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K-RAS GTPase- and B-RAF kinase–mediated T-cell tolerance defects in rheumatoid arthritis

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Autoantibodies to common autoantigens and neoantigens, such as IgG Fc and citrullinated peptides, are immunological hallmarks of rheumatoid arthritis (RA). We examined whether a failure in maintaining tolerance is mediated by defects in T-cell receptor activation threshold settings. RA T cells responded to stimulation with significantly higher ERK phosphorylation (P < 0.001). Gene expression arrays of ERK pathway members suggested a higher expression of KRA and BRAF, which was confirmed by quantitative PCR (P < 0.001). Western blot, and flow cytometry (P < 0.01). Partial silencing of KRA and BRAF lowered activation-induced phosphorylated ERK levels (P < 0.01). In individual cells, levels of these signaling molecules correlated with ERK phosphorylation, attesting that their concentrations are functionally important. In confocal studies, B-RAF/K-RAS clustering was increased in RA T cells 2 min after T-cell receptor stimulation (P < 0.001). Overexpression of B-RAF and K-RAS in normal CD4 T cells amplified polyclonal T-cell proliferation and facilitated responses to citrullinated peptides. We propose that increased expression of B-RAF and K-RAS lowers T-cell activation thresholds in RA T cells, enabling responses to autoantigens.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects individuals in the prime of life and follows a slowly progressive destructive course. The most visible target of the inflammatory process is the synovial membrane; joint pain and swelling, and eventually irreversible damage to cartilage, tendons, and bones dominate the clinical presentation. Disease-related pathologic processes are not limited to synovioctyes, tissue-infiltrating immune cells, and other cell constituents of the arthritic joint, but involve the global immune system. The inflammatory process characteristic of RA affects nonarticular tissues, causing a wide spectrum of disease (1–3). The systemic nature of the disease is reflected in deregulated immune responses. For decades, autoantibodies to IgG Fc have been excellent diagnostic tools for RA. In the past decade, an immune response to citrullinated self-peptides was found to be characteristic for RA (4). Although the roles of these autoimmune responses in the disease process are underdetermined, the nature of the autoantigens suggests that RA patients have a peripheral tolerance defect, in particular to neoantigens created by post-translational protein modifications that accumulate throughout life (5, 6). Such a generalized defect may also explain the therapeutic benefit of CTLA-4Ig treatment (7, 8). CTLA-4Ig blocks the CD28-CD80/86 receptor–ligand interaction and therefore inhibits costimulatory signals that are particularly important for primary T-cell responses and, to some extent, the reactivation of central memory T cells, whereas it does not influence effector T cells that have fewer costimulatory requirements or use alternate pathways (9, 10).

The notion of a T-cell tolerance defect in RA pathogenesis is supported by genetic studies. RA is a polygenic disease. The strongest genetic risk factor is the shared epitope encoded by HLA-DRB1 alleles, followed by a missense mutation of PTPN22 and K-RAS. Expression of these signaling molecules at the signaling cascade after T-cell receptor (TCR) stimulation. Based on the substitution conferring increased phosphatase activity, it was originally considered a gain of function (12). Recent findings, however, have shown that the variant is rapidly degraded, causing T-cell hyperresponsiveness (13). The findings of disease-associated HLA-DRB1 alleles and PTPN22 polymorphisms indicate that T-cell recognition events and TCR threshold calibration are central to RA pathogenesis (13, 14).

To examine this hypothesis, we compared signaling potential after TCR stimulation in patients with RA and age-matched healthy control subjects and found a hyperactive RA T-cell module. The underlying abnormality is an overexpression of B-RAF and/or K-RAS. Expression of these signaling molecules at increased levels overcomes nonresponsiveness to self-antigens. We propose that this mechanism is causing tolerance defects in RA.

