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Immune responses to citrullinated neoantigens and clinical efficacy of costimulation blockade indicate a general defect in maintaining T cell tolerance in rheumatoid arthritis (RA). To examine whether TCR threshold calibration contributes to disease pathogenesis, signaling in RA T cells was quantified. RA patients had a selective increase in ERK phosphorylation compared with demographically matched controls due to a mechanism distal of Ras activation. Increased ERK responses included naive and memory CD4 and CD8 T cells and did not correlate with disease activity. The augmented ERK activity delayed SHP-1 recruitment to the TCR synapse and sustained TCR-induced Zap70 and NF-κB signaling, facilitating responses to suboptimal stimulation. Increased responsiveness of the ERK pathway was also a characteristic finding in the SKG mouse model of RA where it preceded clinical symptoms. Treatment with subtherapeutic doses of a MEK-1/2 inhibitor delayed arthritis onset and reduced severity, suggesting that increased ERK phosphorylation predisposes for autoimmunity and can be targeted to prevent disease. The Journal of Immunology, 2009, 183: 8258–8267.

In rheumatoid arthritis (RA), the adaptive immune system exhibits abnormalities that go beyond the local inflammatory response in the synovium and that are instructive to our understanding of the pathogenesis of the disease (1–3). Although joint-specific Ags entertaining the disease process have only been poorly defined and may not be relevant at all, it is well established that patients with RA have autoimmune responses to common Ags. Autoantibodies to the IgG Fc fragment are a laboratory hallmark of the disease. Even more characteristic is an immune response to citrullinated neoantigens suggesting that patients may have a defect in maintaining tolerance to newly arising Ags (4, 5). Abs to citrullinated peptides appear to function by enhancing disease severity (6). The identification of PTPN22 as a disease risk gene has hinted at a defect in central tolerance even before newly arising Ags (6). PTPN22 is a lymphocyte-specific phosphatase that is involved in terminating TCR signaling and calibrating the T cell activation threshold (7). The PTPN22 polymorphism associated with RA is a gain-in-function mutation that could cause a defect in thymic-negative selection, generating a repertoire with higher affinity to self (10, 11). However, a defect in central tolerance does not explain an autoimmune response to neoantigens nor is it consistent with the finding that RA occurs at an age when thymic production has ceased. In fact, age is one of the strongest risk factors for RA, raising the possibility of an age-dependent defect in peripheral tolerance (12).

T cells in patients with RA exhibit several abnormalities that are best summarized as accelerated aging. Signs of an increased history of proliferation are not limited to T effector cells, but also involve naive CD4 and CD8 cells (13). Naive T cells in RA have shortened telomeres, their repertoire diversities are contracted, and the concentrations of TCR excision circles are age-inappropriately reduced, consistent with reduced thymic production (14, 15). Signs of proliferative stress are evident in the memory compartment which is oligoclonally expanded; memory cells display CD28 loss and gain of regulatory MHC class I-recognizing receptors as markers of an extensive replicative history (16–19). The causes for these abnormalities are unclear; one possible explanation is a history of lymphopenia, either due to insufficient thymic production or to accelerated peripheral cell death that leads to compensatory homeostatic proliferation (14, 20–22). Interestingly, several animal models have shown that lymphopenia and the associated increased turnover undermines peripheral tolerance and precipitates disease, presumably due to TCR recalibration and increased responsiveness to low affinity stimulation (23–26).

In this study, we have examined the hypothesis that RA patients have altered signaling thresholds that predispose them to activate autoreactive T cells. Our results show that RA patients have a selective signal augmentation in the Raf-MEK-ERK module. The increased ERK activity initiates a positive feedback loop delaying SHP-1 recruitment to the TCR signaling complex which sustains signaling and facilitates immune responses to suboptimal stimulation. A similar abnormality of increased ERK phosphorylation was identified in the SKG mouse model of RA even before onset of disease. Subtherapeutic doses of MEK-1/2 inhibitor normalized this abnormality and significantly delayed disease onset and reduced disease expression. Our data suggest that an activated amplification loop in the ERK pathway calibrates the TCR activation threshold, which may contribute to disease initiation and progression.
Table I. Demographic characteristics of RA patients and control populations

<table>
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<th>Controls</th>
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<tr>
<td>n</td>
<td>67</td>
<td>54</td>
<td></td>
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<td>49.4 ± 10.2</td>
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<td>43/11</td>
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<td>27/22/1/4</td>
<td>0.471</td>
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</table>

* AA, African American; C, Caucasian; H, Hispanic; A, Asian.

