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Dysregulation of Human Toll-like Receptor Function in Aging

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Abstract

Studies addressing immunosenescence in the immune system have expanded to focus on the innate as well as the adaptive responses. In particular, aging results in alterations in the function of Toll-like receptors (TLRs), the first described pattern recognition receptor family of the innate immune system. Recent studies have begun to elucidate the consequences of aging on TLR function in human cohorts and add to existing findings performed in animal models. In general, these studies show that human TLR function is impaired in the context of aging, and in addition there is evidence for inappropriate persistence of TLR activation in specific systems. These findings are consistent with an overarching theme of age-associated dysregulation of TLR signaling that likely contributes to the increased morbidity and mortality from infectious diseases found in geriatric patients.

1. Introduction

Interest in understanding the effects of aging on host defense against infection is driven in part by the realities of current and future demographic trends. In particular, next year represents a watershed moment in the demographics of the United States: in 2011, the first members of the post-World War II baby boom generation (extending from 1946 to 1965) will turn 65. The number of individuals over age 65 estimated to grow to over 72 million by 2030—19% of the population and more than double the number in 2005 (US Census Bureau, 2008). This “Gray Tsunami” lends particular urgency to understanding mechanisms associated with aging that may affect the health of older individuals. Although individuals over age 65 currently comprise approximately 12% of the population, they account for over 35% of visits to general internists, 34% of prescription drug use, 50% of hospital stays, and 90% of nursing home residents (Cherry et al., 2007).

It has long been evident that older individuals are at increased risk for morbidity and mortality from infectious diseases (Yoshikawa, 1997). While it is likely that comorbid

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conditions contribute to the observed increase in mortality (e.g. relative tissue ischemia from vascular disease exacerbating intestinal track inflammatory disorders), it is clear that impaired host defenses associated with aging also contribute to increased morbidity and mortality. Substantial progress has been made in understanding of the consequences of aging on adaptive immunity (reviewed elsewhere in this issue); however, the effects of aging on the innate immune system remain incompletely understood (Kovacs et al., 2009; Panda et al., 2009). However, recent studies indicate that aging influences the function of pattern recognition receptors mediating innate immunity. Here we review the progress made in our understanding of age-associated alterations in Toll-like receptors (TLR) expression and function, focusing on reports in humans.

2. Overview of Toll-like Receptors, Pattern Recognition Receptors of the Innate Immune System

Innate immunity is mediated by a network of cell types, including monocytes/macrophages, natural killer (NK) and natural killer T (NKT) cells, dendritic cells, eosinophils and basophils. Activation of the innate immune system in general results in diverse cellular processes including phagocytosis and the elaboration of mediators such as reactive oxygen and nitrogen species, defensins, complement, and pro-inflammatory cytokines and chemokines that mediate the initial host response to pathogens.

The existence of pattern recognition receptors in the innate immune system was predicted over twenty years ago by Janeway, who proposed that pathogen sensing was mediated by a set of germline-encoded receptors that detects conserved products of microbial biosynthetic pathways (known as pathogen-associated molecular patterns (PAMPs)) (Janeway, 1989). Janeway further pointed out that this form of immune recognition must be evolutionarily related to the immune systems of invertebrates, which lack adaptive immunity, and suggested that the purpose of innate immune activation was to induce costimulatory protein expression on antigen presenting cells. Finally, he postulated that most adjuvants augmented immune responses by triggering the receptors of the innate immune system and inducing costimulatory signals by mimicking microbial infection. Though made purely on theoretical grounds, this remarkable set of predictions proved to be correct, and heralded a new era in the biology of innate immunity.

Toll-like receptors are now counted among the key pattern recognition receptors that alert the immune system to the presence of microbial infections. They are named for their similarity to Toll, a gene first identified in the fruit fly *Drosophila melanogaster*, and originally known for its function in the specification of dorso-ventral polarity. The gene was named when Christiane Nusslein-Volhard is said to have exclaimed, "Toll!" (a German slang expression roughly translated as "cool" or "far-out") when Eric Wieschaus showed her the abnormal cuticle pattern of the mutant fly embryos (Anderson, 2000).

