Published in final edited form as:

Clin Immunol. 2012 April ; 143(1): 73–82. doi:10.1016/j.clim.2012.01.007.

Dampened ERK Signaling in Hematopoietic Progenitor Cells in Rheumatoid Arthritis

Inés Colmegna^{1,2}, Sergey Pryshchep^{1,3}, Hisashi Oishi^{1,4}, Jörg J. Goronzy¹, and Cornelia M. Weyand^{1,*}

¹Department of Medicine, Division of Immunology and Rheumatology, Stanford University

Abstract

In rheumatoid arthritis (RA), hematopoietic progenitor cells (HPC) have age-inappropriate telomeric shortening suggesting premature senescence and possible restriction of proliferative capacity. In response to hematopoietic growth factors RA-derived CD34⁺ HPC expanded significantly less than age-matched controls. Cell surface receptors for stem cell factor (SCF), Flt 3-Ligand, IL-3 and IL-6 were intact in RA HPC but the cells had lower transcript levels of cell cycle genes, compatible with insufficient signal strength in the ERK pathway. Cytokine-induced phosphorylation of ERK1/2 was diminished in RA HPC whereas phosphorylated STAT3 and STAT5 molecules accumulated to a similar extent as in controls. Confocal microscopy demonstrated that the membrane-proximal colocalization of K-Ras and B-Raf were less efficient in RA-derived CD34⁺ cells. Thus, hyporesponsiveness of RA HPC to growth factors results from dampening of the ERK signaling pathways; with a defect localized in the very early steps of the ERK signaling cascade.

Keywords

rheumatoid arthritis; hematopoietic progenitor cells; CD34; ERK signaling; STAT signaling

1. Introduction

Rheumatoid arthritis (RA) is a prototypic autoimmune disease in which chronic inflammation of the synovium leads to irreversible joint damage. Joint inflammation is associated with a systemic inflammatory syndrome, acceleration of atherosclerosis, and shortening of life expectancy. Signaling and effector pathways involving components of both the innate and adaptive immune system have been implicated in the pathophysiology of RA as well as in the cluster of associated immune-inflammatory conditions, i.e. coronary artery disease and lymphoma susceptibility [1]. A common denominator in RA immunobiology is that of a premature immune senescence phenotype with accelerated

© 2012 Elsevier Inc. All rights reserved.

*Corresponding author: Cornelia M. Weyand, M.D., Ph.D., Stanford University School of Medicine, Division of Immunology and Rheumatology, 269 Campus Drive West, Stanford, CA 94305-5166; cweyand@stanford.edu, Telephone: (650) 723-9027, Fax: (650) 721-1251.

²Present Address: Department of Medicine, McGill University, Montreal Canada.

³Present Address: Georgia Institute of Technology, Atlanta, GA

⁴Present Address: University of Tsukuba, Ibaraki, Japan

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

telomeric attrition, a surrogate marker for cellular aging [2]. Age inappropriate telomeric shortening in RA is found not only in lineage committed immune cells but also in their progenitors [3].

The daily production of billions of blood cells is essential to maintain the integrity of the human hematopoietic system. Hematopoietic stem cells, the best-characterized type of adult stem cells, give rise to all blood cell lineages [4]. In humans, these cells are contained within the subset of CD34⁺ hematopoietic progenitor cells (HPC). Functionally, a strict regulation of HPC cell cycle entry and proliferation is required to maintain homeostasis, with HPC proliferation being under tight control of hematopoietic cytokines [5].

In RA, the frequencies of bone marrow and circulating CD34⁺ HPC are reduced [3, 6, 7], the median apoptotic index of early bone marrow myeloid precursors is significantly higher than in controls [6, 8] and their proliferative activity is impaired [3, 7]. These defects seem to be independent of disease activity, severity or the chronic use of immunosuppressive drugs [3, 7]. Factors related to the depletion of circulating HPC in RA include a reduced bone marrow reserve, increased HPC attrition [7] and a “pseudo-reduction” secondary to relocation of HPC to peripheral tissues [9]. Reduced colony formation by highly purified RA CD34⁺ cells may be linked to an intrinsic defect in the clonogenic potential of RA progenitors; the molecular pathways responsible for this abnormality remain undetermined.

Herein, we explored mechanisms that underlie the impairment of HPC proliferation following activation with synergistic cytokines. We found that the HPC surface expression of membrane receptors that initiate cytokine induced-intracellular signaling is well maintained. To assess signal transduction cascades that originate from hematopoietin receptor activation, the accumulation of phosphorylated extracellular-signal activated kinase (pERK1/2) and signal transducer and activator of transcription (pSTAT3 and pSTAT5) were quantified. Findings of reduced pERK accumulation in RA CD34 cells lead to explorations of proximal signaling events, which demonstrated defects in RAS/RAF clustering into the signalosome.

2. Patients and Methods

2.1. Study subjects

The Institutional Review Board approved the study, and all participants provided written informed consent. Fifty milliliters of blood were collected, coded, and those who performed the experiments were not aware of the disease/control status of the subjects from whom the samples were obtained.

