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Inverse Rap1 and Phospho-ERK Expression Discriminate the Maintenance Phase of Tolerance and Priming of Antigen-Specific CD4$^+$ T Cells In Vitro and In Vivo[1]

Angela M. Morton, Barbara McManus, Paul Garside,[2] Allan McI. Mowat, and Margaret M. Harnett[3]

T cell recognition of Ag can result in priming or tolerance depending on the context in which Ag is recognized. Previously, we have reported that these distinct functional outcomes are associated with marked differences in the amplitude, kinetics, and cellular localization of activated, pERK signals at the level of individual Ag-specific T cells in vitro. Here, we show that the GTPase Rap1, which can antagonize the generation of such pERK signals and has been reported to accumulate in tolerant cells, exhibits an inverse pattern of expression to pERK in individual Ag-specific primed and tolerized T cells. Although pERK is expressed by more primed than tolerized T cells when rechallenged with Ag in vitro, Rap1 is expressed by higher percentages of tolerant compared with primed Ag-specific T cells. Moreover, whereas pERK localizes to the TCR and lipid rafts in primed cells, but exhibits a diffuse cellular distribution in tolerized cells, Rap1 colocalizes with the TCR and lipid raft structures under conditions of tolerance, but not priming, in vitro. This inverse relationship between Rap1 and pERK expression is physiologically relevant, given that we observed the same patterns in Ag-specific T cells in situ, following induction of priming and tolerance in vivo. Together, these data suggest that the maintenance of tolerance of individual Ag-specific T cells may reflect the recruitment of up-regulated Rap1 to the immune synapse, potentially resulting in sequestration of Raf-1 and uncoupling of the TCR from the Ras-ERK-MAPK cascade. ***The Journal of Immunology,*** **2007, 179: 8026–8034.**

Peripheral tolerance is a state of Ag-specific immune hyporesponsiveness (1) which, once it is induced, can suppress many aspects of the immune response including lymphocyte proliferation and cytokine production in vitro and Ab production and delayed-type hypersensitivity in vivo (2). Such peripheral tolerance is necessary not only because central tolerance is incomplete but also because it is required to prevent immunopathology against self Ags that are only encountered in the periphery or harmless Ag such as food or commensals. The precise mechanisms involved are not clear, but candidates include apoptotic deletion, regulation by cytokines or CTLA-4, ignorance, and anergy. Relating to the latter, it is well established that TCR ligation in the absence of costimulation results in the induction of a form of cellular unresponsiveness known as anergy (3, 4). This reflects the fact that productive T cell priming requires at least two signals from the APC: signal 1 being recognition of antigenic peptide in the context of MHC by the TCR; and signal 2 being costimulation supplied by interaction of CD80/CD86 on the APC with CD28 on the T cell (5). Anergy results when the T cells receive only signal 1.

This state of anergy has been suggested to be a direct result of disrupted TCR-coupling to MAPK signaling pathways (6–12). For example, in many models, productive priming of CD4$^+$ T cells in vitro is associated with Lck/Fyn-mediated tyrosine phosphorylation of the CD3-$\zeta\zeta$ complex which leads to recruitment and activation of ZAP-70 and the PLCγ1/calcium and Ras-ERK signaling cascades, resulting in formation and cooperation of active transcription complexes at the IL-2 promoter site including NF-AT (via calcineurin) and AP-1 (via ERK) (13). In contrast, anergized T cells fail to transcribe IL-2 which is secondary to a lack of ERK and AP-1 activation and is accompanied by accumulation of Rap1 (13–15). Further studies indicated that such Rap1 accumulation disrupted TCR-ERK activation by sequestering Raf-1 and hence directly antagonizing Ras-Raf-1-ERK coupling (16). Consistent with this, accumulation of active Rap1 was reported to play a role in the maintenance of anergy in human T cell clones (16, 17), with tolerant cells displaying reduced ERK activation due to recruitment of a Fyn-Cbl-CrkL-C3G-Rap1 signaling complex not found in their primed counterparts (16). Furthermore, an inverse relationship between ERK and Rap1 activation has been shown in activated T cell lines (18), in which CD28 signaling abolishes TCR-coupled Rap1 activity (13, 19, 20). However, controversy surrounds the role of Rap1, given that it has been shown to play both negative and positive regulatory roles in T cell activation (21, 22). Moreover, many of these events associated with Rap1 accumulation and activation in anergized T cells appear to be downstream consequences of, as yet poorly defined, defects in