Results

Molecular Mechanisms of Increased ERK Activation in RA T Cells. T cells from patients with RA respond to stimulation with elevated ERK phosphorylation compared with healthy controls (16). PhosFlow studies in a cohort of 65 patients with RA and 54 healthy control subjects documented a significantly higher basal ERK phosphorylation in RA CD4 and CD8 T cells (P < 0.0001), whereas no difference was seen for total ERK (Table 1). After CD3/CD28 cross-linking, the differences in phosphorylated ERK (pERK) between RA and control T cells widened; elevated ERK phosphorylation was seen for RA CD4 and CD8 T cells at all time points examined (P < 0.0001). CD3 expression was used as a system control to exclude cytometry artifacts or higher expression of the CD3/TCR complex; no differences were seen.

We explored the hypothesis that altered transcription of one or more constituents of the ERK pathway explains the increased responsiveness of RA T cells to stimulation. Our previous studies...
suggested that proximal TCR signaling is not different between patients with RA and control subjects, and that increased ERK phosphorylation in RA T cells is caused at, or distal of, RAS/RAF activation. We used a gene expression array (MAP Kinase Signaling Pathway PCR Array; SABiosciences) to screen for abnormal transcription of ERK pathway constituents in RA T cells. Transcripts with an increase or decrease of more than 1.5-fold were considered candidate genes. The array also included ERK target genes, many of which were found to be overexpressed, consistent with the observed increased phosphorylation of ERK at baseline (Table 2). Of the signaling members of the ERK pathway present in the array, KRAS, BRAF, and MEK1 were transcribed at higher levels (1.73-fold, 2.05-fold, and 1.55-fold, respectively) in RA compared with control T cells (Table 2), whereas no difference was seen for the majority of the ERK pathway components. The array did not include several important components of the ERK pathway that were therefore quantified by quantitative PCR (qPCR). Initial RAS-RAF association after TCR stimulation is controlled by RasGRP1 and SOS-1. The qPCR studies showed a slightly higher SOS1 level in RA patients (P = 0.04) and no significant difference for RasGRP1 (Fig. S1 A and B). DUSP5 and DUSP6 are two phosphatases that are expressed in nonactivated T cells and that dephosphorylate pERK (17). No difference in their transcription was seen (Fig. S1 C and D).

We focused on those two molecules that were most convincingly overexpressed in the screening assays, B-RAF and K-RAS. Overexpression was confirmed by qPCR at the transcriptional level and by Western blot and flow cytometry at the protein level (Fig. 1). KRAS (P = 0.003) and BRAF transcripts (P = 0.003) were significantly increased in RA T cells. In the flow cytometric studies, N-RAS and C-RAF, not differentially expressed in the array, were stained as controls. RA T cells expressed significantly higher levels of K-RAS (P < 0.001) and B-RAF (P = 0.006 for CD4, P < 0.001 for CD8 T cells) while having similar levels of N-RAS and C-RAF.

Elevated B-RAF and K-RAS Expression Does Not Correlate with Functional T-Cell Subsets. Data so far were derived from total CD4 and CD8 T-cell populations. To determine whether the upregulation of KRAS and BRAF transcription reflects the expansion or activation of a T-cell subset, we compared K-RAS and B-RAF expression in naive CD45RA+CD28+ and CD45RA−CD45RO+ memory CD4 and CD8 T cells of eight or nine patients with RA and seven healthy control subjects by flow cytometry. Representative histograms of CD4 T cells from a healthy individual and a patient with RA are shown in Fig. 2A; results are summarized in Fig. 2B. No subset-specific differences in expression levels were seen in T cells from patients with RA or from healthy controls. B-RAF expression was equal in naive and memory CD4 T cells; K-RAS expression may even be slightly lower in memory than naive T cells. Based on the cytometric analysis, the observed increased gene expression in patients with RA was not caused by a T effector cell population that may have been activated and expanded as part of the disease process. These data are consistent with our finding that increased ERK responsiveness in RA is seen in naive, memory, and effector T cells (16). This interpretation was supported by Western blot analysis. CD45RA−CD45RO−CD28+ and CD45RA+CD45RO+ memory CD4 and CD8 T cells of eight or nine patients with RA and seven healthy control subjects were sorted, and lysates from two or three healthy donors or patients with RA were pooled and analyzed. A representative experiment is shown in Fig. 2C. B-RAF was clearly overexpressed in naive and memory RA CD4 T cells. In these samples, K-RAS expression was not different or Western blots were not sensitive enough to pick up the difference that, by flow cytometry, in some cohorts, was in the order of 25% (Fig. 2B). However, similar to the flow data, K-RAS expression again tended to be lower in memory than naive T cells, not supporting the view that the findings in RA T cells are a consequence of the accumulation of a disease-relevant effector population.