Samples from one hundred and seven consecutive adult patients attending the Rheumatology Clinic with the diagnosis of seropositive RA (American College of Rheumatology 1987 revised criteria) were studied. The study protocol required that the patients had unequivocal evidence for rheumatoid factor with levels higher than 40 units/ml (measured by nephelometry) considered positive. Patients were accepted for enrollment if previous autoantibody testing was documented in the records. Most of the patients enrolled into the study were participants in the CLEAR registry [10]. As previously reported, 85–87% of patients enrolled in the CLEAR cohort have positive anti-CCP antibodies and more than 96% are positive for rheumatoid factors. More than 80% of CLEAR patients are positive for both autoantibodies. One hundred and three demographically matched healthy volunteers (controls) were enrolled. Pregnancy, history of cancer, sarcoidosis, active or chronic infection, and crystal arthropathy were exclusion criteria.

The demographic characteristics of RA patients and healthy controls as well as the clinical characteristics of the patients are summarized in Supplementary Table 1. As CD34⁺ HPC represent a low-frequency population not all phenotypic and functional experiments were performed in each sample. The demographic features of patients and controls from each experimental set were matched and did not differ between different experimental groups.

2.2. CD34⁺ cells isolation

Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation (Mediatech, Herndon, VA). CD34⁺ cells were purified using the CD34 Microbead Kit (Miltenyi Biotec, Auburn, CA) and sorted by magnetic-activated cell sorting analysis (AutoMACS, Miltenyi Biotec). The number of HPC per sample was 60,000 to 110,000 cells. Assessment of cell viability was made by Trypan blue exclusion. CD34⁺ cell purity evaluated by flow cytometry was higher than 85% in all experiments.

2.3. Carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye labeling

Freshly isolated CD34⁺ cells were stained with CFSE and expanded for four days in a defined medium for hematopoietic cells (StemSpan H3000; StemCell Technologies, Vancouver, BC, Canada) supplemented with Flt 3-Ligand (Flt 3-L, 100 ng/ml), stem cell factor (SCF, 100 ng/ml), interleukin-3 (IL-3, 20 ng/ml), and IL-6 (20 ng/ml) (StemSpan CC100; StemCell Technologies). At day 4, the percentage of CD34⁺ cells within different generations was compared between patients and controls.

2.4. Flow cytometric detection of cell surface cytokine receptors

The expression of IL-3 receptor alpha and beta subunits, c-Kit (SCF receptor), Flt 3 (Flt 3-L receptor) and IL-6 receptor alpha and gp130 subunits on the cell surface of purified CD34⁺ was determined by flow cytometry (BD LSRII; Becton Dickinson Co, Mountain View, CA). CD34⁺ cells were first treated with Fc receptor blocking reagent (Miltenyi Biotec) and subsequently incubated with an immunoglobulin (Ig) isotype-matched control or a monoclonal antibody specific for the receptor [fluorescein isothiocyanate (FITC)-anti-CD123 (Miltenyi Biotec), phycoerythrin (PE)-anti-CD131 (eBioscience, Inc, San Diego, CA), allophycocyanin (APC)-anti-CD34, PE-anti-CD117, PE-anti-CD135, PE-anti-CD130 and Alexa Fluor 488 labeled anti-CD126 – all from BD Biosciences]. Data were collected and analyzed using FlowJo software gating specifically on cells that expressed the CD34 surface marker.

2.5. Flow cytometric detection of intracellular phospho-protein expression (phosphoflow)

Healthy control and RA samples were run in pairs to decrease the interexperiment variation. In each experiment, controls and patients were matched for age $\pm$ 3 years. Isotype controls were used to account for nonspecific phospho-antibody binding (Supplementary Figure 1A). Unstimulated samples stained with the phospho-specific antibodies were used as negative controls to assess changes in phospho-epitope expression. For validation of the specificity of the ERK1/2 phospho-antibody in HPC, CD34⁺ cells were preincubated with 10 μ M of the MEK inhibitor U0126 for 30 minutes prior to cytokine stimulation (Supplementary Figure 1B). Kinetics of protein phosphorylation by reverse time course were performed to ascertain the optimal stimulation time (Supplementary Figure 1C). Baseline (unstimulated) and postcytokine activation phosphorylation states of ERK1/2, STAT3 and STAT5 were determined by single-cell phospho-protein analysis by flow cytometry (phosphoflow) using a previously described procedure with modifications [11]. Aliquots of each sample were either kept unstimulated or treated with a cytokine cocktail composed by rh Flt 3-L (100 ng/ml), rh SCF (100 ng/ml), rh IL-3 (20 ng/ml) and rh IL-6 (20 ng/ml) (Stem Cell Technologies) for a previously established optimal stimulation time. Cells were fixed with

4% paraformaldehyde (Fixation Buffer, BD Cytotfix, BD Biosciences) for 10 minutes at 37°C, washed twice and permeabilized with ice-cold 70% methanol (Perm Buffer II, BD PhosFlow, BD Biosciences) for 30 minutes, and stained with surface and phosphospecific antibodies (all from BD-Pharmingen, San Diego, CA): mouse anti-human CD34:APC, anti-phospho-ERK1/2 (T202/Y204):PE, anti-Phospho-Stat3 (Y705):Alexa Fluor 488, anti-phospho-Stat5 (Y694):PE. Flow cytometry was performed on a BD LSRII flow cytometer. After acquisition, data were analyzed using FlowJo software gating specifically on cells that expressed the CD34 surface marker to generate MFI values for each phosphoprotein (Supplementary Figure 1).

