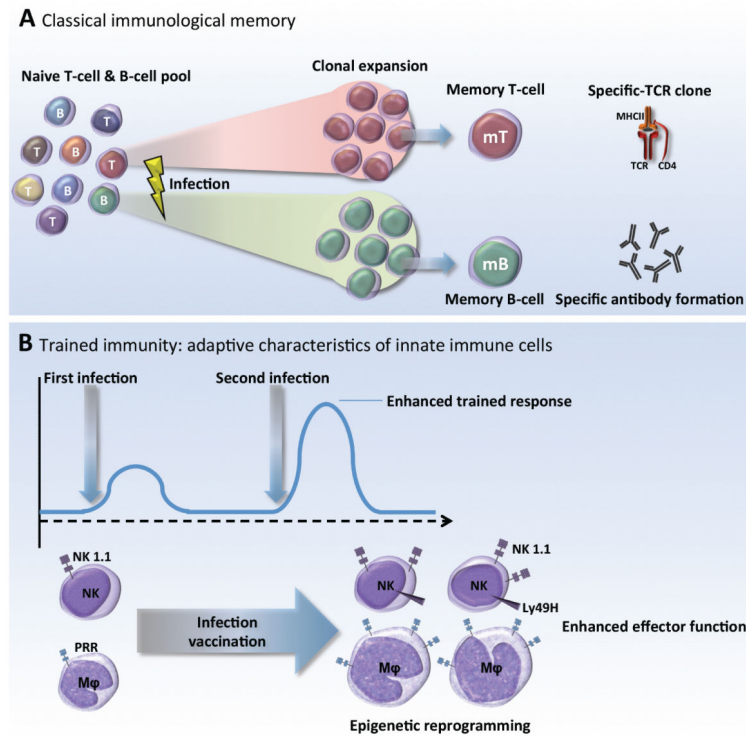
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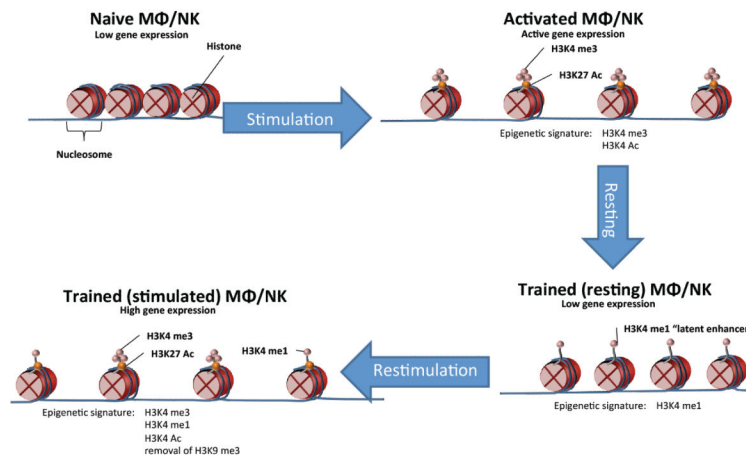
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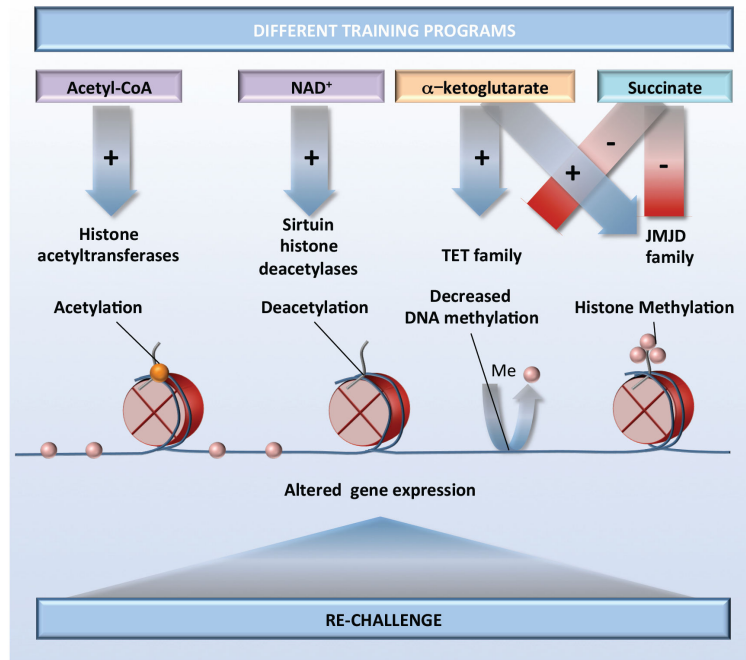
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**Figure 1.** A. Classical adaptive immunological memory involves gene recombination in B- and T-lymphocytes, which confers high specificity and very often long-term, pathogen-specific protection (up to decades). B. Trained immunity defines a *de-facto* innate immune memory that induces enhanced inflammatory and antimicrobial properties in innate immune cells, responsible for an increased non-specific response to subsequent infections and improved survival of the host.



**Figure 2.** Epigenetic rewiring underlies the adaptive characteristics of innate immune cells during trained immunity. Initial activation of gene transcription is accompanied by the acquisition of specific chromatin marks, which are only partially lost after elimination of the stimulus. The enhanced epigenetic status of the innate immune cells, illustrated by the persistence of histone marks such as H3K4me1 characterizing ‘latent enhancers’, results in a stronger response to secondary stimuli upon re-challenge.



**Figure 3.** Stimulation of innate immune cells with training stimuli induces changes in cellular metabolism. Various metabolites function as co-factors for epigenetic enzymes, which in turn induce chromatin and DNA modifications, modulate gene transcription and result in different trained immunity programs.

**Table 1**

Overview of innate immune memory mechanisms described for various types of innate immune cells.

Innate immune cell type	Primary challenge	Type of memory	Pathway involved	Mechanism	References
Monocytes - macrophages	LPS	tolerance / trained immunity	TLR4/MAPK-dependent ATF7-dependent	Epigenetic changes: latent enhancers (H3K4me1), other modifications (H3K4me3, H2K27me, H3K9me2)	Foster & Medzhitov (50) Ostuni et al (74) Yoshida et al (79)
Monocytes - macrophages	$\beta$ -glucan, <i>Candida</i> infection	trained immunity	Dectin-1/Raf1/Akt-dependent STAT1-dependent	Epigenetic changes (H3K4me1, H3K4me3, H2K27Ac, H3K9me2), metabolic rewiring	Quintin et al (26) Saeed et al (51) Cheng et al (78) Yoshida et al (79)
NK-cells	Hapten-induced Influenza A Vaccinia virus HIV-1 infection	antigen-specific		CXCR6-dependent NRG2D-dependent	O'Leary et al (30) Paust et al. (57) Gillard et al (58) Reeves et al (63)
NK-cells	CMV infection	antigen-dependent	Atg3-mediated mitophagy	BNIP3-/BNIP3L-dependent	Sun et al (31) O'Sullivan et al (59)
NK-cells	CMV infection	trained immunity	Stable downregulation of adaptors and transcription factors (e.g. Syk, PLZF)	Epigenetic modification of gene promoters DNA methylation	Lee et al (64) Schlums et al (34)