Early on, extensive homology was observed between the cytoplasmic domain of Toll in *Drosophila* and the interleukin-1 (IL-1) receptor in mammals (Gay and Keith, 1991), though postulated roles in development were naturally the focus of initial characterization of mammalian Toll homologs (Nomura et al., 1994; Taguchi et al., 1996). However, in 1996, Hoffmann and colleagues reported that the same Toll-dependent pathway controlling the establishment of dorsoventral polarity during embryonic development mediated the synthesis of antimicrobial peptides in adult flies, and an essential role in host defense against fungal infection (Lemaitre et al., 1996). In 1997, Medzhitov and Janeway showed that a constitutively active version of a TLR (now known as TLR4) could induce NF- κ B activation and CD80 expression (Medzhitov et al., 1997). This crucial insight was achieved without knowledge of the natural ligand of TLR4; its function as an LPS sensing receptor

was reported by Beutler and colleagues (Poltorak et al., 1998), who used positional cloning to demonstrate that mice that could not respond to LPS had mutations that abolished the function of TLR4.

From these initial studies, the TLR family of evolutionarily conserved, germline encoded type I transmembrane proteins emerged. TLRs are expressed by monocytes, NK cells, DCs, and other cells of the innate immune system as well as in B and T lymphocytes (Medzhitov, 2001; Takeda et al., 2003). To date, 13 different TLRs (TLR1–13) have been identified in mammals (Kawai and Akira, 2010), and 10 of these (TLRs 1–10) are functional in humans. PAMPs serving as TLR ligands include LPS on gram-negative bacteria (TLR4); diacylated (TLR2/6) and triacylated (TLR1/2) lipopeptides, peptidoglycan (TLR2), bacterial flagellin (TLR5), nucleic acids and double-stranded RNA (TLR3) single-stranded RNA (TLR7 and TLR8), and unmethylated CpG oligodeoxynucleotides (TLR9) (Alexopoulou et al., 2001; Alexopoulou et al., 2002; Heil et al., 2004; Lund et al., 2004; Takeuchi et al., 2002). Recognition of microbial components by TLRs initiates MyD88 or TRIF-dependent signal transduction pathways that culminate in both the elaboration of proinflammatory cytokine responses (via NF- κ B-dependent pathways) and the upregulation of Type I interferons and interferon-dependent genes (Kawai and Akira, 2007). Thus, TLRs integrate innate immune responses mediated by pro-inflammatory cytokines and Type I interferons that shape the adaptive T and B cell immune response (van Duin et al., 2006).

3. Human Studies of TLR Function in Aging

3.1. Earlier Studies Focusing on LPS stimulation of PBMCs

A number of studies initially evaluated the effects of aging on LPS-mediated cytokine responses by human monocytes. Some of those studies were conducted even before TLR4 was characterized as a component of the LPS receptor (van Duin and Shaw, 2007). These studies are in general conflicting, as some studies show an age-related increase in LPS-induced cytokine secretion (Clark and Peterson, 1994), while others show unchanged or decreased secretion (Delpedro et al., 1998; Gon et al., 1996; Mariani et al., 2002). The differences in experimental results for these studies likely reflect factors such as different cell enrichment protocols and variation in preparations of LPS. Many of these studies employed enzyme-linked immunosorbent assays (ELISA) to determine cytokine production in bulk mixed-cell populations of peripheral blood mononuclear cells (PBMCs) or whole blood, which does not allow correction for individual differences in representation of specific cell lineages responding to TLRs. Differences in assay conditions may also be relevant; for example, LPS-induced cytokine production measured by ELISA in samples from older, compared to young individuals was increased using a whole blood assay and decreased using isolated PBMCs (Gabriel et al., 2002). With these caveats, studies of LPS stimulation of PBMCs have also begun to evaluate the intersection between immunosenescence and functional status; for example, studies of a Dutch cohort of 551 individuals over the age of 85 showed decreased LPS-induced cytokine production in PBMCs that was associated with an increase in mortality risk that remained significant after adjustment for comorbidity (van den Biggelaar et al., 2004). On the other hand, increased activation of LPS-induced inflammatory gene pathways was observed in a small cohort of individuals meeting criteria for the geriatric syndrome of frailty, compared to non-frail elderly individuals—reflecting the potential contribution of inflamm-aging (Qu et al., 2009).