The finding that the elevated B-RAF and K-RAS expression is not linked to T-cell differentiation raised the possibility that cytokines produced in RA regulate the transcription of KRAS or BRAF. T cells globally express TNF-α and IL-1β receptors and receptors for various homeostatic cytokines, including IL-15, which is elevated in RA. We have recently shown that the

Table 1. Increased responsiveness of the ERK pathway in RA T cells compared with healthy control

<table>
<thead>
<tr>
<th>Response</th>
<th>Control</th>
<th>RA</th>
<th>P value</th>
<th>Control</th>
<th>RA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>24,311 (5,380)</td>
<td>23,204 (5,670)</td>
<td>0.552</td>
<td>19,349 (3651)</td>
<td>17,379 (3,850)</td>
<td>0.125</td>
</tr>
<tr>
<td>Total ERK</td>
<td>2,433 (1,211)</td>
<td>2,436 (1,100)</td>
<td>0.993</td>
<td>2,497 (1,100)</td>
<td>2,656 (1,100)</td>
<td>0.657</td>
</tr>
<tr>
<td>p-ERK at 0 min</td>
<td>403 (71)</td>
<td>504 (92)</td>
<td>&lt;0.0001</td>
<td>475 (88)</td>
<td>644 (152)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>p-ERK at 5 min</td>
<td>1,114 (263)</td>
<td>1,501 (447)</td>
<td>&lt;0.0001</td>
<td>1,751 (446)</td>
<td>2,512 (923)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>p-ERK at 10 min</td>
<td>813 (177)</td>
<td>1,021 (222)</td>
<td>&lt;0.0001</td>
<td>1,236 (327)</td>
<td>1,947 (586)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>p-ERK at 30 min</td>
<td>504 (90)</td>
<td>632 (109)</td>
<td>&lt;0.0001</td>
<td>798 (177)</td>
<td>1,168 (394)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 2. Expression of ERK-dependent and ERK pathway genes in T cells from RA patients and healthy controls (HC)

<table>
<thead>
<tr>
<th>Gene</th>
<th>RA/control, fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK-dependent</td>
<td></td>
</tr>
<tr>
<td>CCNA1</td>
<td>3.28</td>
</tr>
<tr>
<td>CCNA2</td>
<td>1.74</td>
</tr>
<tr>
<td>CCNB1</td>
<td>1.98</td>
</tr>
<tr>
<td>CCNB2</td>
<td>2.48</td>
</tr>
<tr>
<td>CDK6</td>
<td>1.50</td>
</tr>
<tr>
<td>CDKN1C</td>
<td>2.19</td>
</tr>
<tr>
<td>CDKN2D</td>
<td>1.54</td>
</tr>
<tr>
<td>EGFR</td>
<td>1.82</td>
</tr>
<tr>
<td>ETS2</td>
<td>2.22</td>
</tr>
<tr>
<td>FO8</td>
<td>1.77</td>
</tr>
<tr>
<td>MEF2C</td>
<td>1.71</td>
</tr>
<tr>
<td>SMAD4</td>
<td>1.91</td>
</tr>
<tr>
<td>ERK pathway</td>
<td></td>
</tr>
<tr>
<td>GBR2</td>
<td>1.04</td>
</tr>
<tr>
<td>HRAS</td>
<td>−1.32</td>
</tr>
<tr>
<td>KRAS</td>
<td>1.73</td>
</tr>
<tr>
<td>NRAS</td>
<td>−1.05</td>
</tr>
<tr>
<td>KSR1</td>
<td>1.30</td>
</tr>
<tr>
<td>ARAF</td>
<td>−1.12</td>
</tr>
<tr>
<td>BRAF</td>
<td>2.05</td>
</tr>
<tr>
<td>CRAF</td>
<td>1.01</td>
</tr>
<tr>
<td>MEK1</td>
<td>1.55</td>
</tr>
<tr>
<td>MEK2</td>
<td>−1.45</td>
</tr>
<tr>
<td>ERK1</td>
<td>−1.40</td>
</tr>
<tr>
<td>ERK2</td>
<td>−1.41</td>
</tr>
</tbody>
</table>
overproduction of TNF-α accounts in part for the reduced CD28 expression in RA, supporting the notion that T cells are a target cell for TNF-α in RA (18). Incubation of T cells from healthy individuals with TNF-α or IL-1β did not sensitize T cells to respond to TCR stimulation with increased ERK phosphorylation (Fig. S2A), consistent with published reports that TNF-α impairs TCR-induced signaling (19). In contrast, IL-15 preincubation had such an effect, but this effect was not mediated by increased B-RAF or K-RAS (Fig. S2B).