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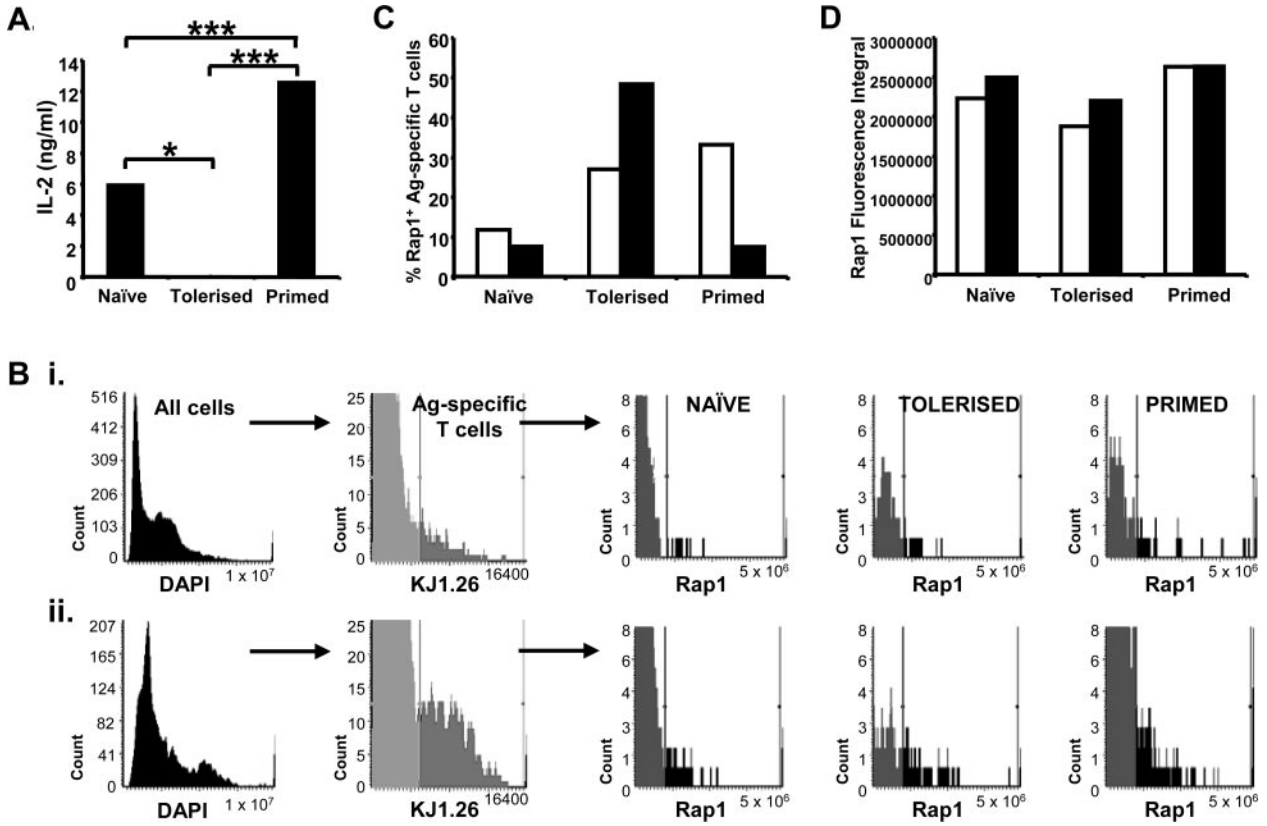

FIGURE 1. Quantitation of Rap1 expression in primed and tolerized Ag-specific T cells at the single-cell level by LSC. Naive T cells were freshly isolated from resting mice, whereas tolerized or primed Tg Ag-specific T cells were generated in vitro using anti-CD3 ± anti-CD28, as described in *Materials and Methods*. The cells were then restimulated with DC alone (□) or DC + $OVA_{323–339}$ (■) for 20 h. *A*, IL-2 production was determined by analysis of culture supernatants by ELISA. ELISA results shown are the mean ± SD of triplicate cultures. *B*, Examplar LSC histograms showing cells restimulated with DC alone (*Bi*) or DC + $OVA_{323–339}$ (*Bii*) depict how cells were gated on for analysis. From these, it was clear that although the level of Rap1 expression was similar in all groups, the percentage of tolerized KJ1-26$^+$ T cells expressing Rap1 was greater compared with naive and primed KJ1.26$^+$ T cells upon restimulation with Ag (*Bii*.). All data are representative of at least three individual experiments with quantitative population statistical analysis being performed on at least 200 KJ1-26$^+$ T cells in each group. The proportion of Ag-specific (KJ1-26$^+$) T cells expressing Rap1 (*C*) and the total cellular level of Rap1 expression (*D*) in Rap1$^+$ Ag-specific (KJ1-26$^+$) T cells was determined by LSC analysis (*B*).

events proximal to TCR signaling and have mainly been characterized using in vitro models that may not reflect the responses of primary Ag-specific CD4$^+$ T cells in vivo. As a result, the role(s) of Rap1 in the molecular processes that distinguish primed and tolerized T cells in intact lymphoid tissues remains uncertain.

Consistent with the proposal that induction of tolerance reflects disrupted coupling of the TCR to ERK activation, we have shown previously that there are marked differences in the amplitude and cellular localization of phosphorylated ERK (p42ERK2/p44ERK1) signals when naive and in vitro-primed and tolerized T cells respond to Ag (23). Here, we investigate whether Rap1 plays a role in mediating these differences in ERK signaling under conditions of tolerance and priming in a physiological setting, by comparing the expression and cellular localization of Rap1 in tolerized and primed Ag-specific T cells both in vitro and in vivo. For the first time, we demonstrate that Rap1 is expressed by higher percentages of tolerant compared with primed Ag-specific T cells in response to challenge with Ag, both in vitro and in vivo. Moreover, in vivo, such tolerized T cells express Rap1 at a significantly higher level relative to primed and naive groups. Finally, because Rap1 and activated pERK were found to display inverse patterns of expression and subcellular localization in primed and tolerized cells, these data provide support for the proposal that Rap1 may play a role in the maintenance of Ag-specific tolerance by disrupting TCR coupling to ERK MAPK.