Materials and Methods

Study population and cells

T cells from RA patients and demographically matched healthy controls (Table I) meeting the 1988 American College of Rheumatology Criteria for seropositive RA were isolated by negative selection using the RosetteSep human T cell enrichment mixture (StemCell Technology). All subjects gave written informed consent as per the protocol approved by the Emory University Institutional Review Board. Patients were considered to have active disease when they fulfilled the Food and Drug Administration criteria of morning stiffness >45 min, tender joints >6, swollen joints >3, and erythrocyte sedimentation rate (ESR) >28 (27). For dendritic cell (DC) generation, CD14+ cells isolated from healthy PBMC from anti-CD14-coated magnetic beads (Miltenyi Biotec) were cultured for 6 days in the presence of IL-4 (1000 IU/ml) and GM-CSF (800 IU/ml). Immature DC were activated with LPS (1.0 μg/ml) for 4 h just before use.

Antibodies

Anti-CD3-allophycocyanin Cy7, CD4-PerCP, CD8-PE Cy7, CD45RA-FITC, CD28-PE, CD25-PE, CD69-allophycocyanin, CD137-allophycocyanin, CD154-PE, and Alexa Fluor 647-conjugated anti-phospho-ERK1/2, phospho-Zap70, and phospho-NF-κB were from BD Biosciences. In addition, the following Abs were used: anti-phospho-ERK1/2 and anti-ERK1/2 (Cell Signaling Technology), Abs to N-Ras, K-Ras, Raf-1, and actin (Santa Cruz Biotechnology); anti-SHP-1 (Millipore); and anti-CD3 (BD Biosciences). Zenon Ab labeling kits with Alexa Fluor 488 and Alexa Fluor 546 (Invitrogen) were used to conjugate primary Abs.

Flow cytometry

One × 10⁶ T cells were stimulated with anti-CD3/CD28 mAb (1 μg/ml each), fixed in BD Cytofix buffer; permeabilized by BD Perm Buffer II, and stained for CD3, CD4, CD8, CD28, and CD45RA and the indicated signaling molecules. In selected experiments, control and RA T cells were preincubated for 1 h with 10 μM ERK1/2 inhibitor FR180204 (EMD Biosciences) followed by activation with anti-CD3/anti-CD28 Ab cross-linking in the continuous presence of the inhibitor. Data were acquired and analyzed on an LSR II flow cytometer (BD Biosciences) with FACSDiva software. Alternatively, cells were activated using CD3 and CD28 Ab-coated beads for up to 24 h and stained for activation markers CD25, CD69, CD137, and CD154.

Western blotting

T cells stimulated as described above were lysed in a cell extraction buffer (Invitrogen) supplemented with 1 mM PMSF and a protease inhibitor mixture (Millipore). Membranes were stripped and reprobed for total ERK1/2 expression is dependent on sustained ERK and JNK signaling and a common feature of all T cells, not limited to in vivo-activated cells, but not different in patients and controls.

Confocal microscopy

T cells from controls and RA patients stimulated and fixed as described above were stained with Alexa Fluor 488-labeled anti-CD3 and Alexa Fluor 488-labeled anti-Raf-1 Abs; images were captured by a Zeiss confocal laser-scanning LSM 510 META Axiovert-200 microscope. Amount and localization of K-Ras, N-Ras, and Raf-1 were quantified by using Image J software. Alternatively, control and RA T cells were stained with Alexa Fluor 488-labeled anti-CD3 Ab and mixed with toxic shock syndrome toxin (1.0 ng/ml)-coated DC at a 5:1 ratio. Following centrifugation at 800 rpm for one minute at 4°C, cells were incubated at 37°C for indicated times. Cells were fixed, permeabilized, and stained with Alexa Fluor 488-labeled anti-SHP-1 Ab.