**K-RAS and B-RAF Concentrations in RA T Cells Influence ERK Activation.** Depending on cell type and subcellular context, the formation of different RAS/RAF complexes involving A-RAF, B-RAF, C-RAF, H-RAS, K-RAS, and N-RAS initiates the activation of the ERK pathway. In T cells, C-RAF is dominant whereas B-RAF was thought to be completely absent and irrelevant or at least only relevant for selected functions in thymic selection (20). To determine whether B-RAF and K-RAS are critical for ERK phosphorylation in T cells, we silenced either

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**Fig. 1.** Increased expression of K-RAS and B-RAF in RA T cells. Differential expression of K-RAS and B-RAF in the array (Table 2) was confirmed by qPCR, Western blot, and flow cytometry. (A) KRAS mRNA expression in T cells from 15 control individuals (HC) and 15 patients with RA was determined by qPCR (Top Left). A representative Western blot of K-RAS and β-actin is shown (Top Right) with density readings of the K-RAS band relative to β-actin indicated. Flow cytometric assessments of K-RAS expression in CD4 (Left) and CD8 T cells (Right) are shown as representative histograms from a control individual (dashed line) and from a patient with RA (bold line) compared with isotype control antibody (filled histogram); data from 17 patients and 16 control subjects are summarized in box plots. (B) N-RAS expression is shown as representative Western blots and histograms; flow cytometric results from 20 controls and 18 RA patients are summarized as box plots. (C) BRAF transcripts were quantified by qPCR in T cells from 15 controls and 15 patients with RA (Top Left). B-RAF protein expression was quantified by Western blots and by flow cytometry. Representative Western blots (Top Right) and histograms (lane 2) for CD4 (Left) and CD8 T cells (Right) from a control (dashed line) and a patient with RA (bold line) are shown. Lane 3 summarizes B-RAF expression in 32 controls and 34 patients with RA as box plots. (D) For comparison, representative C-RAF Western blots, histograms from flow cytometry, and summarized cytometry results of 20 controls and 18 patients with RA are shown.
memory T-cell populations were analyzed for B-RAF and K-RAS expression. Representative histograms of B-RAF (left) and K-RAS (right) staining are shown for naive and memory CD4 T cells from patients with RA (solid line) and healthy controls (dotted line); FMO, fluorescence minus one.

(8) Mean fluorescence intensity of B-RAF (Upper) and K-RAS (Lower) expression for seven healthy control subjects and eight or nine patients with RA is shown. Naive and memory populations from the same individual are joined with a line. CD8 populations showed the same trend. (C) Naive and memory CD4 T cells were isolated by FACS based on the expression of the above markers. Cells from two or three different healthy controls or two or three different patients with RA were pooled and analyzed by Western blot for B-RAF and K-RAS expression. Expression levels of β-tubulin were used as a loading control. Blots are representative of three independent experiments.