2.6. RT-PCR

Total RNA from CD34⁺ HPC was extracted with TRIzol, and cDNA was synthesized with AMV-reverse transcriptase (Roche) and random hexamers (Roche). PCR was performed with 200 nM of sense and anti-sense primer using a Stratagene Mx3000P (Agilent Technologies, Cedar Creek, TX); 3 mM MgCl₂, 0.2X SYBR Green; and 0.75 U Platinum Taq (Invitrogen, Carlsbad, CA). Gene primer sequences are shown in Supplementary Table 2. Copy numbers were calculated by comparing each sample with a standard curve generated by purified PCR products and serial dilutions. The samples copy numbers were adjusted to β -actin expression.

2.7. Ras/Raf colocalization

Isolated CD34⁺ cells were aliquoted and either stimulated with a cytokine cocktail (StemSpan CC100, StemCell Technologies) or left untreated. After 10 minutes cells were fixed, permeabilized and stained with the following antibodies: mouse anti-human CD34:APC, K-Ras (F234) sc-30 mouse monoclonal (Santa Cruz Biotechnology) labeled with Alexa Fluor 546 anti-mouse IgG2A (Invitrogen), and B-Raf (H-145) sc-9002 rabbit polyclonal (Santa Cruz Biotechnology) labeled with Alexa Fluor 488 rabbit IgG (Invitrogen). The samples were analyzed by a Zeiss laser-scanning LSM 510 META confocal microscope (Carl Zeiss, Thornwood, NY) using a Plan-Apochromat 63x/1.4 Oil DIC objective. Z-series of optical sections were performed at 0.2 μ m. Cross-correlation coefficients (colocalization coefficients) of K-Ras/B-Raf in CD34⁺ cells' membrane were analyzed by Imaris software (Bitplane, St. Paul, MN) in a minimum of 20 cells per sample.

2.8. Statistical analysis

For quantitative variables, differences between groups were analyzed by Mann-Whitney U test or paired T-test as appropriate. The significance of differences between categorical variables was determined by chi-square analysis. With the exception of Figure 1D, data are presented as scattered dot plots with lines representing the median. In Figure 1D, means and SEM are shown. Statistical analysis and graphics were performed using STATA/SE version 8.0 and GraphPad Prism version 5 software respectively.

3. Results

3.1. Reduced proliferative capacity of RA CD34⁺ HPC

Regulation of HPC proliferative capacity is a critical component in the homeostatic control of the hematopoietic system. Growth factors are required for the survival and proliferation of hematopoietic cells at all stages of development. Flt 3-L, SCF, IL-3, and IL-6 are early acting hematopoietins that synergistically stimulate the proliferation and differentiation of HPC [12]. In aged hematopoietic progenitors, proliferative defects result in inadequate ability to cope with the demands of blood production and alter lineage-commitment [13, 14]. Since features of premature immune senescence affect the RA immune system [2, 15], we

examined whether premature aging also involves the RA HPC compartment. Hematopoietin-induced HPC proliferative responses were evaluated in RA and matched controls by CFSE assay (Figure 1A). The percentage of cells that performed 1 to >4 cell cycles within 96 hours of cytokine activation were measured as shown in Figure 1B. Four days after stimulating with a cocktail of four hematopoietic growth factors (SCF, Flt 3-Ligand, IL-3, IL-6), almost all control CD34⁺ cells had entered the cell cycle. In contrast, in more than half of the patients a portion of CD34 cells failed to respond to hematopoietic growth factors and remained undivided ($p=0.04$) (Figure 1C). Not only cell cycle entry but also progression was impaired in RA HPC with a higher frequency of patients' CD34 cells performing only one and two divisions in contrast to control HPC that mostly achieved four or more cycles (Figure 1D).

3.2. Intact expression of cell surface growth factor receptors

Since cellular responsiveness is largely determined by the regulated expression of ligand-specific receptors [16], we examined whether the reduced proliferative response of RA HPC was linked to altered expression of cytokine receptors. The basal expression of IL-3 receptor alpha (CD123) and beta (CD131) subunits, IL-6 receptor alpha (CD126) and beta (CD130) subunits, SCF receptor (c-Kit=CD117) and Flt 3 (CD 135) on CD34⁺ cells was assessed by flow cytometry (Figure 2). The frequency of cells expressing each of the different hematopoietin receptor subunits as well as the mean fluorescence intensity (MFI) of those receptors on CD34⁺ cells was similar in RA and controls.

3.3. Reduced expression of ERK-transcriptional targets involved in proliferative responses of RA HPC

To understand why RA HPC responded less vigorously to growth factor signals we analyzed signaling pathways that regulate HPC proliferation. Binding of hematopoietic growth factors to HPC essentially triggers ERK and STAT signaling cascades. ERK directly activates c-Myc, which by upregulating all three D-cyclins impacts the cell cycle machinery and maintains the proliferation and expansion of HPC [17, 18].

Pilot experiments screening RA and control CD34⁺ cells by microarray analysis showed that the expression of *c-Myc* was down-regulated in RA. Transcript levels for *c-Myc* and its effectors (*Cyclin D1*, *D2* and *D3*) were quantified in freshly isolated HPC by real time PCR. *c-Myc*-specific transcripts were markedly reduced in RA HPC (Figure 3). *Cyclin D1* mRNA was expressed at low abundance in both control and RA HPC. The c-Myc-dependent cell cycle regulators *Cyclin D2* and *D3* were detected in cells from controls and patients but mRNA levels were significantly decreased in RA HPC (Figure 3).