Mice

SKG mice were obtained from Dr. W. E. Seaman (University of California, San Francisco, CA) and housed and bred in the animal facility of Emory University. The experimental protocol was approved by the Emory Institutional Animal Care and Use Committee. Spleen cells isolated from 8-wk-old SKG mice and age-matched BALB/c mice were stimulated with 50 ng/ml PMA and stained for phospho-ERK1/2, CD3, CD4, and CD8. In the treatment studies, 8-wk-old SKG mice (12 per group) were given i.p. injections of the MEK-1/2 inhibitor U0126 (1 mg/kg of body weight; Promega) twice per week or vehicle control; joint inflammation was scored weekly by a person blinded to the treatment. The scoring system suggested by Sakaguchi et al. (0.1 for a small and 0.5 for a large joint) was used (28). In a parallel treatment group, mice were sacrificed after 4 wk of treatment; ankle joints of hind limbs were fixed with 10% buffered formalin for 24 h, immersed in 5% EDTA for 2 days for decalcification, and embedded in paraffin blocks. The sections were stained with H&E and evaluated by light microscopy. Joint sections from control and MEK-1/2 inhibitor-treated mice were stained with H&E and leukocyte infiltration into the joint tissue was compared between the two groups. Synovitis was scored on a graded scale of 0–3 (0, no significant change; 1, mild inflammatory infiltration; 2, pannus formation; and 3, heavy inflammatory infiltration and bone destruction).

Statistical analysis

Mean fluorescence intensities of RA and control T cells were compared using the two-tailed Mann-Whitney U test. The relationship between categorical clinical variables and demographics and pERK levels was examined using the Kruskal-Wallis nonparametric one-way ANOVA; to compare two continuous variables, linear regression analysis was applied. Treatment responses in the SKG mice were assessed using two-way ANOVA.

Results

Increased responsiveness of peripheral RA T cells to TCR stimulation

To screen for differences in T cell activation thresholds, T cells from RA patients and age-matched controls were stimulated with suboptimal concentrations of anti-CD3/anti-CD28-coated beads, and the induction of the activation markers CD25, CD69, CD137, and CD154 were assessed by flow cytometry at 8 and 24 h. Although the frequency of cells expressing activation markers was not different at either time point, RA CD4 naive and memory T cells expressed increased cell surface densities of CD69 and CD154 (Fig. 1). Similar differences between RA patients and controls were found for CD69 expression on CD8 T cells. In contrast, cell surface expression of CD25 was not significantly different. Expression of CD137 was ~5-fold higher in CD8 than CD4 T cells, but not different in patients and controls.

Activation of the ERK signaling pathway in RA T cells

CD69 is a T cell activation marker that is very sensitive to the activity of the dual-specific phosphatase DUSP4 (our unpublished observations). We therefore examined RA T cells for their ERK signaling. Initial studies by Western blotting showed increased anti-CD3-induced ERK phosphorylation in RA patients (Fig. 2A). In a subsequent larger study, we used PhosFlow which has the additional advantage of separately analyzing T cell subsets. T cells from 33 patients with RA and 33 demographically matched controls were purified by negative selection and maintained overnight to exclude effects of cytokines that may be present in RA serum. Basal pERK levels were slightly but significantly increased in RA patients (Fig. 2, B and C). The increased baseline pERK was a common feature of all T cells, not limited to in vivo-activated.
cells and not reduced by overnight culture. The difference in pERK levels was more pronounced in T cells after CD3/CD28 cross-linking (Fig. 2, B and C). In contrast to ERK, p38 phosphorylation did not differ at any of the time points analyzed (Fig. 2, B and D).