Fig. 2. B-RAF and K-RAS expression in T-cell subsets. PBMCs from patients with RA and healthy controls (HC) were isolated, stained with antibodies against CD3, CD4, CD8, CD45RA, CD45RO, CD28, B-RAF, and K-RAS, and analyzed by flow cytometry. (A) Naive (CD45RA+CD28+) and memory (CD45RA+CD45RO+) CD4 T-cell populations were analyzed for B-RAF and K-RAS expression. Representative histograms of B-RAF (left) and K-RAS (right) staining are shown for naive and memory CD4 T cells from patients with RA (solid line) and healthy controls (dotted line); FMO, fluorescence minus one. (B) Mean fluorescence intensity of B-RAF (Upper) and K-RAS (Lower) expression for seven healthy control subjects and eight or nine patients with RA is shown. Naive and memory populations from the same individual are joined with a line. CD8 populations showed the same trend. (C) Naive and memory CD4 T cells were isolated by FACS based on the expression of the above markers. Cells from two or three different healthy controls or two or three different patients with RA were pooled and analyzed by Western blot for B-RAF and K-RAS expression. Expression levels of β-tubulin were used as a loading control. Blots are representative of three independent experiments.

silencing experiments showed that major reductions in B-RAF and K-RAS concentrations reduce but do not fully abrogate ERK phosphorylation. To address whether the smaller differences in expression observed between patients with RA and healthy controls are of functional relevance, we used flow cytometry to correlate protein levels of signaling molecules and phosphorylation events (21). Based on B-RAF MFI, the T-cell population was divided into six equal segments, and the ex-
pression of pERK in each gate was determined. CD4 and CD8 staining, respectively, were used as internal controls. As shown in Fig. 4, CD4 and CD8 T cells with higher B-RAF expression had increased basal levels of pERK and a more pronounced ERK response upon stimulation. This correlation was specific, as we did not see any correlation between B-RAF and CD4 levels in CD4 T cells or B-RAF and CD8 levels in CD8 T cells (Fig. 4).

**RAS-RAF Complex Formation in Patients with RA.** To monitor RAF activation, we examined RAS-RAF complex formation at the cell membrane of T cells stimulated with anti-CD3/CD28 antibodies. T cells were fixed at indicated times after TCR stimulation and stained with N-RAS or K-RAS (red) and B-RAF (green) specific antibodies.

Membrane-close fluorescence was quantified in T cells; representative images are shown in Fig. 5A. Coefficients of colocalization were determined by regression analysis of the red and green pixel fluorescence and are summarized for five patients with RA and five normal control subjects in Fig. 5B. In RA T cells, the coefficient for B-RAF/K-RAS was slightly increased before stimulation compared with healthy controls and exhibited a more pronounced response that was sustained over time. In contrast, colocalization of N-RAS and B-RAF was induced to a lesser extent by CD3 stimulation and was not different between control individuals and patients with RA at early time points, but reached increased levels in patients with RA at 5 min. The pattern of K-RAS and N-RAS clustering with C-RAF was identical and followed similar kinetics as N-RAS/B-RAF; again, a difference was seen for complex formation after 5 min, but not at the early time points as was the case with B-RAF/K-RAS (16). These data suggest a model of initially increased K-RAS/B-RAF amplification loops in T cells; representative images are shown in Fig. 5A. Coefficients of colocalization are summarized for five patients with RA.

**Activation of pERK Amplification Loops in RA T Cells.** The confocal studies suggested that the difference between patients with RA and healthy controls can be mapped to increased B-RAF and subsequently sustained C-RAF activation in the patients. This model was supported by Western blotting for serine phosphorylated C-RAF. C-RAF phosphorylation was indistinguishable at 5 min, but higher in patients with RA at subsequent time points. Bioavailability of C-RAF is controlled by several binding proteins, such as 14–3-3 and RKIP (22, 23). RKIP is involved in an important ERK amplification loop; RKIP binds to C-RAF, preventing its activation. If phosphorylated, RKIP dissociates from C-RAF, which initiates a positive feedback loop of the ERK pathway. RKIP is phosphorylated by PKC or pERK. Increased pERK levels therefore can free up more C-RAF in a positive feedback loop (24). In contrast, RKIP does not sequester B-RAF. Western blots showed that patients with RA have higher basal phosphorylated RKIP levels compared with control individuals while having similar total RKIP levels (Fig. 6A). RKIP was immunoprecipitated and the precipitates were examined for C-RAF by Western blotting. The amount of RKIP-bound C-RAF was higher in normal individuals than in patients with RA before and after stimulation (Fig. 6B), suggesting that the activation of this positive feedback loop is increased in patients with RA.