3.2 Approaches to variability in human immunologic studies

These contradictory results illustrate a fundamental problem in human aging research: how does one account for variation between young and older study participants, which must encompass not only genetic heterogeneity inherent in any human study (and not encountered

in genetically defined murine systems), but also heterogeneity in infectious exposures, medication use, comorbid medical conditions and other factors that accrue during a lifespan? One approach is a restrictive enrollment procedure such as the SENIEUR Protocol, which was developed in 1984 by the working party of the EURAGE concerted Action Programme on Aging of the European Community (Ligthart et al., 1984) to minimize conflicting results between studies. SENIEUR subjects must meet strict admission criteria for and tend to have more homogeneous immune responses than those not satisfying the protocol. However, these strict exclusion criteria excluded at least 70% of community dwelling older adults in one study (Wick and Grubeck-Loebenstein, 1997) and likely select for an uncommonly healthy, successfully aged subset of older individuals. Thus, insights obtained from studies of successful aging are not generalizable to patient populations, but complement those that evaluate older individuals with comorbid illness or alterations in functional status.

However, studies of immunologic data from young and older humans that do not employ strict exclusion criteria, such as SENIEUR, need to use statistical methods that can control for the confounding effects of other comorbid conditions or characteristics such as gender and race that are associated with differences in immunologic function. Few studies have applied linear mixed models (LMM) that allow for adjustment for confounding or address the correlation among multiple immunologic measures. In the absence of adjustment for confounding and correlation, the statistical significance of reported age-associated differences and their standard errors may be biased and Type I error inflated. Moreover, when analyzing several separate models of immunological responses (such as LPS-induced production of several cytokines) few reports correct for multiple comparisons, again increasing the Type I error rate and inflating the number of statistically significant differences.

3.3 Studies of aging and TLR function in human monocytes/macrophages

To apply some of these principles, van Duin et al. evaluated a wide range of TLR ligands in peripheral blood mononuclear cell (PBMC) samples from 79 young (age 21–30) and 80 older (age 65) adults (van Duin et al., 2007b). To minimize manipulation of cells, PBMCs in suspension were stimulated with TLR ligands in round-bottom wells, and cytokine production was detected via flow cytometry and intracellular staining on gated populations of monocytes. The study revealed an age-associated reduction in tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6) production in monocytes after stimulation of the TLR1/2 heterodimer. An age-associated decrease in ssRNA-induced (TLR7) IL-6 production was also observed; TNF- α and IL-6 production after engagement of TLR2/6 heterodimer, TLR4 and TLR5 appeared grossly intact, although it remains possible that alterations in functions of these TLRs could be uncovered at different ligand concentrations. An age-associated decrease in TLR1/2-induced p38 MAP kinase phosphorylation was observed in purified monocytes from older individuals as well. To account for health and demographic covariates and the correlation of TLRs within each person, the study used LMM statistical analysis, and this observed defect in TLR1/2 function remained statistically significant using a LMM. Moreover, the reduction in cytokine production was strongly associated with a decrease in surface expression of TLR1, but not TLR2, on the surface of monocytes from older, compared with young individuals. Notably, intracellular TLR1 expression was unchanged between younger and older individuals, suggesting a post-translational alteration in surface TLR1 expression in monocytes from older individuals. These findings were confirmed in a recent study of 17 young and 17 older individuals that also employed intracellular cytokine staining, but used stimulation of whole blood instead of isolated PBMCs (Nyugen et al.). Of note, this study reported age-associated alterations in monocyte subpopulations stratified on the basis of CD14 and CD16 expression, and found that alterations in cytokine production

and TLR1 expression associated with impaired ERK1/2 phosphorylation in cells from older individuals had a greater association with CD16+ monocytes (Nyugen et al., 2010).

The influence of human aging on TLR-induced changes in expression of costimulatory markers CD80 and CD86 on monocytes showed a generalized, highly significant decrease in TLR-induced upregulation of CD80 in older, compared to young adults for all TLR ligands tested (engaging TLR1/2, TLR2/6, TLR4, TLR5 and TLR7/8), though the magnitude of decrease was largest for TLR1/2 (van Duin et al., 2007a). These differences remained statistically significant after adjustment for covariates in the LMM. Moreover, in contrast to TLR-induced cytokine production in monocytes, TLR-induced changes in the expression of CD80 were strongly associated with both seroconversion (a four-fold increase in antibody titer) and seroprotection (a post-vaccine antibody titer of 1:64) to the trivalent influenza vaccine. Under the experimental conditions used, TLR stimulation resulted in downregulation of CD86 expression on monocytes. While the extent of CD86 downregulation in young vs. older individuals was only significant following flagellin (TLR5) or poly (U) (TLR7/8) stimulation, the extent of CD86 downregulation had a highly significant association with seroconversion to influenza vaccine (for which efficacy is known to be markedly decreased in old individuals).

