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Review

Extralymphocytic Flexible Immune Recognition: a New Angle on Inflammation and Aging

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ABSTRACT: Longstanding immunological dogma holds that flexible immune recognition, which forms the mechanistic basis of adaptive immunity, is strictly confined to the lymphocyte lineage. In higher vertebrates, flexible immune recognition is represented by recombinatorial antigen receptors of enormous diversity known as immunoglobulins, expressed by B lymphocytes, and the T cell receptor (TCR), expressed by T lymphocytes. The recent discovery of recombinatorial immune receptors that are structurally based on the TCR (referred to as TCR-like immunoreceptors, "TCRL") in myeloid phagocytes such as neutrophils and monocytes/macrophages now challenges the lymphocentric paradigm of flexible immunity. Here, we introduce the emerging concept of "extralymphocytic flexible immune recognition" and discuss its implications for inflammation and aging.

Key words: TCR, macrophage, neutrophil, immunosenescence

The lymphocentric paradigm of adaptive immunity

Vertebrate host defense is commonly subdivided into two arms – innate immunity and adaptive immunity [1]. Historically, this concept has its origin in Elie Metchnikoff's discovery of phagocytosis, i.e. the ability of cells to ingest solid particles, as a basic principle of innate host defense in 1883 [2] and the identification of antibodies by Behring and Kitasato seven years later as the defining component of adaptive immunity [3]. Two major populations of professional phagocytes, neutrophils and monocytes/macrophages, are the founding pillars of the innate immune response and, as such, constitute the first line of host defense against infections [4,5]. It is widely accepted that these ancient immune cells initiate inflammatory responses, phagocytose and kill pathogens, recruit natural killer cells (NK), and engage dendritic cells that in return trigger the adaptive immune response.

Longstanding immunological dogma holds that the molecular machinery of adaptive immune recognition in higher vertebrates, which is represented by immunoglobulins and the T cell receptor (TCR), is restricted to effector cells of the lymphocyte lineage [1,6]. This lymphocentric concept of variable immunity is based on Fagraeus' groundbreaking observation in 1947 that plasma cells are the cellular source of immunoglobulins [7]. The identification of the hypothesized, yet long-elusive, second variable immune receptor in T lymphocytes in the mid 1980s [8] has corroborated the concept that TCR expression in vertebrates is a prerequisite of the T lymphoid lineage (hence designated "T cell receptor"). In hindsight, however, the general acceptance of this concept contrasts strikingly with the complete absence of reports from the literature that provide explicit or systematic experimental proof that immune cells beyond the T cell lineage are indeed incapable of expressing recombinatorial T cell receptors.

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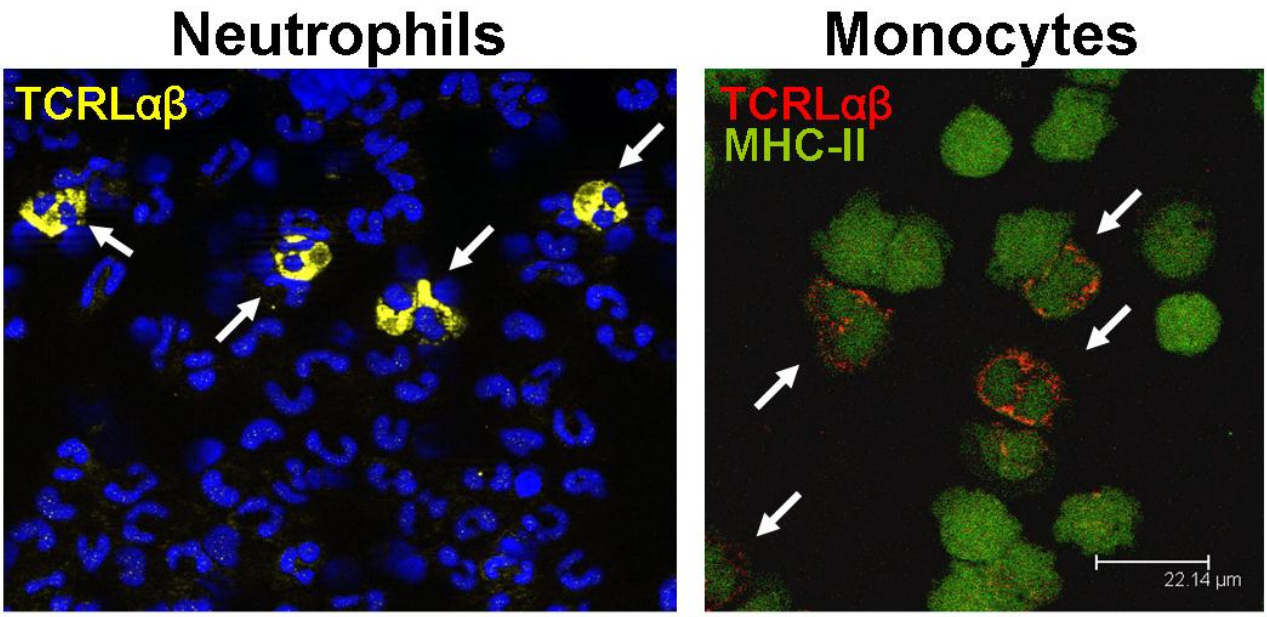