Increased ERK phosphorylation does not correlate with disease activity

Data in Fig. 2 were analyzed to address the question of whether pERK in RA T cells correlated with demographic and/or clinical characteristics of the RA population; results are summarized in Table II. Importantly, the disease activity score and the laboratory disease activity markers ESR and C-reactive protein failed to correlate with pERK levels at 5 and 10 min after activation. Treatment with TNF-α-neutralizing reagents (etanercept, infliximab, or adalimumab) correlated with increased pERK levels but inversely and only in CD8 T cells at 5 min; patients on anti-TNF-α were even more different from healthy controls, consistent with previous studies that TNF-α impairs T cell function (30, 31). Methotrexate treatment did not influence stimulation of the ERK pathway. Other treatment regimens were too infrequent to analyze. These data suggest that heightened activation-induced pERK is not a disease activity marker. ERK phosphorylation in CD8 T cells correlated with age; however, this correlation was entirely due to a CD8 subpopulation of CD28− T cells that is increased in the elderly (19). The age-related increase in CD28− T cells with age is also found in healthy controls and is not specific for RA.

Activation of the ERK signaling pathway involves naive and memory T cells from RA patients

ERK pathway calibration may change with T cell activation and differentiation, and the decline in naive T cells and expansion of effector T cell populations have been shown in RA (32, 33). To determine whether the increased ERK activity was due to differential representation of T cell subsets or a higher frequency of activated T cells, we quantified pERK in naive and memory CD4 and CD8 T cells. Representative histograms are shown in supplemental Fig. 2; data from 33 patients and 33 controls are summarized in Fig. 3. Because the fixation need for PhosFlow interferes with CCR5 and CCR7 staining, we used coexpression of CD45RA and CD28 to identify naive cells and loss of CD28 to define end-differentiated effector cells which frequently regain CD45RA expression. ERK phosphorylation was more pronounced but less sustained in naive compared with memory T cells, with the exception of CD8+CD28− effector T cells which exhibited a strong ERK response (Fig. 3). CD4+CD28− T cells were too infrequent in normal individuals to be analyzed. The increased ERK responsiveness in RA was a feature of all CD4 and CD8 T cells including naive and memory T cell subsets.

Signal abnormalities in RA map distal from early TCR signaling

To map the difference in signaling events that distinguishes RA from control T cells, we quantified phosphorylation of Zap70 as a proximal signaling event after TCR engagement which peaks earlier than pERK. Zap70 phosphorylation was indistinguishable between T cells from RA patients and controls up to 5 min after activation (supplemental Fig. 3 and Fig. 4A). There was a trend for RA CD4 T cells to have higher pZap70 at 10 min, which reached significance at 30 min. In CD8 T cells, an increase in Zap70 phosphorylation in RA reached significance at 10 and 30 min. This finding of normal Zap70 phosphorylation early after activation raised the possibility that the increased ERK phosphorylation is not primarily due to increased TCR signaling but may induce a positive feedback loop that sustains Zap70 phosphorylation at later time points in RA T cells. Supporting results for this interpretation were obtained from calcium influx and NF-κB studies. No differences in calcium fluxes were found in the first 10 min after stimulation (data not shown). RA patients and controls did not have different baseline levels of pNF-κB (Fig. 4B). There was no evidence for increased NF-κB phosphorylation in RA T cells in the first 10 min; a difference was only seen at 30 min for CD8 T cells.

Results so far have indicated that the difference between RA patients and normal controls maps to the ERK pathway distal from TCR signaling and that the observed differences in Zap70 and NF-κB activation are secondary events. To address this issue, we examined ERK phosphorylation in RA patients and controls after PMA stimulation. PMA activates protein kinase C, which controls one amplification loop in ERK phosphorylation and RasGRP, which directly stimulates Ras. As seen with TCR stimulation, pERK was increased in RA patients after PMA stimulation (Fig. 4C). Again, naive and memory CD4 and CD8 cells were affected (data not shown).