Materials and Methods

Mice

DO11.10 TCR-transgenic (Tg)[4] mice on a BALB/c background were used as donors of T cells for both in vitro and in vivo adoptive transfer studies. These Tg T cells recognize $OVA_{323–339}$/I-A^d and are detected using the clonotypic mAb KJ1-26 (24). BALB/c (H-$2^{d/d}$) mice were used as recipients. All animals were specified pathogen free and were maintained under standard animal house conditions in accordance with local and Home Office regulations.

Generation of dendritic cells (DC)

Bone marrow-derived DCs (25) were generated by culture in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 0.05 mM 2-ME + 5% GM-CSF for 9 days before maturation with 1 μg/ml LPS (*Salmonella abortus*; Sigma-Aldrich) for 24 h. $OVA_{323–339}$ (1 μg/ml; Genosys; Sigma-Aldrich) was added for 3–4 h to the matured DCs, which were then washed extensively before use.

Preparation of cell suspensions and identification of Tg TCR by flow cytometry

Peripheral (axillary, brachial, inguinal, cervical) lymph nodes (PLN) were removed from D011.10 TCR-Tg mice, pooled, and forced through Nitex (Cadisch Precision Meshes) to generate single-cell suspensions. The cells were washed in sterile RPMI 1640, and the percentage of KJ1-26$^+$CD4$^+$ DO11.10 T cells was determined by incubation with PE-conjugated anti-CD4 (BD Pharmingen) and biotinylated clonotypic anti-TCR Abs, KJ1-26

[4] Abbreviations used in this paper: Tg, transgenic; DC, dendritic cell; PLN, peripheral lymph node; LSC, laser scanning cytometry; PKC, protein kinase C.