Figure 1. Circulating human neutrophils and monocytes express αβT cell receptor-like recombinatorial immune receptors (TCRLαβ). The confocal fluorescence immunocytochemistry images show ~5-8% subpopulations of circulating neutrophils (left) and monocytes (right) that express the TCRLαβ (arrows). TCRLαβ positive neutrophils and monocytes display yellow and red fluorescence, respectively. Nuclei are counterstained in the left image (blue). $CD15^+$ and $CD14^+$ purified peripheral blood neutrophils and monocytes, respectively, were isolated from representative healthy donors and immunostained using antibodies against the TCRαβ (yellow, neutrophils; red; monocytes). Monoytes were costained for MHC-II (green). Adapted from Puellmann *et al.* [9] and Beham *et al*. [13].

Recombinatorial TCR-like receptors (TCRL)

Recent studies in myeloid phagocytes now challenge the lymphocentric paradigm of adaptive immunity (Fig. 1). The initial observation came from work performed in our laboratories which demonstrated that peripheral blood neutrophils from healthy humans and mice possess rearranged T cell receptors [9,10]. A 5-8% fraction of neutrophils was identified in the circulation that constitutively expresses variable immune receptors that are composed of the TCR α- and β-chain. These TCR-based immunoreceptors (TCR-like immune receptors, "TCRL") are expressed across the entire human life-span [11]. Circulating human neutrophils were also shown to constitutively express the TCR γ- and δ-ligand binding subunits and all critical components of the TCR signaling complex [9]. These unexpected findings provided evidence, for the first time, that neutrophil granulocytes express a flexible antigen recognition machinery that is homologous to that present in T cells. The presence of TCRL in the granulocyte lineage was confirmed by a subsequent study which demonstrated expression of a functional TCRγδ in circulating eosinophil granulocytes from healthy individuals [12].

Consistent with the identification of TCRL expressing granulocytes, a most recent study provides compelling evidence that subpopulations of peripheral blood monocytes, which next to neutrophils constitute the second professional phagocyte population in the circulation, also possess TCRαβ-based combinatorial receptors [13]. These TCR-like receptors were also identified in monocyte-derived macrophages that had been differentiated under *in vitro* conditions and resident macrophages from tissue of healthy donors indicating that both monocytic phenotypes are capable of expressing TCRL.

TCRLαβ immunoprofiling in peripheral blood neutrophils and monocytes from healthy individuals reveals expression of diverse and individual-specific TCRLαβ repertoires. This indicates that TCRL represent a flexible immune receptor system [9,13]. Consistent with this, rearrangement analyses of expressed TCRαβ variants in neutrophils, monocytes/macrophages and eosinophils have routinely shown V(D)J recombination of the TCR α/β loci and TCR γ/δ loci, respectively, in these myeloid cells. Furthermore, GM-CSF stem cell progenitor experiments established that rearrangement of the TCRL Vβ locus is an early event during myeloid lineage development [9,12,13].