3.4. Decreased ERK phosphorylation in RA CD34⁺ cells

In CD34 cells binding of SCF and Flt 3-L induces rapid phosphorylation of the Ras/Raf/MEK/ERK signal transduction cascade, whereas IL-3 elicits activation of the STAT5 pathway and IL-6 activates the STAT3 pathway [19–23]. To interrogate and compare cytokine evoked signaling responses in CD34 cells we used phosphospecific flow cytometry (Supplementary Figure 1). The involvement of MEK-specific activation of the pERK response in HPC was confirmed by exposing primary samples to a MEK inhibitor prior to cytokine stimulation, which abrogated pERK response (Supplementary Figure 1B). To define optimal stimulation times, kinetics of ERK phosphorylation were assessed in RA and controls HPC by reverse time course following IL-3, IL-6, Flt 3-L, and SCF activation (Supplementary Figure 1C). HPC phosphorylation states of ERK1/2 were tested at baseline and maximal activation (10 and 15 minutes post-cytokine stimulation) in 12 RA and control pairs. At all three time points, the levels of pERK in CD34⁺ cells were significantly lower in RA patients (Figure 4A–C).

3.5. STAT3 and STAT5 tyrosine phosphorylation is preserved in RA HPC

To examine whether the functioning of intracellular signaling networks was broadly impaired in RA HPC or whether the defect in accumulating pERK was pathway-specific, we assessed cytokine-induced phosphorylation of STAT3 and STAT5 proteins [24]. STAT3 and STAT5 phosphorylation kinetics were determined in RA and control HPC by reverse time course following IL-3, IL-6, Flt 3-L, and SCF activation. Baseline and maximal activation (10 and 20 minutes post-cytokine stimulation) of pSTAT3 and STAT5 were tested in HPC from 12 RA and control pairs (Figure 5). Results demonstrated that the activation of the STAT pathway was intact in RA HPC (Figure 5A–F). Baseline pSTAT3 was higher in RA HPC (Figure 5A), suggesting ongoing STAT3 activation even under resting conditions.

3.6. Defects in MAPK pathway proximal signaling events

To further localize the defect leading to insufficient ERK phosphorylation, we analyzed proximal events responsible for the activation of the MAPK pathway. Following cytokine-receptor interaction, GTP bound active Ras recruits Raf to the cell membrane with Ras/Raf spatial interactions being a prerequisite for MAPK pathway activation. Among the four Ras isoforms, K-Ras is localized exclusively to the plasma membrane and does not participate in signaling from intracellular compartments such as endosomes or the Golgi complex. In addition K-Ras is the isoform that most efficiently activates Raf [21, 25]. Among the Raf family members, B-Raf is the stronger inducer of the Raf/MEK/ERK cascade [26]. We analyzed K-Ras/B-Raf colocalization in resting and cytokine activated CD34⁺ cells by confocal microscopy (Figure 6A). Baseline Ras/Raf colocalization did not differ between RA and controls and in both groups increased following activation. However, cytokine-induced Ras/Raf colocalization in RA CD34 was significantly lower than that in HC (Figure 6B). Following receptor ligation Ras-GTP is immobilized in membrane microclusters to then recruit B-Raf to the membrane [27]. Thus, the impairment in Ras/Raf colocalization in RA HPC implicates a membrane-proximal event in the defective ERK phosphorylation.

4. Discussion

The pathognomonic lesions of RA are localized in the synovial membrane supporting the classical paradigm that recognition of an arthritogenic antigen initiates and sustains inflammation. Yet cellular and molecular abnormalities broadly affect the immune system of RA patients, including immune cell populations that have never been involved in antigen-specific immune responses. The principle of system-wide immune abnormalities is exemplified by the concept that RA patients have premature immune senescence with several phenotypic and functional defects recapitulating changes otherwise present in the healthy immune system of much older individuals [28, 29]. Here we add support to the notion that prematurity of immune aging broadly affects the immune system of RA patients, including the hematopoietic progenitor cells that give rise to all lineages of myeloid and lymphoid cells. Specifically, HPC isolated from RA patients are hyporesponsive to hematopoietic growth factors and fail to expand appropriately. This defect appears specifically connected to the ERK signaling pathway, a signaling cascade critically involved in cell cycle entry and progression of progenitor cells. Data reported here extend the spectrum of molecular abnormalities of RA HPC which have previously been reported to have age-inappropriate erosion of telomeres [3] and emphasize a potentially important role of the bone marrow in the tolerance defect that characterizes RA.

Insufficient cellular expansion of HPC, dampened ERK phosphorylation and diminished production of c-myc are all consistent with a proliferation defect seen in senescent cells. The proliferative and regenerative capacity of human HPC diminishes with advancing age, a loss-of-function that has been implicated in the decreased disease-free survival of bone

marrow recipients transplanted with grafts from aged donors [14]. In RA, similar to aged donors, HPC resist entering the cell cycle and are delayed in their progression through subsequent cell divisions. Precocious aging of HPC has a number of implications for the patients including insufficiency of regenerating hematopoietic cells. Considering the daily turnover of blood cells, it would be expected that RA HPC proliferative defects may adversely affect immune regeneration contributing to cytopenias (anemia and, less frequently, to neutropenia).