Ras-Raf complex formation in RA patients

To monitor Raf activation, we examined Ras-Raf-1 complex formation at the cell membrane of T cells stimulated with anti-CD3/anti-CD28. T cells were fixed at the indicated times after TCR stimulation and stained with N-Ras- or K-Ras (red) and Raf-1 (green)-specific Abs (Fig. 5, A and B). Membrane-close fluorescence was quantified; results for five patients and five controls are summarized in Fig. 5, C–H. K-Ras baseline levels tended to be slightly but not significantly higher in RA. After stimulation, neither K-Ras nor N-Ras recruitment was different.
between patients and controls. In contrast, membrane-close Raf-1 concentrations, which were similar at 0 and 2 min, were increased at 5 min in RA T cells, suggesting increased Raf-1 recruitment. The results for Raf-1 were similar regardless of whether it was examined along with K-Ras (Fig. 5D) or N-Ras (Fig. 5G) staining. In Fig. 5, E and H, we used regression analysis of

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Increased activation of the ERK pathway in RA T cells. A, T cells from healthy controls and RA patients were activated by CD3/CD28 cross-linking. Cell lysates were probed for total and phosphorylated ERK by Western blotting. Actin was probed as a loading control. Results are representative of six patients and six controls. B, pERK and p-p38 concentrations in T cells were determined by PhosFlow before and after stimulation with anti-CD3/anti-CD28 cross-linking. Representative histograms of RA (solid lines) and healthy control T cells (dotted lines) are shown; gray fill indicates isotype control. C, Results of 33 healthy controls (open boxes) and 33 RA patients (shaded boxes) are shown as box plots with medians, 25th, and 75th percentiles as boxes and 10th and 90th percentiles as whiskers. D, p-p38 levels after CD3/anti-CD28 cross-linking is shown for 15 patients and 12 controls. E, Five-minute pERK concentrations as shown in C were corrected for baseline levels by subtracting basal pERK from the 5-min pERK levels. Statistical analysis was done using two-tailed Mann-Whitney U test: **, p < 0.01 and ***, p < 0.001. MFI, Mean fluorescence intensity.

### Table II. Influence of demographic/clinical characteristics on pERK levels in RA patients

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<tr>
<th>Characteristic</th>
<th>CD4+ pERK 5 min r</th>
<th>CD4+ pERK 5 min p</th>
<th>CD4+ pERK 10 min r</th>
<th>CD4+ pERK 10 min p</th>
<th>CD8+ pERK 5 min r</th>
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<th>CD8+ pERK 10 min p</th>
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<tr>
<td>Gender</td>
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<td>n/a</td>
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<td>0.83</td>
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<td>Age (years)</td>
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<td>n/a</td>
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<td>−0.07</td>
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<td>0.13</td>
<td>0.49</td>
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<td>0.95</td>
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<td>ESR</td>
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<td>0.33</td>
<td>−0.05</td>
<td>0.79</td>
<td>0.13</td>
<td>0.46</td>
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<td>C-reactive protein</td>
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<tr>
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*The relationship between clinical and demographic characteristics and pERK levels at 5- and 10-min after stimulation in the RA cohort were evaluated. Kruskal-Wallis nonparametric one-way ANOVA was used to compare a categorical and a continuous variable; to compare two continuous variables, linear regression analysis was applied.

bEtanercept, infliximab, and adalimumab.
the red and green pixel fluorescence to determine a coefficient of colocalization. In healthy individuals, the coefficient peaked after 2 min. In contrast, colocalization continued to increase in the RA patient over the first 5 min. The confocal studies suggested that the difference between RA patients and healthy controls can be mapped to sustained Raf-1 activation in the patient. In support of this model, serine-phosphorylated Raf-1 as determined by Western blot was only different at later time points and not immediately after stimulation (Fig. 5).