Infection of macrophages with the bacterial pathogen mycobacterium *BCG* induces changes of the expressed TCRLαβ repertoires and leads to a significant

induction of the macrophage-TCRL [13]. TCRL repertoires are also dynamically regulated in response to non-infectious exogenous stimuli. For example, *in vivo* G-CSF administration leads to transient suppression of TCRL repertoire diversity in human neutrophils and exposure of cultivated macrophages to IL-4 or IFNγ induces distinct repertoires changes *in vitro* [9,13]. Taken together, these findings clearly demonstrate that TCRL meet two cardinal criteria of adaptive immune systems - repertoire flexibility and responsiveness to exogenous stimuli.

Hidden in the shadow of T cells

Given that the existence of the TCR has already been hypothesized and proven in the early 1970s and 1980s [14,8], respectively, the question inevitably arises why the discovery of the TCRL in myeloid immune cells did not occur sooner. In all likelyhood, it was an unfavorable constellation of conceptual and technical hindrances that may have obivated earlier detection of TCR-based receptors outside the T lymphocyte lineage. The first major obstacle was the absence of a theoretical concept of non-lymphoid TCR expression. Long before its molecular identification the postulated TCR was believed to be the prototypic feature of T cells and thus by definition absent from the other branch of recombinatorial immunity represented by B cells. This explains why in all the initial TCR cloning studies a specific effort was made to exclude that the identified TCR chains were not of B cell origin and no systematic investigation of TCR expression in other tissues was conducted [15-23]. Besides this conceptual bias, a series of technical shortcomings and methodological intricacies contributed to prevent earlier identification of TCRL. These include the non-availability of reverse transcription PCR until 1988 [24] which allows for highly sensitive detection of gene expression and the fact that fluorescence-based flow cytometry routinely fails to unequivocally identify TCR bearing leukocyte populations outside lymphocytes [25]. Moreover, gene ablation studies in mice in which integral components of the TCR machinery had been deleted (e.g. rag1/rag2 knockouts, TCRαβ/γδ null mice) also did not give overt clues for the existence of TCRL. Most likely this is owing to the fact that TCR ablation massively compromises the development and function of T cells [26-32], a dominant biological effect that may have largely masked phenotypic alterations associated with defective TCRL in neutrophils and macrophages. At the turn of the millenium, the advent of large-scale gene expression microarrays another quantum leap technology became available to researchers that may have potentially facilitated identification of TCR expression in myeloid cells. Of note, several microarray-based expression profiling studies have indeed documented gene expression of integral components of the TCR ligand binding and signaling complex in neutrophils and macrophages [33-39]. In retrospect, it is puzzling, however, that this trail of clues for the existence of TCR-based recognition molecules beyond T cells has been completely ignored.

Potential functions of TCRL

Due to the recentness of the identification of combinatorial TCRL in phagocytes only little is currently known on what cellular function they serve in host defense. The finding that canonical CD3/CD28 costimulation of the neutrophil-TCRL induces the release of the major neutrophil chemoattractant CXCL8 (IL-8) suggests roles for the TCRL in neutrophil self-recruitment [9]. Consistent with this, CD3 mediated engagement of the macrophage-TCRL results in selective secretion of the monocyte chemoattractant CCL2 (MCP-1) by macrophages [13]. Similarly as in T cells, activation of TCR-based receptors appears to exert antiapoptotic functions. This is evidenced by the demonstration that CD3/CD28 costimulation of human neutrophils leads to upregulation of the antiapoptotic protein bcl-x_L and promotes neutrophil survival [9]. It is therefore likely that activation of the TCRL prolongs the functional life span of neutrophils at sites of inflammation. Considering that TCRL are expressed by cells that function as professional phagocytes, the obvious question is whether these recognition proteins interfere with the process of phagocytosis. Indeed, bait targeting experiments in human proinflammatory M1 macrophages demonstrate that TCRL facilitate phagocytosis [13]. Of note, the observed positive effect on phagocytosis was in the same order of magnitude as that mediated by the potent mediator of phagocytosis complement receptor 3 (CR3). Further support for the implication of the TCRL in phagocytosis comes from experiments in rag1 knockout mice which demonstrate that macrophages deficient in functional TCRL have reduced phagocytosis capacity [13]. Together, available evidence suggests potential roles of the TCRL as a modulator of cell survival, self-recruitment and phagocytosis. More evidence, however, is required to assess whether this also holds true when TCRL are activated by specific agonists *in vivo*.