Experiments presented here were designed to address the underlying molecular defect of the sluggish proliferative response of the RA HPC. Analysis of mice in which spontaneous or engineered mutations prevent the appropriate expression of cytokine receptors or their intracellular signals [30] have unequivocally confirmed an indispensable physiological role for cytokine action in hematopoietic stem cell regulation. Substantial defects in the numbers or function of stem cells are clearly evident in mice lacking signals from TPO, SCF, IL-6 or LIF, with redundancy in the actions of these cytokines assuring hematopoietic stem cells homeostasis [31–33]. In RA HPC, the frequency of IL-3, IL-6, SCF and Flt 3 receptors and their density on the cell surface is intact.

We subsequently addressed the question of whether cytoplasmic signaling cascades were maintained in RA HPC. The STAT family of proteins, specifically STAT3 and STAT5, are important mediators of IL-3 and IL-6 receptor signals [19, 34]. Activation induced pSTAT3 and STAT5 are fully preserved in RA, making the association between these pathways and the HPC proliferative defects unlikely. Interestingly, baseline phosphorylation levels of STAT3 are higher in RA HPC. Constitutive activation of STAT3 may reflect the action of the cytokine milieu in the patient, especially the chronic presence of elevated levels of IL-6. IL-6 mediated gp130 signaling has been linked to defects in hematopoietic differentiation ability of bone marrow precursors [35].

Reduced levels of the critical cell cycle regulator c-myc, in a gene expression microarray that compared spontaneous gene expression profiles in RA and control HPC, provided a clue that the ERK signaling pathway was reduced in efficiency. Myc induces the expression of Cyclin D2 and D3 and promotes the growth of HPC, in contrast downregulation of the level of D cyclins contributes to G1 arrest in HPC [36]. The generic MAPK signaling pathway is shared by four different cascades (ERK1/2, JNK1/2/3, p38-MAPK and ERK5) with growth factors being the major regulators of the ERK1/2 cascade [37]. The ERK pathway involves the sequential phosphorylation of Raf, MAPK/ERK kinase (MEK) and ERK leading to the modulation of cell cycle progression, proliferation, cytokinesis, transcription, differentiation, senescence, cell death, migration, GAP junction formation, actin and microtubule networks, neurite extension and cell adhesion [38]. ERK is spontaneously active in over 50% of acute myeloid leukemias and acute lymphocytic leukemias, and its pharmacological blockade impairs leukemic cell proliferation and clonogenic growth [39, 40]. The induction of D-type cyclins and the stabilization/induction of c-Myc are two key mechanisms involved in ERK1/2 pathway regulation of G1-phase progression. The isolated Raf/MEK/ERK signal transduction cascade is sufficient to induce the expression of the *c-Myc* gene, and the inhibition of the MEK/ERK signaling pathway leads to downregulation of c-Myc expression and reduced cell proliferation. In RA-HPC, baseline and post-activation levels of pERK, as well as the transcript expression of pERK regulated genes (*c-Myc*, *Cyclin D2* and *D3*), are reduced. The defect in MAPK activation in RA HPC was further supported by the upregulation of ERK dependent antiproliferative genes *Tob1*, *Ddit3* and *JunD* in RA CD34 microarrays (data not shown), genes known to be downregulated upon activation of ERK1/2 [41].

To understand the ERK1/2 pathway defect, proximal events were evaluated. Ras proteins are signal-switch molecules that modulate cell fates by cycling between inactive GDP-bound and active GTP-bound conformations. Once activated, Ras interacts with the Raf/MEK/ERK pathway. Data presented here shows that in RA, CD34⁺ cells activation-induced K-Ras/B-Raf colocalization is impaired, supporting the concept of a proximal defect in the ERK pathway.

In the patient cohort examined here, African American patients represented the majority, raising the question whether the observed abnormalities in the ERK signaling pathway are transferrable to other patient populations. In preliminary studies we have tested Caucasian patients with autoantibody positive RA and have confirmed the blunted accumulation of pERK when CD34⁺ cells were stimulated with hematopoietic growth factors (data not shown).

The defective ERK phosphorylation only involves CD34⁺ HPC since effector cells in RA (CD4 and CD8 T-cell subsets) are characterized by a spontaneous activation of the ERK signaling pathway and enhanced responses after CD3/CD28 cross-linking [42]. Thus, dampening of the ERK pathway in HPC is a cell-type specific effect, likely a result of microenvironmental stresses or exposure to inflammatory stimuli. Among the inflammatory cytokines, TNF- α has been considered to have a central pathogenic role in RA. A recent study by Bluml et al. came to the conclusion that the net effect of TNF- α on hematopoietic cells is actually anti-inflammatory [43]. Addition of anti-TNF antibodies to cultures of CD34 cells undergoing expansion did not affect proliferation or apoptosis (data not shown). The possibility remains that other proinflammatory cytokines alter HPC function. In a previous study [3], decreased frequencies of CD34⁺ cells in RA did not correlate to disease activity, disease duration and treatment. Taken together, these data suggest that HPC in RA patients have defects that are not simply an epiphenomenon of inflammation.

Other signaling pathways have been involved in the premature senescence phenotype of RA immune cells. Specifically, chronic activation of the JNK signaling pathway has been associated with chronic cellular stress evoked by DNA breakage and insufficient repair of DNA double strand breaks [44]. In RA T cells, chronic activity of the DNA repair machinery appears to be the upstream event sustaining cellular stress responses and setting the threshold for apoptosis [44, 45]. The defect in maintaining genomic stability and the functional integrity of immune cells also extends to impaired telomeric maintenance [29]. Remarkably, all these molecular defects affect naïve CD4 T cells, reiterating that RA patients have molecular abnormalities that are clearly distant from the memory immune responses underlying chronic synovitis.