An additional example of a clinically relevant age-associated impairment in immune response is the decline in delayed-type hypersensitivity (DTH) responses, such as to the tuberculin purified protein derivative (PPD) used in skin testing to detect exposure to *Mycobacterium tuberculosis*. Such impaired DTH responses were recently found to be associated with diminished production of TNF- α by human dermal macrophages from older, compared to young individuals; notably, *ex vivo* stimulation of such macrophages with TLR1/2 or TLR4 agonists revealed comparable TNF- α levels in cells from young and older subjects—suggesting a still incompletely understood inhibitory microenvironment in older adults that may result in part from an increased proportion of cutaneous regulatory T cells in older individuals (Agius et al., 2009). Taken together, these results indicate that human aging results in immunosenescent phenotypes in the innate immune system and demonstrate the link between innate immune activation and adaptive immune responses.

In some circumstances, age-related altered TLR responses may contribute to risk of infectious disease in older individuals. A recent study by Kong et al. provided evidence for this point in studies of age-dependent TLR function in human macrophages in the context of viral infection. In studies using West Nile virus (WNV), a mosquito-borne flavivirus recently introduced to North America with disproportionate morbidity (particularly from meningoencephalitis) and mortality in older individuals, the authors demonstrated that interaction of WNV envelope protein with the DC-SIGN lectin on the surface of macrophages results in diminished TLR3 expression in macrophages but only in macrophages of young donors (Kong et al., 2008). The decrease in TLR3, and a concomitant decrease in STAT1 phosphorylation and signaling were deficient in macrophages from older individuals. This dysregulation of TLR3, if present *in vivo*, may result in elevated inflammatory responses that may contribute to the excess in morbidity of WNV infections seen in elderly individuals. It is attractive to speculate that this STAT1-dependent pathway might also result in inappropriately sustained TLR3 engagement during other viral infections as well.

3.4 Aging and TLR function in human dendritic cell populations

Studies of TLR function in human dendritic cell populations have also revealed evidence for age-associated dysfunction. Such studies encompass myeloid dendritic cells (mDCs), which express a broad range of TLRs and play a critical role in facilitating TH1 responses, for example via the TLR-induced production of IL-12; and on plasmacytoid (pDC) dendritic

cells, which express a more restricted range of TLRs (principally TLR7 and TLR9) and are particularly adept at type I interferon production in response to viral infections. However, study of such primary DC populations in humans is challenging, because of their rare abundance in peripheral blood and the difficulties in obtaining human lymphoid tissue via biopsy for analyses. An alternative approach is to utilize monocyte-derived DCs (MDDCs), in which monocytes are induced to differentiate via treatment with specific growth factors and cytokines (a typical combination would be IL-4 and GM-CSF). MDDCs generated in this way most closely resemble myeloid DCs, and may be obtained from a relatively minimal volume of blood in far greater numbers for analyses than primary DCs. Evidence that DCs can be generated from monocytes, particularly in inflammatory contexts, suggests a potential *in vivo* parallel for MDDCs. The numbers and baseline characteristics of MDDCs appear grossly similar when derived from young versus older adults (Agrawal et al., 2007; Lung et al., 2000; Steger et al., 1996); however, the use of growth factors for *in vitro* differentiation of MDDCs, and their potential effect on attenuating age-specific phenotypes, is a consideration in evaluating results from young and older adults.