**Functional Consequences of Increased B-RAF and K-RAS Expression.** CD4 T cells were transfected to overexpress B-RAF or K-RAS at about the same level as seen in RA T cells (Fig. 7A). Transfected cells were stimulated by CD3 or CD3 and CD28 cross-linking, and proliferative responses were assessed by [³H]thymidine incorporation. Under the conditions used, CD3 cross-linking alone only induced suboptimal proliferation in control transfected T cells, which was clearly enhanced in CD4 T cells overexpressing K-RAS or B-RAF (Fig. 7B). Costimulation with anti-CD28
antibodies boosted T-cell activation, but again significantly more so in B-RAF or K-RAS than control transfected T cells. To determine whether B-RAF and/or K-RAS overexpression could overcome tolerance, the ability of CD4 T cells from healthy HLA-DRB1*04+ individuals to respond to a normal or a citrullinated vimentin, implicated in RA as autoantigen, was examined (25). Control transfected T cells only minimally responded to the unmodified and citrullinated vimentin peptide. Overexpression of either B-RAF or K-RAS enabled the autoreactive response to the unmodified and even more so to the citrullinated vimentin peptide (Fig. 7C).

**Discussion**

Production of autoantibodies to ubiquitous autoantigens such as citrullinated proteins and IgG Fc epitopes in RA suggests a general defect in maintaining tolerance (4, 5). Here, we find that T cells from RA patients have increased transcription of BRAF and KRAS. Increased cytoplasmic concentrations of these signaling molecules cause hyperreactivity of the ERK pathway upon TCR stimulation. Transcriptional activation cannot be reproduced by incubation with TNF-α, known to be elevated in RA, consistent with the observation that increased ERK phosphorylation does not correlate with disease activity (16). ERK activity has been shown to be an important regulator of TCR threshold calibration (26, 27). B-RAF is of additional interest because its expression levels in T cells are normally minute and increased expression alleviates the need for costimulation and impairs anergy induction (28, 29). We propose that the increased expression of B-RAF and K-RAS accounts for the autoreactivity characteristic of RA. In support of this interpretation, NRAS germline mutations cause a selective syndrome of autoimmunity and lymphoproliferation (30). KRAS or BRAF polymorphisms have not yet been associated with autoimmunity, possibly because germline mutations in KRAS or BRAF cause complex and severe developmental anomalies such as the Noonan and cardiofaciocutaneous syndromes.

Improved understanding of the factors driving RA has reinforced the notion that T-cell function is central to disease pathogenesis (6, 31). In addition to rheumatoid factor, autoantibody responses to citrullinated epitopes on several proteins including vimentin, fibrinogen, collagen type II, and α-enolase have been identified (5). Autoantibody responses are HLA- and T-cell-dependent. Abatacept (CTLA-4Ig), which binds to CD80 and CD86 and blocks CD28-mediated costimulation, has emerged as a successful treatment intervention (7). In addition to effector activities, studies into T-cell function have described abnormal findings in the naive T-cell compartment including defective DNA repair mechanisms, telomeric loss, and repertoire contraction, suggesting a primary role of T cells (2, 32–34). Finally, several disease risk genes are concerned with T-cell function, in particular, the two genes that carry the highest risk, HLA-DRB1 and PTPN22.
As is generally the case in human disease-association studies, the question of whether increased B-RAF and K-RAS expression in T cells is a cause or consequence of the disease cannot be completely resolved. Several findings argue against the latter possibility, although this evidence is indirect and therefore not conclusive. As shown in Fig. 2, increased expression of B-RAF or K-RAS is not a typical feature of T-cell differentiation, excluding the possibility that the findings reflect the accumulation of end-differentiated or activated T cells generated as part of the inflammatory disease process. However, even naive T cells are not completely normal in RA. They have evidence of DNA damage (32), shortened telomeres (33), increased expression of CD45RB and CD45RO (35, 36) and reduced expression levels of T-cell receptor rearrangement excision circles (33), all of them more indicative of increased replicative history rather than the activity of inflammatory cytokines. Furthermore, cytokines commonly produced as a consequence of RA did not reproduce the B-RAF or K-RAS expression patterns. Possibly the most convincing evidence that the expression level of either of these signaling molecules could principally be involved in pathogenesis comes from the in vitro forced overexpression experiment. Autoreactive T-cell responses to citrullinated peptides were enabled and cellular tolerance was impaired by transfecting cells with B-RAF or K-RAS.