An unanswered question is whether the abnormalities reported here as well as the previously reported telomeric structural defects in RA HPC are a consequence of the chronic disease process. Long-lasting exposure of cells in the bone marrow to chronically elevated levels of cytokines may well have an impact on the turn-over of such cells. Changes in cellular demand and excessive attrition of end-differentiated cells of the immune system could also have indirect effects on hematopoiesis.

In summary, RA HPC have a defect in the assembly of the proximal components responsible for the activation of ERK; are not efficient in activating ERK following hematopoietin-induced stimulation; and express decreased cell-cycle related ERK-targeted genes. Our findings in RA patients are consistent with a model that multiple cell types of the immune system have a signature of premature aging. Whether and how HPC aging is connected to the prematurity in the aging process of lymphocyte precursors remains to be elucidated. Also, it would be informative to know whether HPC abnormalities occur in other

inflammatory conditions and what their precise relationship is to the inflammatory milieu. Dampening of the ERK signaling pathways and insufficient production of c-myc may have profound consequences for the regenerative capacity of the hematopoietic system. A better understanding of the membrane-close events that reduce the efficiency of hematopoietic growth factors in driving expansion of RA HPC could hold valuable information in how this cell population can be protected. Similarly, it may be informative to understand how current therapeutic interventions affect HPC signaling and function. Finally, the CD34 population may include cell types that are relevant for other aspects of the rheumatoid disease process, such as endothelial precursor cells. Pinning down the precise molecular events that disrupt the ERK signaling cascade at the level of membrane-proximal steps could have far reaching implications for the understanding of RA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this research was made possible by a grant from the ACR Research and Education Foundation Within Our Reach: Finding a Cure for Rheumatoid Arthritis campaign and from the National Institutes of Health (R01 AI044142 and R01 AR042527).

References

1. Gorman CL, Cope AP. Immune-mediated pathways in chronic inflammatory arthritis. *Best Pract Res Clin Rheumatol*. 2008; 22:221–238. [PubMed: 18455681]
2. Goronzy JJ, Weyand CM. Developments in the scientific understanding of rheumatoid arthritis. *Arthritis Res Ther*. 2009; 11:249. [PubMed: 19835638]
3. Colmegna I, Diaz-Borjon A, Fujii H, Schaefer L, Goronzy JJ, Weyand CM. Defective proliferative capacity and accelerated telomeric loss of hematopoietic progenitor cells in rheumatoid arthritis. *Arthritis Rheum*. 2008; 58:990–1000. [PubMed: 18383391]
4. Martinez-Agosto JA, Mikkola HK, Hartenstein V, Banerjee U. The hematopoietic stem cell and its niche: a comparative view. *Genes Dev*. 2007; 21:3044–3060. [PubMed: 18056420]
5. Metcalf D. Hematopoietic cytokines. *Blood*. 2008; 111:485–491. [PubMed: 18182579]
6. Snowden JA, Nink V, Cooley M, Zaunders J, Keir M, Wright L, Milliken ST, Brooks PM, Biggs JC. Composition and function of peripheral blood stem and progenitor cell harvests from patients with severe active rheumatoid arthritis. *Br J Haematol*. 1998; 103:601–609. [PubMed: 9858207]
7. Porta C, Caporali R, Epis O, Ramaioli I, Invernizzi R, Rovati B, Comolli G, Danova M, Montecucco C. Impaired bone marrow hematopoietic progenitor cell function in rheumatoid arthritis patients candidate to autologous hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2004; 33:721–728. [PubMed: 14743200]
8. Papadaki HA, Marsh JC, Eliopoulos GD. Bone marrow stem cells and stromal cells in autoimmune cytopenias. *Leuk Lymphoma*. 2002; 43:753–760. [PubMed: 12153161]
9. Ruger B, Giurea A, Wanivenhaus AH, Zehetgruber H, Hollemann D, Yanagida G, Groger M, Petzelbauer P, Smolen JS, Hoecker P, Fischer MB. Endothelial precursor cells in the synovial tissue of patients with rheumatoid arthritis and osteoarthritis. *Arthritis Rheum*. 2004; 50:2157–2166. [PubMed: 15248213]
10. Hughes LB, Reynolds RJ, Brown EE, Kelley JM, Thomson B, Conn DL, Jonas BL, Westfall AO, Padilla MA, Callahan LF, Smith EA, Brasington RD, Edberg JC, Kimberly RP, Moreland LW, Plenge RM, Bridges SL Jr. Most common single-nucleotide polymorphisms associated with rheumatoid arthritis in persons of European ancestry confer risk of rheumatoid arthritis in African Americans. *Arthritis Rheum*. 2010; 62:3547–3553. [PubMed: 21120996]