TCRL extend the basis of variable immunity

Current evidence indicates the presence of TCRLαβ or TCRLγδ in four populations of myeloid immune effector cells: neutrophils, eosinophils, monocytes and

macrophages. The identification of flexible recognition molecules in these professional phagocytes has two fundamental implications for our understanding of the workings of innate and acquired immunity. First, the demonstration of immune receptors beyond the realm of lymphocytes reveals that variable immune recognition in higher vertebrates is built on a broader cellular foundation than commonly thought. This gives formal proof for the existence of a molecular platform for extralymphocytic flexible immune recognition (Fig. 2). Moreover, it predicts adaptive immune mechanisms in vertebrates outside T cells that rely on the TCRL ("extralymphocytic adaptive immunity") [25].

The second major implication of the existence of TCRL is that it demonstrates, for the first time, the coexistence of phagocytosis and flexible immune recognition in mammalian immune cells. Both fundamental principles have been previously thought to be mutually exclusive cellular functions. Their presence in phagocytes thus unifies the imunological armamentarium of myeloid cells with that of lymhpoid cells. It has been proposed that TCRL expressing phagocytes may represent a host defense system that is positioned between the innate and the adaptive branch of immunity which may bridge both arms of the immune system [40,41,25]. From a teleological viewpoint, a fast-acting phagocytic and at the same time flexible immunological intervention system would certainly make sense, because it fills the conceptual gap between first-line invariant host defense and the T cell based flexible immune response which occurs with a considerable temporal delay [1].

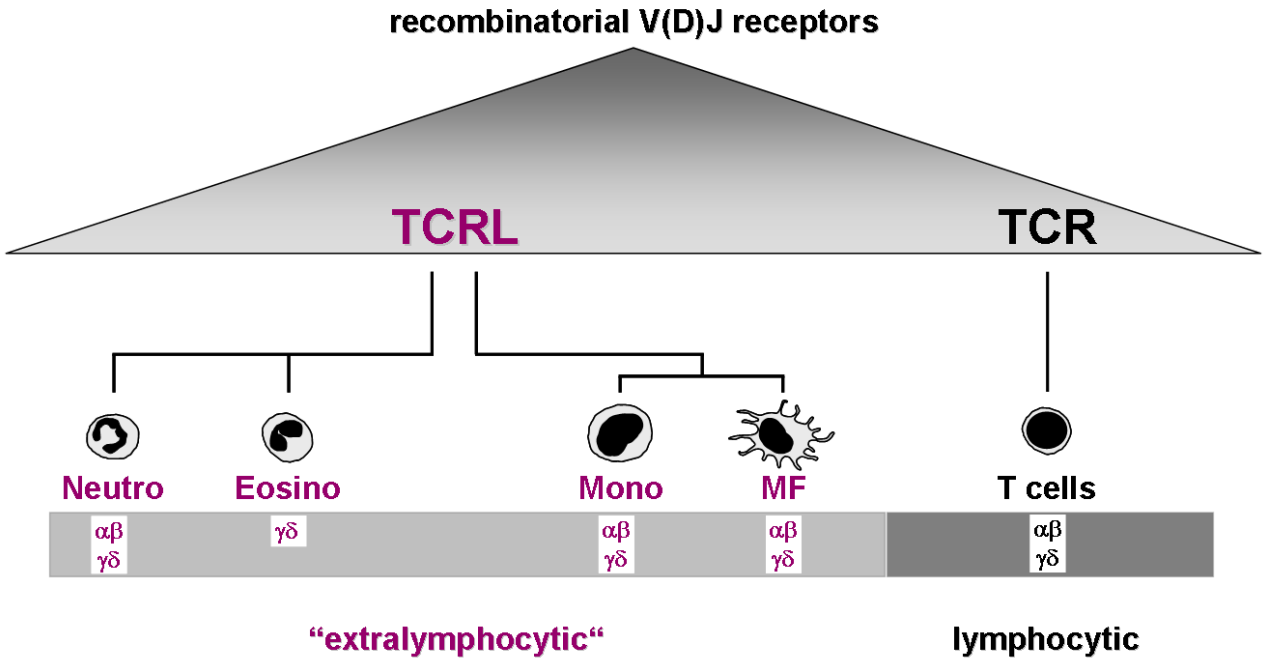

Figure 2. TCRL expression in phagocytes. The V(D)J recombined TCRL variable chains (α-δ) that have thus far been identified in each phagocyte population are indicated. The existence of TCRL extends the cellular basis for flexible immune recognition in jawed vertebrates beyond T cells and provides a molecular platform for extralymphocytic flexible immune recognition. This diagram was adopted from a previous publication [25].