Abnormal antigen receptor signaling has been implicated in the pathogenesis of several autoimmune diseases (37, 38) including RA and systemic lupus erythematosus. In murine models, mutations in signaling molecules conferring B-cell receptor hyperresponsiveness are associated with a lupus-like disease. Frequently, the mutations involve a negative regulator such as SHP-1, CD22, or FCyRIIb (39–41); overexpression of a positive regulator such as CD19 or a mutation in CD45 that controls the activity of src kinases can also break tolerance and cause autoantibody production (42). Patients with systemic lupus erythematosus have a subset of T cells with FcγR-Syk replacing ζ-ZAP70 in the TCR signaling complex, which induces a preferential calcium signal (43). The best example of T-cell hyperresponsiveness causing autoimmune disease is Cbl/Cbl-b double-deficient mice (44). Interestingly, one characteristic finding in T cells from these double-KO mice is prolonged ERK phosphorylation after TCR stimulation. Conversely, impaired TCR signaling can also induce autoimmunity as best illustrated by the SKG mouse that has a spontaneous ZAP70 mutation and develops arthritis (45). A failure in negative selection has been implicated, which, however, does not explain how potentially autoreactive T cells are activated in the periphery. We have observed increased basal and induced ERK phosphorylation in SKG T cells after PKC activation. Treatment with subtherapeutic doses of a MEK-1 inhibitor delayed and ameliorated disease supporting the notion that ERK activation may compromise peripheral tolerance (16). Similarly, the LAT Y136F mutation that causes a multigorgan inflammatory disease is characterized by relatively preserved ERK phosphorylation in the presence of severely impaired calcium flux (46, 47), again consistent with the model that qualitative disturbances in TCR signaling predispose for autoimmunity.

ERK activity is pivotal in regulating T-cell sensitivity to antigenic stimulation. Li et al. (48) identified miR-181a as an intrinsic modulator of TCR threshold calibration. miR-181a regulates the translation of several phosphatases, including DUSP5 and DUSP6, which dampen ERK activity. When activated, pERK sustains several positive feedback loops. pERK phosphorylates RKIP, thereby releasing C-RAF, and, in the presence of active RAS, supports further ERK activation (23). As shown in Fig. 6, this loop is activated in RA T cells. pERK also sequesters, but does not phosphorylate and activate C-RAF. TCR activation in anergic T cells for defective RAF activation. RAP1 sequesters, but does not phosphorylate and activate C-RAF. TCR stimulation alone induces RAP1 but poorly activates RAS, whereas CD28 costimulation activates RAS and inhibits RAP1 activation, consistent with the notion that costimulation prevents energy induction (50). Our findings in RA T cells provide evidence that this conceptual framework of a central role of ERK activation in the pathogenesis of autoimmunity is relevant for a human autoimmune disease.