Increased ERK activation in RA T cells sustains TCR signaling by delaying SHP-1 recruitment

Germain and colleagues (34, 35) proposed a model that assigns pERK, an important function in regulating TCR threshold. In this model, serine phosphorylation of Lck by pERK prevents Lck inactivation and amplifies TCR signaling. To examine whether the increased Zap70 phosphorylation seen in RA T cells at later time points after activation is a primary event due to increased TCR signaling or is due to a secondary feedback loop initiated by the increased ERK phosphorylation, we used a specific ERK inhibitor, FR180204. This inhibitor inhibits the kinase activity of the phosphorylated ERK1/2 and therefore does not inhibit ERK1/2 phosphorylation. Initial induction of ERK phosphorylation was not suppressed by the inhibitor, neither in control subjects nor in RA patients (Fig. 6A). However, ERK activity inhibition reduced pERK in RA patients after 30 min to about the same level as in controls. ERK activity inhibition also had an inhibitory effect on Zap70 phosphorylation in RA patients (Fig. 6B). A trend was already seen 10 min after stimulation and reached significance at 30 min. These data confirm that proximal TCR signaling is normal and that a positive amplification loop involving pERK sustains Zap70 phosphorylation in RA patients.

To examine whether SHP-1 recruitment to the TCR signaling complex is delayed in RA, we used confocal imaging of CD4 T cells interacting with toxic shock syndrome toxin-loaded DC. SHP-1 and CD3 fluorescence in the TCR-DC contact region was quantified (Fig. 6, C–E). CD3 fluorescence increased, reflecting the TCR clustering. SHP-1 recruitment was delayed in RA patients and significantly reduced in the first minutes after T cell activation. To determine whether the delayed SHP-1 recruitment is associated with a lowered TCR threshold in RA patients, ERK phosphorylation was quantified after suboptimal stimulation with anti-CD3/anti-CD28. CD4 and CD8 T cells from RA patients responded to lower Ab doses. The dose-response curve from RA patients had a more sigmoid pattern and drifted apart from the controls at anti-CD3 concentrations of <1 ng/ml (Fig. 6F).

ERK phosphorylation is increased in SKG mice compared with BALB/c mice

Our preliminary analysis did not find an association of increased pERK levels with disease activity (Table II). We, therefore, wanted to explore the possibility that the increased responsiveness preceded disease onset and predisposed for disease. To examine the preclinical situation, we turned to an animal model of RA which has been shown to be T cell dependent. The SKG mouse has a mutation in Zap70 and develops arthritis similar to RA (28). We examined ERK phosphorylation in T cells at an age before the mice develop disease. Results are shown in Fig. 7A.
in RA patients, pERK levels were increased in T cells from 2-mo-old nonarthritic SKG mice compared with normal BALB/c mice. To examine whether the increased ERK responsiveness predisposes for disease, SKG mice (12 mice/group) were treated with solvent control or a MEK-1/2 inhibitor, U0126 (1 mg/kg/injection, twice per week) starting at the age of 8 wk. This dose is subtherapeutic; the normal dosing in cancer treatment trials is 25 mg/kg two times daily. Control-injected mice started to develop disease 2 wk after initiation of treatment. In the MEK-1/2 inhibitor-treated mice, disease onset was delayed and activity was reduced (Fig. 7B). Representative histologies from treated and control mice are shown in Fig. 7C.

FIGURE 5. Sustained Ras-Raf complex formation in RA T cells. T cells were stimulated, fixed, and stained with Abs for signaling molecules. Representative cells stained for Raf-1 and K-Ras (A) or N-Ras (B) are shown. C, D, F, and G, Fluorescence intensities in membrane-gated regions were quantified; results are expressed as fold difference compared with unstimulated control cells and are shown as box plots of 75 cells from 5 patients (shaded boxes) and 5 controls (open boxes). E and H, Colocalization of green and red fluorescence (indicated by yellow in A and B) was examined. Fluorescence for each pixel was correlated and the data are shown as the correlation coefficients. I, T cells were stimulated by CD3 cross-linking; cell lysates were obtained at indicated time points and analyzed by Western blotting for p-c-Raf. Results are representative of three experiments with nine RA patients and nine controls. *, p < 0.05 and **, p < 0.01 by two-tailed Mann-Whitney U test.
Discussion
RA is an autoimmune disease with autoantibodies against common autoantigens such as IgG Fc and citrullinated peptides, a posttranslational modification that increases with progressive age. In this study, we report that RA T cells have a lowered threshold to respond to stimulation. The underlying abnormality is a hyperactive Ras/Raf-MEK-ERK module that delays SHP-1 recruitment to the TCR synapse and sustains and amplifies TCR signaling. Our data are consistent with the model that an increased responsiveness of the ERK module enables an autoimmune response, eventually leading to RA. Resetting the TCR threshold in individuals at risk emerges as an attractive prophylactic intervention to prevent disease.