Immunosenescence of neutrophils and macrophages

A large body of evidence as accumulated in the past decade to suggest that aging has a profound impact on the phenotype and function of various immune cells [43,44]. Neutrophils and macrophages play a key role in the innate immune system in that they constitute the first line of defense against tissue damage and invading pathogens [4,5]. Immunosenescence, that is the decline of diverse immune functions at old age, has been well established for neutrophils [43,45]. For example, neutrophils from old individuals have reduced phagocytic capacity, generation of reactive oxygen species, intracellular killing and degranulation [46]. In

contrast, several studies have established that neutrophil counts in the blood are not lowered in the elderly [47,48].

Similar to neutrophils and T cells, functional changes have also been reported for monocytes and macrophages from aged humans, rats and mice [49-53]. endocytic These include the phagocytic ability, the capacity to release chemokines and the function of invariant immune recognition receptors. However, there is little information as to whether structural components of the phagosome or receptors, which are implicated in the phagocytic process, undergo changes during aging.

Impaired function of immune recognition receptors in aged macrophages has been evidenced for toll-like pattern recognition receptors (TLR) [54,55]. For example, alveolar macrophages from aged rats show a significant decrease in the production of reactive oxygen species in response to the TLR4 ligand LPS [55]. On the other hand, bone marrow macrophages from aged mice have increased susceptibility to oxidants and an accumulation of intracellular reactive oxygen species which is paralleled by telomere shortening [56]. Expression of TLR 1-9 genes is reduced in splenic macrophages and in peritoneal macrophages from old relative to young C57BL/6 mice [57]. Furthermore, evidence for the implication of the TCR immune recognition machinery has been reported for M1 polarized macrophages from aged mice which display decreased cell surface expression of the MHC class II IA complex [58]. Whether the observed age-associated dysfunction of macrophages is rather the result of their functional adaptation to age-related changes in tissue than a primary decline of physiological function [52] is an intriguing, yet unsolved question.

TCRL undergo immunosenescence

Recent work from our groups now demonstrates that TCRL are implicated in immunosenescence. We observed that peripheral blood neutrophils display a 3-4 fold reduction in neutrophil TCRL repertoire diversity of their TCRL Vβ repertoire diversity in >70 year old individuals relative to young adults. The decline of the neutrophil TCRL Vβ repertoires is characterized by a strikingly predominant usage of a few selected Vβ chains and a high degree of clonotype sharing in the elderly [11]. A similar contraction of TCR repertoire diversity has been reported in T cells at old age in humans and mice [59,60]. These findings strongly suggest that TCR-based immunoreceptors in neutrophils and T cells undergo a concurrent decline during aging (Fig. 3). However, the dramatic TCRL repertoire contraction observed in aged neutrophils appears to be less pronounced compared to that of the TCR in $CD4^+$ T lymphocytes for which a >90% contraction of TCR repertoire diversity has been reported [61]. It is presently unknown whether the TCRL in monocytes/macrophages also undergoes age-related repertoire narrowing. By analogy, however, this appears likely, given that age-dependent repertoire contraction of the TCR-based immunoreceptors occurs both in neutrophils and T lymphocytes. Experiments are underway to address this important question.

At this point, it is unclear whether the decline in neutrophil TCRL repertoire diversity and the striking dominance of a limited number of TCRL Vβ clonotypes is linked to compromised recombination activity ("senescent recombination") or dysregulated transcriptional dynamics of individual TCRL producing neutrophil clones. If one takes into account that the TCRL in neutrophils and macrophages may function as facilitators of phagocytosis, it is possible that the impaired phagocytic activity oberved in aged macrophages and neutrophils is not only the consequence of a compromised function of the structural components of the cellular phagocytosis machinery but also the result of reduced TCRL expression of.

It has been postulated that loss of organized complexity is a characteristic feature of aging and disease [62,63]. In light of this decomplexification theory, the decline of TCRL or TCR repertoire diversity in aged individuals can be viewed as the loss of functional plasticity of the highly complex neutrophil and T cell flexible immune systems. Consistent with this concept, the striking expression of only a few dominant TCRL Vβ clonotypes we noted in aged neutrophils may reflect the emergence of a dominant functional mode. Intriguingly, this phenomenon of decomplexification is often observed in non-linear regulatory systems following the breakdown of complex dynamics [64].