In studies using monocyte-derived DCs (MDDCs), evidence for both augmented and impaired function has emerged from studies in young vs. older individuals. Reports by Agrawal et al. revealed higher levels of cytokines from older, compared to younger individuals for LPS- and single-stranded RNA-induced TNF- α and IL-6 production, as well as increased self-DNA-induced IL-6 and IFN- α production; at the same time, age-associated impairments in phagocytosis and migration *in vitro* were observed (Agrawal et al., 2007; Agrawal et al., 2009). Decreases in the signaling activity of phospho-inositol 3 (PI3)-kinase, as manifested by decreased phosphorylation of AKT, were also reported in MDDCs from older individuals, and were associated with increased levels of phosphorylation of the p38 mitogen-activated protein kinase which inhibits TLR signaling. The potential function of the PI3K-signaling pathway as a positive regulator of phagocytosis and migration, and as a negative regulator of TLR signaling by inducing activation of p38 MAPK, may contribute to the reduced innate immune functioning of DCs from elderly subjects. Notably, studies of MDDCs also reveal an increased expression of another negative regulator of the PI3K-signaling pathway, phosphatase and tensin homolog (PTEN), in DCs from older individuals. The observed hyper-responsiveness to TLR stimulation with concomitant impaired phagocytic and migration functions of MDDCs derived from older individuals could reflect some aspect of DC maturation in inflammatory milieus, and could contribute to the heightened proinflammatory environment—so-called “Inflamm-Aging”—that has been reported in the context of aging humans (Franceschi et al., 2007).

Recently, the effect of aging on MDDC function has been evaluated in a viral infection model. When DCs were infected with WNV *in vitro*, DCs from older donors showed significantly lower production of type I IFN compared to younger donors. Notably, this deficit from older donors was noted in both MDDCs as well as primary pDCs isolated from peripheral blood. While there was no significant difference between younger and older individuals in the signaling molecules that participate in the initial induction of anti-viral responses (including expression and nuclear translocation of the transcription factor IRF3, and the type I IFN receptor or its downstream signaling pathway, JAK-STAT), DCs from older donors had diminished late phase responses such as induction of the transcription factors STAT1 and IRF7, and lower expression of IRF1, suggesting defective positive-feedback regulation of type I IFN expression. Such deficits in critical regulatory pathways in the anti-viral response may contribute to the enhanced susceptibility to viral infections observed in aging (Qian et al, unpublished results).

An analysis of primary DCs from healthy subjects using flow cytometric methods to enumerate and characterize cells directly from blood samples showed an age-associated

decrease in TLR4-induced IL-12 production in mDCs from a subset of 27 young and 27 older individuals analyzed by a paired t test (Della Bella et al., 2007). Similarly, assessment of TLR7- and TLR9-induced IFN- α production via intracellular cytokine staining in pDCs from peripheral blood (n = 18 young and 19 older individuals—a subset of the overall study group) showed an age-associated decrease but no differences were observed in younger vs. older individuals for TLR3-induced IL-12 production in mDCs (Jing et al., 2009). The authors report similar trends when cytokines were detected using ELISAs of stimulated mDCs and pDCs purified via magnetic bead sorting: decreased production of IFN- α , IL-6, IL-8 and TNF- α was observed in influenza virus (TLR7)-stimulated pDCs from older, compared to younger individuals, while no obvious differences were observed for poly (I:C) stimulation of cytokine production in mDCs. However, these ELISA results were obtained using cells pooled from 5–10 young or aged donors, complicating evaluation of statistical significance. In concordance with age-related changes in the function of mDCs and pDCs, it was also found that the proportion of pDCs positive for TLR7 or TLR9 was reduced, whereas the proportions of TLR2 and TLR4 positive mDC were not altered with aging.

The most comprehensive study to date of TLR-induced cytokine production in 52 young (21–30) and 52 older (65) individuals, using intracellular staining to quantify cytokine levels in primary mDCs and pDCs, evaluated the generation of TNF- α , IL-6, and the p40 subunit shared by IL-12 and IL-23 following stimulation with a wide range of TLR ligands (Panda et al., 2010). As in the previously cited studies, an age-associated decrease in TLR7 and TLR9-induced production of IFN- α was observed in pDCs. However, in contrast to previous studies, a generalized age-associated defect in the intracellular production of TNF- α , IL-6 and p40 was observed for virtually all TLRs evaluated (TLR1/2, TLR2/6, TLR3, TLR4, TLR5, and TLR8) in mDCs. To account for both the heterogeneity among young and old adults and the within-person correlation among ligand-specific stimulations for a given cytokine response, the investigators used a LMM to estimate the effect of age group on intracellular cytokine production. The full models tested for the fixed effect of age group (older versus young), ligand, the interaction between age group and ligand, controlled for potential confounding variables by including the covariates gender, race, number of comorbid conditions (heart disease, stroke, peripheral vascular disease), and body mass index. Even after this comprehensive statistical analysis and control for multiple comparisons, these age-associated differences remained significant. Moreover, these age-related decreases were sustained when mDCs and pDCs derived from a subset of the young and older adults were re-evaluated nearly two years after initial enrollment. Notably, the extent of TLR-induced cytokine production in mDCs and pDCs was strongly correlated with the generation of protective influenza vaccine antibody responses, providing additional evidence for the functional consequences of immunosenescence of the innate immune system.