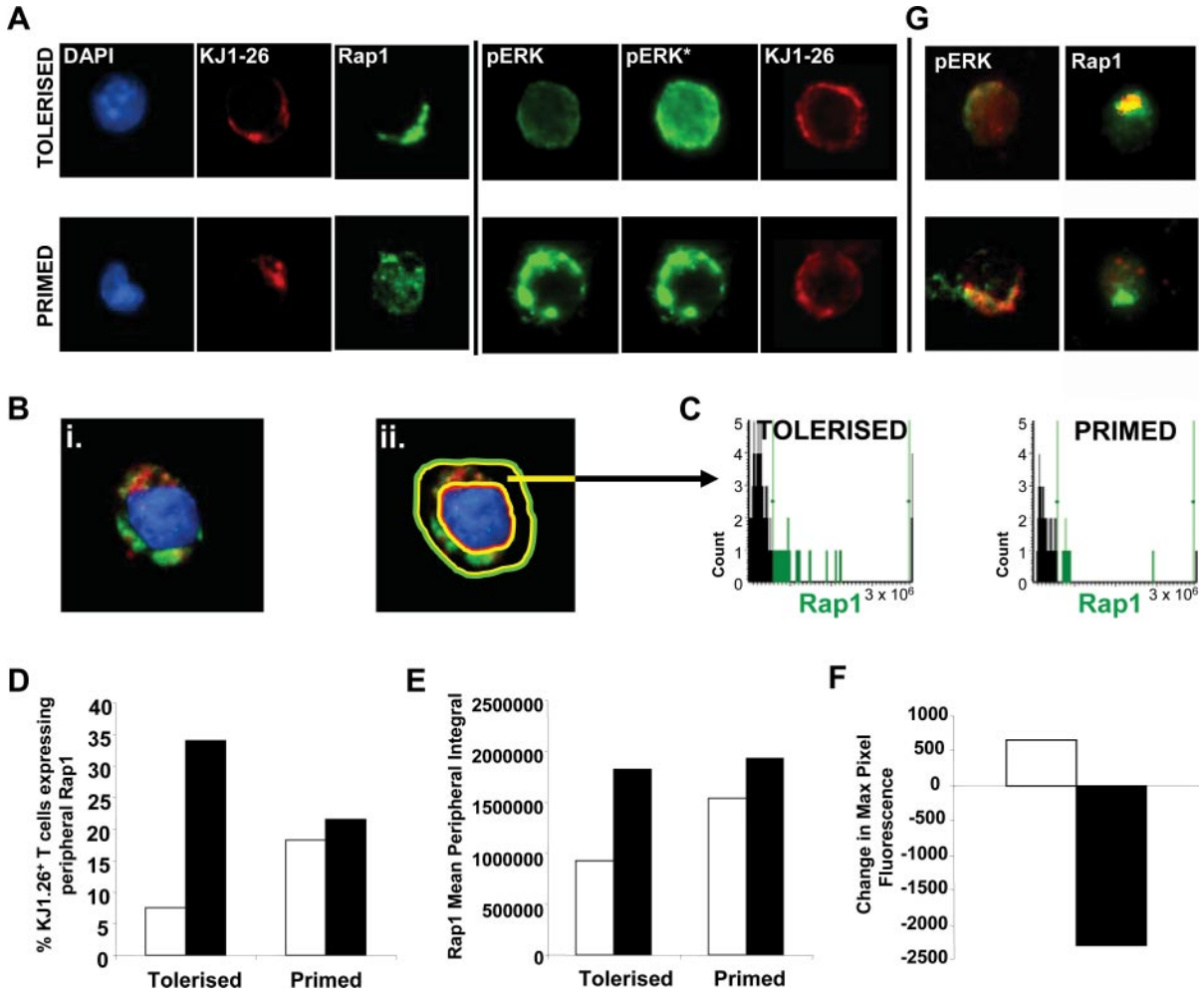

FIGURE 2. Intracellular localization of Rap1 expression in Ag-specific Tg T cells. Tolerized and primed Ag-specific Tg T cells were generated in vitro using anti-CD3 ± anti-CD28, as described in *Materials and Methods*, and restimulated with DC alone (□) or DC + $OVA_{323–339}$ (■) for 20 h. Cells were treated with 4′,6′-diamidino-2-phenylindole (DAPI) to stain the nuclei (blue) and the clonotypic Ab KJ1-26 to visualize the Tg TCR (red). Rap1 or pERK expression was detected using specific Abs (green). KJ1-26$^+$ Rap1$^+$ T cells from tolerized and primed samples were randomly relocated by LSC. Representative individual tolerized and primed cells were identified and imaged as described previously (Ref. 23; *A*) showing the relative levels of expression of Rap1 and pERK in primed and tolerized cell populations. pERK expression in tolerized cells was also imaged using a higher exposure time to demonstrate better the distribution of signal within these cells (pERK*; *A*). *B*, Example of three-color merged images depicting a Rap1$^+$ (green staining) KJ1-26$^+$ (red staining) T cell (*Bi*) with threshold (red), integral (green), and peripheral (yellow) contours applied (*Bii*). Peripheral contours discriminate the periphery of the cell, external to the nucleus, and the fluorescence detected therein was plotted as histograms (*C*). *D*, Percentage of KJ1-26$^+$ T cells expressing Rap1 at their periphery was therefore quantitated by peripheral contouring using LSC. Similarly, the peripheral MFI of Rap1 is shown in *E*, whereas *F* shows the intensity (max pixel value) of Rap1 expression within the periphery, calculated as the difference between the max pixel of cells cultured with DC alone and those stimulated with DC + $OVA_{323–339}$. Rap1 expression was assessed in 200 tolerized (□) and primed (■) Rap1$^+$ KJ1-26$^+$ T cells per sample; results are representative of three replicate experiments. *G*, Tolerized and primed Ag-specific T cells were induced and restimulated with DC + Ag for 20 h. Ag-specific Tg T cells were identified by the clonotypic Ab KJ1-26 (blue), whereas Rap1 or pERK expression was detected by the relevant specific Abs (red) and lipid raft structures were identified using a cholera toxin subunit B-Alexa Fluor 488 conjugate (green). KJ1-26$^+$ pERK$^+$ and KJ1-26$^+$ Rap1$^+$ T cells from tolerized and primed samples were randomly relocated by LSC and the localization of pERK and Rap1 in KJ1-26$^+$ T cells was determined in relation to lipid rafts (KJ1-26 staining not shown). Representative individual tolerized and primed cells were identified and imaged as described previously (23).

for 20 min at 4°C. Cells were then washed in FACS buffer (0.05% sodium azide, 2% FCS in PBS) and incubated with FITC-conjugated streptavidin (Vector Laboratories) for 10 min at 4°C. PE-conjugated rat IgG2a and biotinylated mouse IgG2a (BD Pharmingen) served as isotype-negative controls for PE-conjugated CD4 and biotinylated KJ1-26, respectively. Two-color FACS was performed on 20,000 events using a FACSCalibur and CellQuest software.

Induction of tolerance and priming in T cells in vitro

Single-cell suspensions were prepared as described above. The percentage of Tg T cells was calculated by flow cytometry, and aliquots containing 1×10^6 Tg T cells/ml/well were cultured in complete medium (RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 0.05 mM 2-ME; all Invitrogen) with 1 μg/ml immobilized anti-CD3 (clone 145-2C11) for 48 h in the presence or absence of 1 μg/ml anti-CD28 (clone 37.51; BD Pharmingen) to induce priming or tolerance, respectively. After 48 h, cells were washed twice with RPMI 1640, replated, and rested in complete medium for an additional 48 h (26, 27). Tg T cells were then restimulated by culturing with LPS-matured, $OVA_{323–339}$-loaded DC at a ratio of 1:1 (5×10^5 T cells + 5×10^5 DC in 2 ml of medium/well) for 20 h in 6-well plates; Costar).