Implication in disease

The presence of flexible immune receptors in neutropils and macrophages offers a fascinating new angle on understanding their role in inflammation. Because neutrophils are routinely the first cells at the site of inflammation [4] and macrophages are ubiquitously present in tissues both phagocyte populations are implicated in a wide spectrum of clinical inflammatory diseases. It is thus foreseeable that the neutrophil and macrophage TCRL are involved in numerous clinical pathologies. In keeping with this, efforts to test for the implication of TCRL in inflammatory diseases have as yet consistently shown that TCRL bearing neutrophils and macrophages accumulate at sites of inflammation [13,65]. For example, a recent study in which we tested in detail the potential role of the macrophage-TCRL in

the pathogenesis of tuberculosis revealed that the vast majority of the macrophages that accumulate in the inner host-pathogen contact zone of caseous granulomas from patients with lung tuberculosis express the TCRLαβ [13]. This intriguing finding strongly suggests that TCRLαβ bearing macrophages constitute the front line of cellular defense against mycobacteria. Consistent with this, *in vitro* infection of macrophages with *M. bovis BCG* induces formation of macrophage clusters that express restricted TCRL Vβ repertoires. More importantly, experimental tuberculosis in chimeric rag1$^{-/-}$ mice, which were reconstituted with wild-type T cells, revealed that ablation of the macrophage-TCRL in the presence of intact T cells results in disorganized tuberculous granulomas [13]. Collectively, these experiments strongly suggest that the TCRL in macrophages is critcally involved in the formation of the tuberculous granuloma. Recent work also demonstrates that the neutrophil-TCRL is implicated in inflammatory disease. This is evidenced by the observations that a massive induction of the neutrophil-TCRL occurs in the circulation in autoimmune hemolytic anemia [65] and in the cerebrospinal fluid from patients with acute bacterial meningitis (Fuchs T et al., unpublished).

Based on these initial studies, work is underway to test whether TCRL are implicated in other inflammatory processes. Our attention has focused primarily on two promising candidate diseases – atherosclerosis and cancer – based on their eminent epidemiological importance and the fact that both entities represent paradigmatic diseases of advanced age. Whereas it is well established that macrophages are key players in the pathogenesis of atherosclerosis [66], it was only recently that tumor infiltrating macrophages (commonly known as tumor associated macrophages, TAM) have gained considerable attention owing to evidence demonstrating a pivotal role for these cells in promoting angiogenesis and tumor progression [67-70]. In fact, recent *ex situ* TCRL clonotype analyses in our laboratories consistently reveal expression of TCRL by macrophages in advanced lesions of atherosclerosis and melanoma metastases (Fuchs T et al., unpublished). More work is necessary, however, to substantiate these fascinating preliminary results.