T cells are intimately involved in the pathogenesis of RA. Earlier studies have emphasized the HLA-DRB1*04 association of the disease and the production of T cell-dependent autoantibodies as evidence that the disease is T cell driven (36–38). Although the most prominent disease manifestations occur in the synovial tissues, RA is primarily a systemic disease and the autoantigens best characterized so far are not limited to the synovium (39). More
recent studies have shown that costimulation blockade by CTLA-4lg is able to ameliorate disease activity (40). Furthermore, PTPN22, which in conjunction with Csk controls TCR signal strength and duration, is a disease risk gene for RA (7, 10). In our initial studies probing T cell responsiveness, we found a relatively selective hyperactivity of the ERK pathway after TCR stimulation. This hyperactivity appeared to be a universal feature of RA patients, making it very unlikely that the increased ERK responsive-

ness is genetically determined; the PTPN22 allelic polymorphism is found in 14% of Caucasian RA patients compared with 9% of healthy individuals (41). Furthermore, neither the linkage studies nor the genome-wide association studies have implicated any region that is directly linked to the ERK pathway suggesting that this abnormality is acquired.

The finding that all CD4 and CD8 T cell subsets were equally affected raised the possibility that an exogenous factor, in particular the cytokine milieu in RA, could reset the threshold for ERK activation. However, we did not find any evidence that the increased ERK responsiveness was a disease activity marker. Multivariate analysis in our patient population did not show any correlation with disease activity or acute phase response. In vitro studies confirmed that inflammatory cytokines did not induce increased sensitivity of the ERK module. Exposure of normal or patient T cells to TNF and IL-1β did not have any effect on TCR calibration (data not shown). IL-6 had a limited effect exclusively on naive CD4 T cells and did not reproduce the in vivo pattern that included CD8 and memory T cell subsets. Furthermore, the ERK hyperresponsiveness was not lost when highly purified T cells were kept ex vivo in culture medium overnight (data not shown). Consistent with these in vitro data, in vivo TNF blocking did not reduce ERK responsiveness in T cells (Table II). These data indicate that the inflammatory cytokine milieu present in RA patients is not responsible for sensitizing the ERK pathway.

The signaling abnormality appears to be at the level of or distal of Ras/Raf association. Increased pERK did not correlate with other TCR-induced signals such as Zap70, NF-κB, or p38 phosphorylation. Increased responses in these pathways at later time points could be normalized by pERK kinase inhibition and were therefore secondary to the increased ERK activity. Also, PMA was able to reproduce the ERK hyperresponsiveness in RA patients. Protein kinase C affects the ERK modulation on at least two levels: it directly activates Ras and it also initiates an amplification loop by dissociating Raf-1 from RKIP and increasing the availability of free Raf-1. Confocal studies confirmed that the increased activation of the ERK pathway is distal to Ras-mediated Raf-1 activation. Although in the first 2 min after TCR activation, recruitment of Raf-1 to either N-Ras or K-Ras in the cytoplasmic membrane was not different in RA patients and healthy controls, a sustained Raf-1 recruitment was characteristic for patients.

Taken together, these data are consistent with the interpretation that positive feedback loops of the ERK pathway are activated in RA patients. Activation of Ras in T cells is primarily controlled by RasGRP with son of sevenless playing an important amplification mechanism (29). Both RasGRP and son of sevenless transcripts were not differentially expressed in RA patients and controls (data not shown). Other kinases and phosphatases known to control ERK amplification loops may be distinct in RA patients and controls. Of particular relevance for T cell activation is DUSP6 that dephosphorylates pERK and increases the TCR threshold to respond. DUSP6 is controlled by RasGRP with son of sevenless playing an important role in RA patients and healthy controls, a sustained Raf-1 recruitment was characteristic for patients.