C-RAF has been considered the major RAF kinase in T cells. B-RAF is best known as an oncogene in melanoma and other tumors (51). In contrast to A-RAF and C-RAF, B-RAF is constitutively phosphorylated at position S445 and only requires membrane recruitment but no phosphorylation for activation.
which may explain its unique role as an oncogene (52). B-RAF is independent of two of the negative mechanisms that control C-RAF activation. B-RAF does not bind to, and therefore cannot be inactivated by, RKIP. B-RAF bound to MAP-ERK stimulates the ERK pathway, producing IL-2 and proliferation.

We propose that increased B-RAF expression lowers the TCR activation threshold in T cells from patients with RA and renders these cells more susceptible to activation. The relative contributions of different RAS members to T-cell activation is less well understood than that of RAK kinases; however, overexpression of K-RAS may have a similar net effect. In our functional assays, forced overexpression of B-RAF or K-RAS alone heightened the T-cell response to CD3 and CD28 cross-linking and lowered the TCR threshold sufficiently to permit the activation of self-reactive T cells to native and more so to citrullinated vimentin peptides. Our preliminary studies also identified enhanced transcription of another ERK pathway member, SOS, at least in some patients with RA. SOS is an important amplifier of RAS-GRP–induced RAS activation after T-cell receptor triggering by entailing a positive feedback loop (53). Increased ERK activity in T cells appears to be a common denominator in RA, but may be attained through different pathways.

Increased TCR sensitivity to stimulation with low-affinity antigens and relative resistance to anergy induction could explain many of the T-cell phenomena observed in RA. Increased TCR sensitivity could increase homeostatic proliferation, facilitate differentiation into CD28 effector cells and eventually accelerate cell aging and repertoire contraction, all immunological hallmarks of RA. At the same time, it may facilitate responses to antigens that are controlled by peripheral tolerance mechanisms and not by central negative selection, such as responses to neoantigens including those generated by citrullination. In this model, B-RAF and K-RAS emerge as excellent targets to prevent or treat RA because their inhibition would be selectively inactivated by RKIP. B-RAF bound to RAP-1 continues to be inactivated by RKIP. B-RAF is a positive feedback loop for ERK activation (28). In T cells, B-RAF and MAP-ERK molecules. Data were acquired and analyzed on an LSR II flow cytometer (BD Biosciences) with FACS DIVA software or FlowJo software (Treestar).
the pIRE2-GFP-FP1 empty vector (Clontech). Total CD4 T cells were transfected with empty vector, KRAS-pIRE2-GFP-FP1, or BRAF-pIRE2-GFP-FP1 (4 μg each) using the Amaxa Nucleofector system and the Human T-cell Nucleofector kit (Lonza).

Cytokine Stimulation. Purified T cells were incubated without cytokine or with 10 ng/mL IL-1β, TNF-α, or IL-15 (PeproTech). After 24 h, cytokines were removed by extensive washing with complete media; cells were stimulated by cross-linking of CD3 for 5 min and analyzed for ERK phosphorylation by PhosFlow. Untreated and IL-15-treated T cells were transfected for BRAF and KRAS expression by qPCR.

Proliferative Assays. CD4 T cells from healthy individuals were transfected with empty vector, KRAS-pIRE2-GFP-FP1 or BRAF-pIRE2-GFP-FP1, stimulated at a concentration of 0.5 × 10⁶ cells/mL with 1 μg/mL anti-CD3 Ab cross-linked with rabbit anti-mouse IgG or anti-CD3 in combination with 1 μg/mL anti-CD28 Ab, followed by cross-linking and cultured for 7 d. Alternatively, 2.5 × 10⁵/mL transfected CD4 T cells from healthy HLA-DRB1*04 and HLA-DRB1*01 donors were stimulated for 7 d with 0.625 × 10⁶/mL autologous adherent cells and 25 μg/mL vimentin 65-77 peptide (SAVARSSVPGRV) or VimR70Ct (SAVARCtSSVPGRV) in the presence of 2.5 μg/mL anti-CD28 and anti-CD49d antibodies. Proliferative responses were quantified by [³H]-thymidine incorporation.

Statistical Analysis. Demographic data and results from RA and control T cells were compared by using a two-tailed Mann-Whitney U test or unpaired Student t test as appropriate. A level of P < 0.05 was considered significant.

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