Adoptive transfer and administration of Ag

Cell suspensions containing 3×10^6 Tg TCR T cells/mouse were injected i.v. into BALB/c recipients. For induction of systemic priming or tolerance, 24 h after adoptive transfer mice were exposed to Ag by i.v. injection with

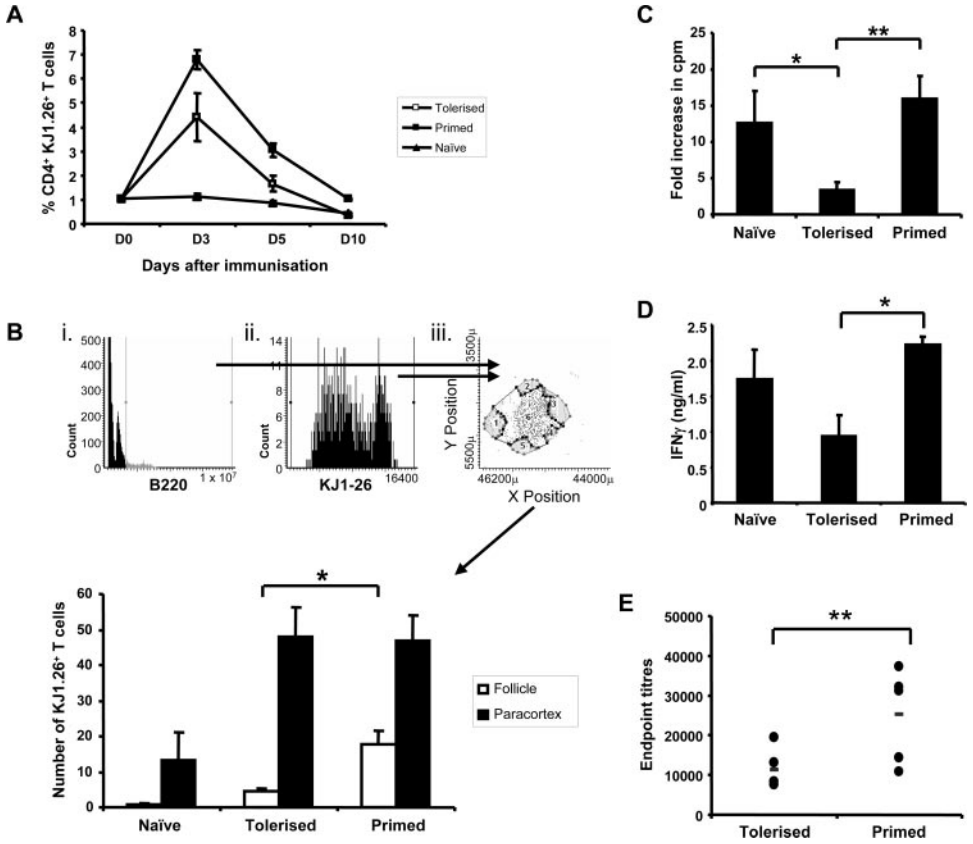

FIGURE 3. Induction of priming and tolerance in Ag-specific $CD4^+$ T cells in vivo. Ag-specific Tg T cells were transferred into naive recipients 24 h before i.v. injection with $OVA_{323–339}$ + LPS (primed) or $OVA_{323–339}$ alone (tolerized). At days 0, 3, 5, and 10 after immunization, PLNs were harvested, and the percentage of Ag-specific Tg T cells assessed by flow cytometry (*A*). In *B*, the percentage of Ag-specific Tg T cells present in follicular and paracortical areas of the PLNs at day 3 after immunization was determined by LSC (32, 76, 77). LSC histograms depicting how $B220^+$ B cells (*Bi*) and KJ1-26^+ T cells (*Bii*) were gated are shown, together with a sample tissue map, which illustrates how KJ1-26^+ T cells situated in follicular and paracortical areas of the lymph node were quantitated (*Biii*). At day 10 after immunization, PLNs were harvested, and single-cell suspensions were restimulated in vitro with or without Ag to assess proliferation (*C*) and IFN-γ production (*D*). Proliferation was assayed by [^{3}H]thymidine uptake at 72 h, and the level of IFN-γ in culture supernatants was detected by ELISA at 48 h after restimulation in vitro. Both proliferation and IFN-γ data are expressed as fold increase in signal from samples restimulated in the presence of Ag compared with the signal from those restimulated with medium alone. Ova-specific IgG1 Ab levels in serum of mice challenged with OVA/CFA 7 days after the induction of priming or tolerance with $OVA_{323–339}$ ± LPS (*E*). Data represent the mean ± SD for three mice per group, and each animal sample was performed in triplicate. *, $p < 0.05$; **, $p < 0.01$.

$OVA_{323–339}$ (100 μg; Sigma-Aldrich) with or without LPS (1 μg; *S. abortus*; Sigma-Aldrich) in 200 μl), respectively. In experiments comparing priming and oral tolerance, mice were either fed 100 mg of OVA (Sigma-Aldrich) to induce tolerance or immunized by s.c. injection with OVA (100 μg) in 100 μl of PBS, 50% CFA (OVA/CFA; Sigma-Aldrich) for induction of priming. To recall secondary responses, mice were challenged i.v. with 100 μg of $OVA_{323–339}$ with or without 1 μg of LPS 7 or 10 days after immunization.