FIGURE 7. Suppression of increased ERK response in T cells from SKG mice attenuates arthritis. A, Splenocytes from male and female SKG mice and from female BALB/c mice were obtained at the age of 2 mo. stimulated with PMA, and analyzed for ERK phosphorylation at the indicated time points. Results are shown as mean ± SD of three to five mice. pERK levels in SKG mice were significantly different from those in control BALB/c mice (p < 0.001 by two-way ANOVA). B, Eight-week-old SKG mice (n = 12 per group) were treated with injections of 1 mg/kg of body weight of the MEK-1/2 inhibitor U0126 or a solvent control twice per week. Mice were monitored for the emergence of joint inflammation. Disease activity was defined as the sum of all inflamed joints with small inflamed joints counting as 0.1 and large joints as 0.5. Statistical analysis was done by two-way ANOVA. Treatment-delayed disease onset (left panel) and reduced disease activity (right panel; p < 0.001). C, Representative histologies from 12-wk-old SKG mice treated for 4 wk with solvent (upper panels) and MEK-1/2 inhibitor (lower panels) after H&E staining are shown. Tissue from the MEK-1/2 inhibitor-treated mouse did not show any inflammation (score 0) while the tissue from the control-treated mouse shows synovial inflammation without cartilage or bone invasion (score 1). Original magnifications of left images ×10 and right images ×20.
The increased ERK responsiveness in RA patients has implications for gene expression profiles. Induction of the ERK-dependent T cell activation markers CD69 and CD154 was increased in RA patients (Fig. 1). Even before stimulation, T cells from RA patients have a signature characteristic for ERK activation. Comparative gene expression arrays of T cells from RA patients and healthy controls showed a 1.5–3.3-fold overexpression of additional ERK pathway-dependent genes (cyclin A1, A2, B1, B2, D1, cyclin-dependent kinase 6, cyclin-dependent kinase inhibitor 2D, c-ETS2, c-FOS, and SMAD4) (data not shown).

More importantly, increased ERK activation lowered the TCR thresholds in RA T cells to respond to antigenic stimulation, a finding that is consistent with a recent model to explain the tolerogenic activity of antagonist peptides (34, 35). Germain and colleagues (34, 35) proposed that serine phosphorylation of Lck by pERK prevents recruitment of SHP-1 and sustains TCR activity to levels that are necessary to phosphorylate Lck. As a consequence, SHP-1 is recruited and the T cell is tolerized and unresponsive to subsequent TCR stimulation. In this study, we propose that this pathway is important in RA. In confocal studies, SHP-1 recruitment to the TCR signaling complex was delayed in RA. Furthermore, patients had evidence of sustained TCR signaling with increased Zap70 and NF-κB phosphorylation at 30 min after stimulation which was sensitive to ERK inhibition. Our data therefore suggest that the constitutive activation of the ERK pathway and its increased responsiveness to TCR stimulation amplifies early TCR signals and lowers the threshold for T cell activation. As a consequence, T cells from patients with RA respond to suboptimal anti-CD3 stimulation (Fig. 6).

Studies in the SKG mouse model of RA illustrate the clinical implications of these findings. Similar to human RA, SKG mice have a hyperresponsive ERK module. The abnormality is distal of early TCR signals; in fact, early TCR signaling is defective in the SKG mouse due to a Zap70 mutation. The increased ERK responsiveness precedes the onset of clinically visible arthritis and is already found in healthy mice. This may also be the case in human disease where production of autoantibodies precede disease onset by many years (43). Tuning of the ERK pathway may therefore not be a marker of, but a risk factor for, the disease and can be therapeutically targeted in patients at risk. Indeed, treatment of SKG mice with minute amounts of a MEK-1/2 inhibitor, initiated only 2–3 wk before disease onset, delayed disease onset and reduced disease severity.

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Disclosures

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References