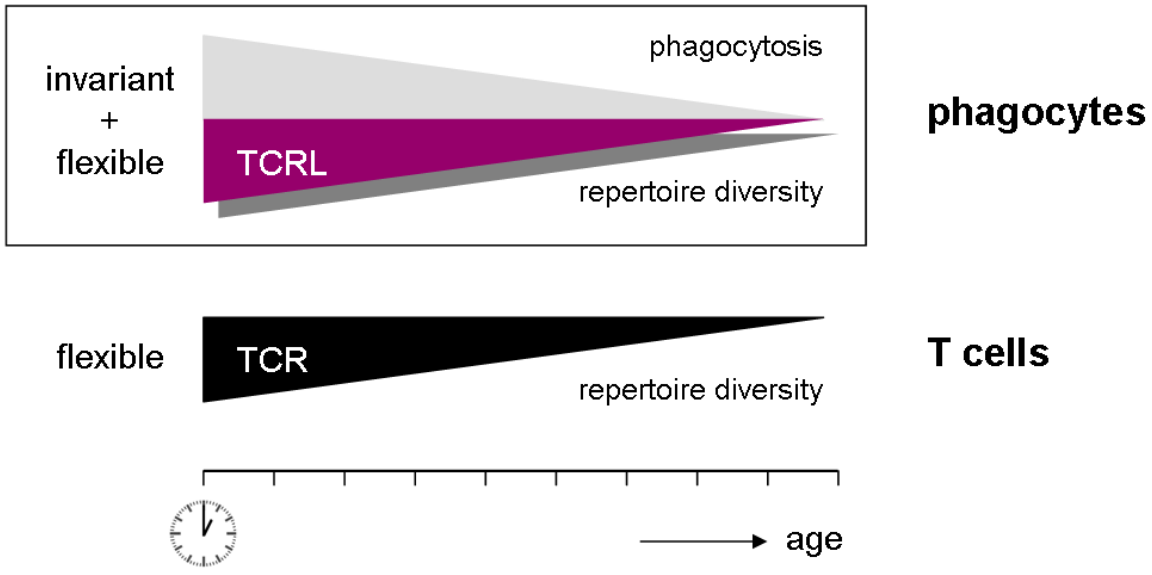

Figure 3. Conccurrent decline of the capacities for phagocytosis and flexible immune recognition in myeloid phagocytes during aging. The myeloid machinery for flexible immune recognition is based on the recently identified recombinatorial TCRL. This process is paralleled by the decline of the T cell receptor (TCR) in the lymphoid lineage.

Perspectives

The discovery of TCR-based recombinatorial receptors in myeloid immune cells comes as a big surprise and without a pre-existing theoretical framework. It challenges the lymphocentric concept of flexible immune recognition and adds a new complexity of unknown dimension to our present understanding of the workings of the vertebrate immune system. The scenario bears somewhat of a resemblance to navigators who have landed on an unknown coast wondering about the size of the land behind it. Theoretically, it could be an island of limited size *viz.* a finding of limited physiologic importance. However, in light of the constellation that (i) neutrophils initiate virtually any form of inflammation, (ii) macrohages are strategically positioned throughout the entire body, (iii) TCRL have persisted in these evolutionarily ancient phagocytes throughout evolution and (iv) TCRL are implicated in major inflammatory syndromes, it is rather likely that a vast uncharted territory of immunology may lie ahead of us. This view is also supported by a growing body of evidence that points to the existence of host response mechanisms in innate immune cells which involve immunologic memory beyond lymphocytes ("innate memory") [71]. With the underlying molecular components for innate memory not yet identified, it is thus tempting to speculate that TCRL form (in part) the mechanistic basis for these phenomena.

A major challenge in the quest to understand the biological significance of TCRL will be the assessment of their true TCRL repertoire diversity under physiological conditions and the elucidation of the dynamics of repertoire changes that are associated with age and disease. With the quantum leap technology of next generation sequencing a powerful tool has recently become available that will allow direct sequencing of entire TCRL transcriptomes [72-74]. Another important question is whether neutrophils, eosinophils and monocytes/macrophages represent the only leukocyte populations that rely on TCRL. It is possible that other cells of myeloid origin are capable of expressing TCRL recognition molecules. Promising candidates include tissue-specific macrophage populations such as Kupffer cells, osteoclasts and dendritic cells. It will be challenging to outline the actual cellular basis of TCRL in non-lymphoid immune cells.

Although our knowledge of the immunological relevance of TCRL is still fairly rudimentary, available evidence links these novel immune receptors to both aging and inflammatory disease. It will thus be rewarding to explore the potential roles of TCRL in age-associated diseases. In particular, atherosclerosis as *the* paradigmatic inflammatory disease of advanced age will be a most attractive target for investigating the pathophysiological role of TCRL in macrophages. Other diseases in which macrophages play a key pathophysiogical role include bone osteoporosis [75], Alzheimer disease [76] and chronic obstructive pulmonary disease (COPD) [77]. The latter will be a particularly interesting candidate disease because its pathogenesis depends critically not only on macrophages but also neutrophils.

The TCR-like immune receptors presented here combine the properties of innate and adaptive immunity and, like these, undergo deterioration during aging. There is an enormous amount of work ahead of us to explore the function and true complexity of this novel myeloid flexible immune system that was hidden in the shadow of lymphocytes for so long. Considering the key role of phagocytes in vertebrate host defense, it is foreseeable that this task will provide a new angle on the pathogenesis of inflammatory disease and help us to better understand the immunologic deficits that arise during aging.

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