The etiology of these age-associated alterations in TLR function remains incompletely understood; in DCs, age-associated decreases in TLR protein expression were observed for TLR1, TLR3, and TLR8 in mDCs but not for TLR9 in pDCs, while qPCR analysis demonstrated modest but statistically significant age-associated decreases in the gene expression of TLR3 and TLR8 in mDCs and of TLR7 in pDCs but not of other TLRs. It is likely that both transcriptional and post-transcriptional mechanisms play roles in TLR dysfunction with aging, and such processes are under active investigation.

This observation of age-associated decreases in TLR-induced cytokine production seemingly contrasts with evidence for increased pro-inflammatory cytokine production in aged humans (Franceschi et al., 2007). A possible explanation emerged from analyses of basal levels of intracellular cytokine production by DCs in the absence of TLR agonist stimulation (Panda et al., 2010). Such basal levels of p40, IL-6 and TNF- α in mDCs and of

IFN- α and TNF- α in pDCs were markedly elevated in older, but not young adults. The authors speculate that because basal levels of cytokines are elevated in DCs from older adults, reflecting dysregulation, further increases following TLR ligand stimulation of such cells will be attenuated, potentially contributing to impaired responses to infectious challenges in the host.

4. Potential mechanisms underlying age-associated TLR dysfunction

As discussed above, recent studies have implicated specific signaling pathways in alterations in TLR function in the context of human aging. These include decreased PI3-kinase activity in MDDCs (associated with increased PTEN phosphorylation) (Agrawal et al., 2007) and an impairment in downregulation of STAT1 phosphorylation in macrophages (Kong et al., 2008). Both of these events result in increased TLR-dependent cytokine production in aging, and while the basis for these alterations in signaling is unclear, these findings illuminate specific pathways that can be manipulated pharmacologically for future study.

At the same time, decreases in human TLR-dependent signaling, such as impaired MAP kinase phosphorylation, have been observed in the context of decreased TLR protein expression (van Duin et al., 2007b), and in this regard, age-associated declines in TLR expression and signal transduction have been reported in murine systems as well (Kovacs et al., 2009). In particular, an age-associated decrease in TLR1 surface expression has been observed in human monocytes, with decreased TLR1, TLR3 and TLR8 protein expression observed in mDCs (Panda et al., 2010; van Duin et al., 2007b). Both decreases in TLR gene expression and post-transcriptional mechanisms likely contribute to the observed TLR dysfunction (Panda et al., 2010). The observation that TLR1 surface expression on monocytes is impaired in older adults, while the total pool of intracellular TLR1 protein is not significantly different from levels in monocytes from younger adults, is consistent with this notion (van Duin et al., 2007b), and raises the potential importance of TLR localization and trafficking in mediating aging-related phenotypes. In this regard, several critical chaperone proteins have been identified that have overlapping, but incompletely understood, associations with TLR proteins. These proteins include gp96, which has been implicated in the localization of TLRs 2, 4, 5, 7, and 9 (Randow and Seed, 2001; Yang et al., 2007) and PRAT4a, an ER protein which, when deleted in the germline of mice, results in impaired responses to TLR1/2, TLR2/6, TLR4, TLR7 and TLR9 activation (Takahashi et al., 2007). PRAT4a has been implicated in the trafficking of endosomal TLRs such as TLR9 from the ER to the lysosome, a function it appears to share with another ER chaperone protein, UNC93B1, which associates with the (endosomally localized) TLRs 3, 7 and 9 (Fukui et al., 2009). Notably, UNC93B1 deficiency in humans leads to susceptibility to herpes simplex virus encephalitis and may influence autoimmune susceptibility via differential viral recognition by TLR 7 and 9 (Casrouge et al., 2006; Zhang et al., 2007). A further layer of complexity to the post-transcriptional regulation of TLRs is introduced by the recent demonstration of post-translational cleavage of TLR7 and TLR9 (Ewald et al., 2008; Sepulveda et al., 2009). Downstream signal transduction via TLR9 was found to be activated by the cleaved and not the full-length receptor. Conceivably, such post-translational localization and processing steps could contribute not only to impairment in TLR function, but also could influence the downregulation of signaling after receptor activation and potential dysregulation of TLR-induced outcomes such as cytokine secretion—another potential interface with aging of the innate immune system.