In vitro proliferation and cytokine assays

Ten days after immunization, PLNs were restimulated in vitro in the presence or absence of 1 μg/ml $OVA_{323–339}$ at 4×10^5 cells/well in 96-well plates (Costar) for 72 h before being pulsed with 1 μCi/well [^{3}H]thymidine (Western Infirmary) and harvested after an additional 16 h. In parallel cultures, culture supernatants were harvested 48 h after restimulation in vitro, and the presence of IL-2 and IFN-γ detected by ELISA as described previously (23).

Immunofluorescence, imaging, and laser scanning cytometry (LSC)

Intracellular staining of cells in vitro. Cytospins were prepared as described previously (23). Briefly, cytospins were fixed and stained with biotinylated KJ1-26 and streptavidin-HRP and then biotinylated-tyramide (both TSA Biotin system; PerkinElmer Life Sciences) followed by streptavidin-Alexa Fluor 647 (Invitrogen). Next, the cells were permeabilized with 2% FCS, 2 mM EDTA (pH 8.0), 0.1% w/v saponin in PBS and stained with rabbit anti-Rap1 or rabbit anti-dually phosphorylated ERK and then with anti-rabbit IgG-HRP conjugate (all New England Biolabs) and subsequently with Alexa Fluor 488 or Pacific blue tyramide. Cholera toxin subunit B-Alexa Fluor 488 was used to stain the glycosphingolipid GM1-containing lipid raft structures (28, 29). Finally, the cells were mounted in Vectashield with or without DAPI (Vector Laboratories). Biotinylated mouse IgG2a and rabbit IgG (Sigma-Aldrich) served as isotype negative controls for biotinylated KJ1-26 and anti-pERK or anti-Rap1, respectively. The anti-Rap1 Ab (SC-65; Santa Cruz Biotechnology) is an affinity-purified rabbit polyclonal Ab raised against an epitope contained between aa 110 and 160 of Rap1 (Swiss protein number P62834). Basic local alignment search tool searches of this sequence show 100% identity with Rap1B and 95% identity with the RAP1A isoform, but no homology with any other protein. Confirmation that this sequence is unique and specific to Rap1 comes from Western blot analysis of DO.11.10 lymph node cell lysates which reveals a single band of ~21 kDa commensurate with Rap1 (results not shown). Moreover, preincubation of the Ab with a 10-fold excess of blocking peptide (Santa Cruz Biotechnology; SC-65P) completely blocks staining of Rap1 in lymph node tissue (results not shown).

Tissue staining. Inguinal lymph nodes were removed 1 or 24 h after challenge and then fixed in 1% paraformaldehyde at 4°C for 24 h. They were then transferred into 30% sucrose in PBS for a further 48 h before being frozen in liquid nitrogen in OCT embedding medium (Bayer) and stored at −70°C. Sections (6 μm) were cut and subsequently stored at −20°C for up to 72 h before staining. Sections were stained as described previously (30). In brief, tissue sections were further fixed in acetone and stained with biotinylated KJ1-26 as described above. Next, the sections were permeabilized with 3% BSA, 0.1% Triton X-100 in PBS and stained with rabbit anti-Rap1 or rabbit anti-pERK for 16 h, then anti-rabbit IgG-HRP conjugate together with anti-CD45R/B220-FITC conjugate, and subsequently with Pacific blue tyramide (kit T-20940; Invitrogen). Finally, the sections