11. Perez OD, Nolan GP. Phospho-proteomic immune analysis by flow cytometry: from mechanism to translational medicine at the single-cell level. *Immunol Rev.* 2006; 210:208–228. [PubMed: 16623773]
12. Bradley HL, Hawley TS, Bunting KD. Cell intrinsic defects in cytokine responsiveness of STAT5-deficient hematopoietic stem cells. *Blood.* 2002; 100:3983–3989. [PubMed: 12393407]
13. Chambers SM, Shaw CA, Gatz C, Fisk CJ, Donehower LA, Goodell MA. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. *PLoS Biol.* 2007; 5:e201. [PubMed: 17676974]
14. Rossi DJ, Jamieson CH, Weissman IL. Stems cells and the pathways to aging and cancer. *Cell.* 2008; 132:681–696. [PubMed: 18295583]
15. Weyand CM, Fujii H, Shao L, Goronzy JJ. Rejuvenating the immune system in rheumatoid arthritis. *Nat Rev Rheumatol.* 2009; 5:583–588. [PubMed: 19798035]
16. Tajima S, Tsuji K, Ebihara Y, Sui X, Tanaka R, Muraoka K, Yoshida M, Yamada K, Yasukawa K, Taga T, Kishimoto T, Nakahata T. Analysis of interleukin 6 receptor and gp130 expressions and proliferative capability of human CD34+ cells. *J Exp Med.* 1996; 184:1357–1364. [PubMed: 8879208]
17. Yu Q, Ciemerych MA, Sicinski P. Ras and Myc can drive oncogenic cell proliferation through individual D-cyclins. *Oncogene.* 2005; 24:7114–7119. [PubMed: 16103884]
18. Murphy MJ, Wilson A, Trumpp A. More than just proliferation: Myc function in stem cells. *Trends Cell Biol.* 2005; 15:128–137. [PubMed: 15752976]
19. Woost PG, Solchaga LA, Meyerson HJ, Shankey TV, Goolsby CL, Jacobberger JW. High-resolution kinetics of cytokine signaling in human CD34/CD117-positive cells in unfractionated bone marrow. *Blood.* 117:e131–141. [PubMed: 21330471]
20. Miranda MB, Xu H, Torchia JA, Johnson DE. Cytokine-induced myeloid differentiation is dependent on activation of the MEK/ERK pathway. *Leuk Res.* 2005; 29:1293–1306. [PubMed: 16164983]
21. Shelton JG, Steelman LS, Lee JT, Knapp SL, Blalock WL, Moye PW, Franklin RA, Pohnert SC, Mirza AM, McMahon M, McCubrey JA. Effects of the RAF/MEK/ERK and PI3K/AKT signal transduction pathways on the abrogation of cytokine-dependence and prevention of apoptosis in hematopoietic cells. *Oncogene.* 2003; 22:2478–2492. [PubMed: 12717425]
22. Chang F, Steelman LS, Lee JT, Shelton JG, Navolanic PM, Blalock WL, Franklin RA, McCubrey JA. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia.* 2003; 17:1263–1293. [PubMed: 12835716]
23. Ronnstrand L. Signal transduction via the stem cell factor receptor/c-Kit. *Cell Mol Life Sci.* 2004; 61:2535–2548. [PubMed: 15526160]
24. Si J, Collins SJ. IL-3-induced enhancement of retinoic acid receptor activity is mediated through Stat5, which physically associates with retinoic acid receptors in an IL-3-dependent manner. *Blood.* 2002; 100:4401–4409. [PubMed: 12393611]
25. Kenworthy AK. Nanoclusters digitize Ras signalling. *Nat Cell Biol.* 2007; 9:875–877. [PubMed: 17671455]
26. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, Stivala F, Libra M, Basecke J, Evangelisti C, Martelli AM, Franklin RA. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta.* 2007; 1773:1263–1284. [PubMed: 17126425]
27. Tian T, Harding A, Inder K, Plowman S, Parton RG, Hancock JF. Plasma membrane nanoswitches generate high-fidelity Ras signal transduction. *Nat Cell Biol.* 2007; 9:905–914. [PubMed: 17618274]
28. Schonland SO, Lopez C, Widmann T, Zimmer J, Bryl E, Goronzy JJ, Weyand CM. Premature telomeric loss in rheumatoid arthritis is genetically determined and involves both myeloid and lymphoid cell lineages. *Proc Natl Acad Sci U S A.* 2003; 100:13471–13476. [PubMed: 14578453]
29. Fujii H, Shao L, Colmegna I, Goronzy JJ, Weyand CM. Telomerase insufficiency in rheumatoid arthritis. *Proc Natl Acad Sci U S A.* 2009; 106:4360–4365. [PubMed: 19255426]

30. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell*. 2000; 103:211–225. [PubMed: 11057895]
31. Lyman SD, Jacobsen SE. c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood*. 1998; 91:1101–1134. [PubMed: 9454740]
32. McKenna HJ, Stocking KL, Miller RE, Brasel K, De Smedt T, Maraskovsky E, Maliszewski CR, Lynch DH, Smith J, Pulendran B, Roux ER, Teepe M, Lyman SD, Peschon JJ. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood*. 2000; 95:3489–3497. [PubMed: 10828034]
33. Thoren LA, Liuba K, Bryder D, Nygren JM, Jensen CT, Qian H, Antonchuk J, Jacobsen SE. Kit regulates maintenance of quiescent hematopoietic stem cells. *J Immunol*. 2008; 180:2045–2053. [PubMed: 18250409]
34. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001; 414:105–111. [PubMed: 11689955]
35. Coppo P, Dusanter-Fourt I, Millot G, Nogueira MM, Dugray A, Bonnet ML, Mitjavila-Garcia MT, Le Pesteur D, Guilhot F, Vainchenker W, Sainteny F, Turhan AG. Constitutive and specific activation of STAT3 by BCR-ABL in embryonic stem cells. *Oncogene*. 2003; 22:4102–4110. [PubMed: 12821944]
36. Ando K, Ajchenbaum-Cymbalista F, Griffin JD. Regulation of G1/S transition by cyclins D2 and D3 in hematopoietic cells. *Proc Natl Acad Sci U S A*. 1993; 90:9571–9575. [PubMed: 8415743]
37. Katz M, Amit I, Yarden Y. Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochim Biophys Acta*. 2007; 1773:1161–1176. [PubMed: 17306385]
38. Balmano K, Cook SJ. Tumour cell survival signalling by the ERK1/2 pathway. *Cell Death Differ*. 2009; 16:368–377. [PubMed: 18846109]
39. Milella M, Konopleva M, Precupanu CM, Tabe Y, Ricciardi MR, Gregorj C, Collins SJ, Carter BZ, D'Angelo C, Petrucci MT, Foa R, Cognetti F, Tafuri A, Andreeff M. MEK blockade converts AML differentiating response to retinoids into extensive apoptosis. *Blood*. 2007; 109:2121–2129. [PubMed: 17077328]
40. Kornblau SM, Womble M, Qiu YH, Jackson CE, Chen W, Konopleva M, Estey EH, Andreeff M. Simultaneous activation of multiple signal transduction pathways confers poor prognosis in acute myelogenous leukemia. *Blood*. 2006; 108:2358–2365. [PubMed: 16763210]
41. Yamamoto T, Ebisuya M, Ashida F, Okamoto K, Yonehara S, Nishida E. Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow cell-cycle progression. *Curr Biol*. 2006; 16:1171–1182. [PubMed: 16782007]
42. Singh K, Deshpande P, Pryshchep S, Colmegna I, Liarski V, Weyand CM, Goronzy JJ. ERK-dependent T cell receptor threshold calibration in rheumatoid arthritis. *J Immunol*. 2009; 183:8258–8267. [PubMed: 20007589]
43. Bluml S, Binder NB, Niederreiter B, Polzer K, Hayer S, Tauber S, Schett G, Scheinecker C, Kollias G, Selzer E, Bilban M, Smolen JS, Superti-Furga G, Redlich K. Antiinflammatory effects of tumor necrosis factor on hematopoietic cells in a murine model of erosive arthritis. *Arthritis Rheum*. 2010; 62:1608–1619. [PubMed: 20155834]
44. Shao L, Goronzy JJ, Weyand CM. DNA-dependent protein kinase catalytic subunit mediates T-cell loss in rheumatoid arthritis. *EMBO Mol Med*. 2:415–427. [PubMed: 20878914]
45. Shao L, Fujii H, Colmegna I, Oishi H, Goronzy JJ, Weyand CM. Deficiency of the DNA repair enzyme ATM in rheumatoid arthritis. *J Exp Med*. 2009; 206:1435–1449. [PubMed: 19451263]

Highlights

- Hypo-proliferation of hematopoietic progenitor cells (HPC) in rheumatoid arthritis
- Reduced c-myc expression in RA progenitor cells
- Intact phosphorylation of STAT3 and STAT5 in RA HPC
- Impaired activation of the ERK signaling pathway in RA HPC
- Insufficient co-localization of K-Ras and B-Raf in proximal ERK signaling

Figure 1. Hyporesponsiveness of RA HPC to growth factor-induced proliferation
(A) Gating strategy to evaluate CD34 proliferation. **(B)** Percentage of CD34 cells that performed 1, 2, 3, 4 or more than 4 cycles at day 4. **(C)** Percentage of CD34⁺ cells that remained undivided at day 4. **(D)** Percentage of cells that undergone the indicated number of cell cycles at day 4 (*control vs. RA $p < 0.001$). Data presented as median $\pm$ interquartile range.

Figure 2. Cytokine receptor expression on control and RA CD34⁺ cells
 CD34 cell surface expression of IL-3 receptor alpha (CD123) and beta (CD131) subunits, IL-6 receptor alpha (CD126) and beta (CD130) subunits, SCF receptor (c-Kit=CD117) and Flt 3-L receptor (CD 135).

Figure 3. Reduced transcript expression of pERK regulated genes

Transcripts of the pERK target genes *c-Myc*, *Cyclin D2* and *D3* were analyzed by real time PCR in CD34 from RA and controls and standardized to β -actin expression.

Figure 4. Phosphorylation of ERK1/2 in resting and cytokine-stimulated CD34⁺ cells
Flow cytometry was applied to measure the intracellular pool of pERK molecules at baseline and 10 and 15 minutes after stimulation in isolated CD34⁺ cells. Results for 12 RA patients and 12 age-matched control donors are shown.

Figure 5. STAT3 and STAT5 activation in RA CD34⁺ cells

Baseline and post-cytokine activation phosphorylation states of STAT3 (A–C) and STAT5 (D–F) were assessed by single-cell phospho-protein analysis. HPC isolated from 12 RA patients and 12 controls were run in pairs and results are expressed as scatter dot plots with lines representing the median.

Figure 6. Proximal events in the activation of the MAPK pathway are defective in RA HPC
 Baseline and post-activation (10 minutes) K-Ras/B-Raf colocalization was determined by confocal microscopy. **(A)** Representative middle plane of the z-axis. **(B)** Colocalization of Ras and Raf molecules quantified by comparing the correlation coefficient of red and green pixels in 100 individual CD34 cells. Data from independent experiments are presented with bars representing medians.