5. TLR Polymorphisms in Aging

Responses to TLR ligands may also be influenced by single nucleotide polymorphisms (SNPs) within TLR genes, though the impact of such SNPs in the context of aging remains

unclear. While it is likely that TLR SNPs favoring survival and fitness for reproduction may be subject to evolutionary selection in young individuals, SNPs in older individuals are less likely to undergo such selective pressure. Thus, a SNP resulting in, for example, enhanced function of a TLR and improved host innate immune defense in young individuals could be maintained in the germline via selection, but might result in a deleterious increase in the pro-inflammatory milieu in older individuals (perhaps exacerbated by age-associated deterioration in other pathways controlling innate immune activation).

Studies of specific TLR SNPs in the context of aging to date have been largely limited to the TLR4 polymorphism Asp299Gly. The effect of this SNP on TLR4 responses remains unclear. For example, in transfection studies of THP-1 cells, this SNP was associated with impaired LPS-induced signaling (Arbour et al., 2000; Ferwerda et al., 2007; Schwartz, 2001); however, other studies in primary human cells from individuals heterozygous for Asp299Gly showed unchanged or increased LPS-induced cytokine responses (Calvano et al., 2006; Erridge et al., 2003; Ferwerda et al., 2007; Schippers et al., 2004). In a cohort of Sicilian subjects, the Asp299Gly SNP was overrepresented in centenarians but underrepresented in individuals with acute myocardial infarction (Balistreri et al., 2004). The authors hypothesized that inflammation, which may contribute to vascular disease, was attenuated in the setting of diminished TLR4 function, such that mutations associated with decreased TLR responsiveness may be associated with longer lifespan. However, another study in a German cohort did not demonstrate these associations (Nebel et al., 2007), and indeed there are contradictory findings with regard to the association of Asp299Gly with the risk of atherosclerosis overall (summarized in (Balistreri et al., 2009)).

Based on the evidence of a role for inflammatory responses to beta-amyloid peptide (among other compounds) in the pathogenesis of Alzheimer's Disease (AD) (Heneka and O'Banion, 2007), investigators have begun to evaluate the potential association between TLR4 SNP Asp299Gly and the risk for AD. To date, two studies suggest that the Asp299Gly SNP is associated with a decreased risk for disease (and conversely, that the Asp299 allele is associated with increased risk) (Balistreri et al., 2008; Minoretti et al., 2006). Important differences are found in all of these studies, most notably variations in allele frequency in the populations evaluated, as well as uncharacterized host or environmental factors that could contribute to the observed phenotypes. It seems likely that large-scale population studies will be needed to clarify the role of key TLR SNPs in aging and aging-related diseases. Indeed, genome-wide association studies of SNPs and of gene expression are increasingly being applied to diseases of aging such as Alzheimer's Disease (Gandhi and Wood, 2010; Seshadri et al., 2010), and coronary artery disease (Musunuru and Kathiresan, 2010). These genome-wide approaches are also being applied to elucidate genes or SNPs associated with response or toxicity to specific drugs—so-called pharmacogenomics (Motsinger-Reif et al., 2010). The application of pharmacogenomics to the geriatric age group remains limited at present, but represents an important tool to mitigate the high rates of treatment failure or toxicity in older individuals (Franceschi et al., 2008).