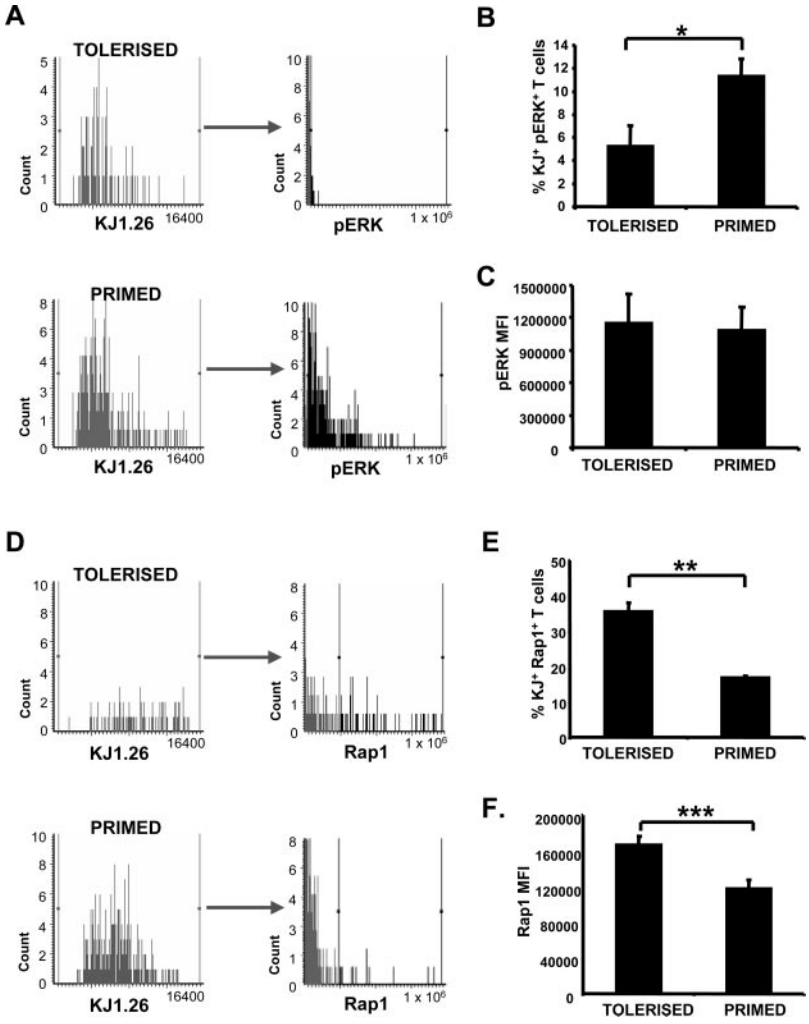

FIGURE 4. Quantitation of Ag-specific pERK and Rap1 expression in primed and tolerized Ag-specific T cells in PLNs in situ by LSC. After induction of priming or tolerance of adoptively transferred Tg TCR T cells in vivo, all groups were challenged with 100 μg of $OVA_{323–339}$/1 μg of LPS 7 days after immunization. Inguinal lymph nodes were harvested 24 h after challenge and stained using the appropriate specific Abs, for TCR Tg T cells (KJ1-26), B cells (B220) and pERK or Rap1. LSC analysis (as described previously (1); *A* and *D*) were used to determine the proportion of Ag-specific T cells expressing pERK (*B*) and Rap1 (*E*) as well as the total cellular levels of pERK (*C*) and Rap1 expression (*F*) in situ. Representative LSC histograms depicting the pERK and Rap1 signaling profiles in tolerized and primed T cells, in situ, are shown in *A* and *D*. Results presented in *B*, *C*, *E*, and *F* are the mean values ± SD of three animals per group. *, $p < 0.05$; ***, $p < 0.01$.

were mounted in Vectashield (Vector Laboratories). Isotype-negative controls and storage conditions were as described above.

Immunofluorescence imaging and laser scanning cytometry. Fluorescence quantitation of cytospins and fluorescence immunohistochemistry was conducted using LSC (Compucyte) as described previously (23, 30). LSC data were analyzed using WinCyte version 3.6 (CompuCyte). Fluorescence images were taken using a connected Hamamatsu Orca ER digital camera and Openlab version 3.0.9 digital imaging program (Improvision).

Statistics

Results are expressed as mean ± SD. To test significance, Student's unpaired *t* test was performed.

Results

Rap1 expression is higher in tolerized than in primed Ag-specific CD4$^+$ T cells in vitro

Priming or tolerance was induced in primary OVA-specific DO11.10 T cells by activation of the TCR in the presence or absence of appropriate costimulation using plate-bound anti-CD3 and soluble anti-CD28. As described previously (23, 26, 27), these conditions induced functional priming or unresponsiveness, evidenced here by analysis of IL-2 production. On restimulation with Ag, primed T cells produced significantly higher levels of IL-2 than naive cells and tolerized T cells produced no IL-2 whatsoever (Fig. 1*A*).

To investigate the potential role(s) of Rap1 in the induction and effector phases of tolerance induction in vitro, Rap1 expression in naive, primed or tolerized T cells was examined by LSC (Fig. 1, *B–D*) 20 h after these T cells had been re-stimulated in the absence or presence of Ag. As expected and consistent with the IL-2 production data (Fig. 1*A*), such LSC analysis confirmed that the in vitro priming and tolerance regimens produced a larger population of Ag-specific (KJ1-26$^+$) T cells in the primed relative to the tolerized groups (Fig. 1*B*). When challenged with LPS-matured DC in the absence of Ag, a similar percentage of tolerized and primed Tg T cells expressed Rap1 and this was higher than that observed for naive cells cultured with DC alone (Fig. 1, *B* and *C*). Upon restimulation with Ag, there was a sharp decrease in the percentage of primed Ag-specific T cells expressing Rap1. Conversely, there was a substantial increase in the percentage of tolerized Ag-specific T cells expressing Rap1 (Fig. 1, *B* and *C*), suggesting that Rap1 expression may be associated with the maintenance phase of tolerance. Quantitation of the Rap1 signal in Rap1$^+$ Ag-specific T cells revealed that, when Rap1 was expressed, it was expressed at similar levels in all groups, regardless of whether they had been restimulated with Ag or not (Fig. 1, *B* and *D*).

Differential intracellular localization of Rap1 expression in tolerized and primed Ag-specific T cells

As we showed previously (23), primed T cells expressed higher levels of activated ERK after challenge with Ag, and this pERK was localized primarily at the cell periphery. Conversely, tolerized T cells expressed lower levels of pERK with a more diffuse cellular distribution (Fig. 2*A*). Rap1 displayed an inverse pattern of cellular localization to that of pERK, being concentrated at the