6. Future Perspectives

6.1 TLR agonists and antagonists

Alterations in TLR function in older adults are particularly relevant in view of the increased development and use of TLR agonists and antagonists as pharmacological agents (reviewed in (Hennessy et al.)). Because at present there is little information on the efficacy of such agents in the context of aged individuals, we will provide a selective overview focused on conditions of potential relevance to human aging. In this regard, several TLR agonists are being used as vaccine adjuvants—particularly relevant for an aging population that generally has a reduced response to immunization (Chen et al., 2009). Influenza vaccine formulations

in clinical trials employ the TLR5 agonist flagellin, including vaccines that incorporate highly conserved motifs that offer promise as universal influenza vaccines that would not require yearly reformulation and administration (Adar et al., 2009; Huleatt et al., 2008; Skountzou et al., 2010). Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs are finding use as TLR9-dependent vaccine adjuvants; for example, the addition of a TLR9 agonist to the 7-valent pneumococcal conjugate vaccine significantly enhanced the proportion of vaccine high responders amongst HIV infected adults (Sogaard et al., 2010). At the same time, so-called immunoregulatory sequences acting as inhibitors of TLR7 and TLR9 may have promise as glucocorticoid-sparing agents in the treatment of autoimmune diseases such as lupus (Barrat et al., 2007; Guiducci et al., 2010). Perhaps the most developed TLR-dependent adjuvant is the TLR4 agonist monophosphoryl lipid A (MPL), a derivative of lipid A from LPS, which is already used as an adjuvant in vaccines against human papillomavirus (Cervarix) and in a hepatitis B vaccine approved in Europe (Fendrix). This decreased toxicity appears to be a consequence of the predominant engagement of TRAM/TRIF-dependent pathways, resulting in the upregulation of costimulatory pathways. By contrast, diphosphoryl lipid A activates both MyD88 and TRAM/TRIF signaling, resulting in activation of not only costimulatory protein expression but also proinflammatory cytokine production that could be associated with clinically adverse effects—a particularly important consideration for vaccine administration to older adults (Mata-Haro et al., 2007). Other lipid A analogs have been identified as TLR4 antagonists; one example is eritoran, a compound which has shown activity in animal models of sepsis, as well as in preliminary human studies (Bennett-Guerrero et al., 2007; Czeslick et al., 2006; Mullarkey et al., 2003). Notably, the crystal structure of TLR4 and MD-2 complexed to eritoran has been solved, and should provide important mechanistic information on eritoran and other TLR-binding compounds (Kim et al., 2007). These approaches to adjuvant design based upon dissection of interactions with specific signaling pathways offer great promise in harnessing TLR pathways for pharmacologic manipulation. The extent to which the activity of TLR agonists or antagonists will be preserved or altered in the context of human aging remains to be elucidated; however, results from murine systems indicate that TLR agonists used as adjuvants can substantially improve adaptive immune responses in aged mice (Maue et al., 2009; Sen et al., 2006; Sharma et al., 2008). The use of similar approaches in aging humans represents a potential means to enhance compromised immune responses, such as those related to vaccination, or inhibit inappropriate innate immune activation in the setting of sepsis or autoimmune disease.

6.2 Conclusion

Understanding of the association between age-associated alterations in TLRs in the elderly and the risk and prognosis of infectious diseases and responsiveness to important vaccines is still emerging, with results that provide evidence for both augmented and impaired function in the context of immunosenescence. The reasons for these discrepancies in part may reflect different experimental methods and heterogeneity of study populations. In this regard, appropriate use of advanced statistical methods in the design and analysis of human aging studies will help to make the data more interpretable and applicable to aging populations.

At the same time, there appears to be substantial evidence for age-associated dysregulation of TLR function, resulting in both inappropriate activation and impairment in TLR signaling. These divergent effects may occur in different cell types and contexts, but could occur concurrently, for example with basal levels of cytokines limiting further induction upon subsequent TLR activation. The mechanisms underlying these changes in TLR biology remain incompletely understood, but are likely to be multifactorial—reflecting changes in TLR expression arising through both transcriptional and post-transcriptional mechanisms, as well as changes in specific pathways regulating TLR signal transduction. Ongoing studies of

pathways implicated from recent findings should reveal new biological insights. In view of the likely overall complexity of immunosenescence of TLR function, additional advances may arise through the use of genomic, proteomic, and other systems biology approaches applied to human aging. Ultimately, the goals of these approaches are to identify processes and pathways amenable to therapeutic manipulation to improve innate immune responses in older individuals, and improve outcomes from infectious diseases and immunization.

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