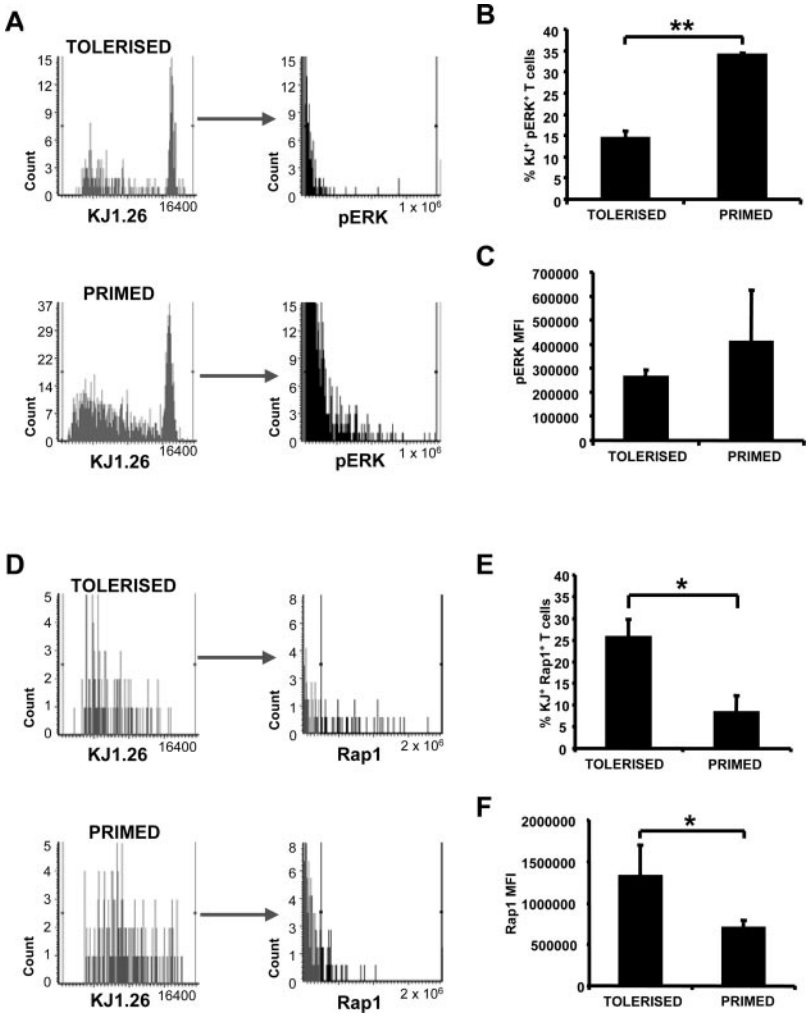

FIGURE 5. Quantitation of Ag-specific pERK and Rap1 expression in primed and orally tolerized Ag-specific T cells in PLNs in situ by LSC. After induction of priming or tolerance of adoptively transferred Tg TCR T cells in vivo, all groups were challenged with 100 μg of $OVA_{323–339}$ 10 days after immunization. Inguinal lymph nodes were harvested 1 h after challenge and stained using the appropriate specific Abs, for TCR Tg T cells (KJ1-26), B cells (B220), and pERK or Rap1. Analysis by LSC (as described previously (Ref. 1; *A* and *D*) were used to determine the proportion of Ag-specific T cells expressing pERK (*B*) and Rap1 (*E*) as well as the total cellular levels of pERK (*C*) and Rap1 expression (*F*) in situ. Representative LSC histograms depicting the pERK and Rap1 signaling profiles in tolerized and primed T cells, in situ, are shown in *A* and *D*. The proportion of Ag-specific T cells expressing pERK (*B*) and Rap1 (*E*) as well as the total cellular level of pERK (*C*) and Rap1 (*F*) expression in situ was assessed, and results are expressed as mean section values ± SD where sections were derived from three animals per group. *, $p < 0.05$; **, $p < 0.01$.

periphery of tolerized T cells, and showing a more diffuse, punctate pattern of expression in primed T cells. Although some foci of Rap1 staining could be seen at the periphery of primed cells, it appeared to be excluded from the vicinity of the TCR (Fig. 2*A*). To quantify the subcellular localization of Rap1 by LSC, we used peripheral contour analysis methods that we established previously (Fig. 2, *B* and *C*, and Refs. 31 and 32). This confirmed that, in addition to a greater percentage of tolerized relative to primed cells expressing Rap1 following challenge with Ag, a higher proportion of tolerized T cells expressed Rap1 peripherally (Fig. 2*D*). Moreover, although the integral levels of such peripheral Rap1 expression appeared similar in both groups of T cells following antigenic challenge (Fig. 2*E*), this showed a more focused pattern of expression in tolerized T cells, as indicated by increased intensity (max pixel) of signal (Fig. 2*F*). This peripheral localization of Rap1 in tolerized cells is again inverse to that of pERK, which we found previously to be expressed at much lower levels in the periphery of tolerized T cells than in primed T cells (23).

Relationship of ERK and Rap1 localization to lipid rafts in primed and tolerized Ag-specific T cells

Given that organized recruitment of the TCR and signaling molecules in lipid rafts to the immunological synapse is one of the principal features of T cell activation and is reported to be defective in tolerized cells (33–36), we examined the intracellular localization of pERK and Rap1 in relation to lipid rafts 20 h after the cells were restimulated with Ag. Consistent with the idea that peripheral pERK was associated with T cell activation, pERK colocalized with lipid raft staining in primed Ag-specific T cells (Fig. 2*G*). Such colocalization was not observed in tolerized Ag-specific T cells, suggesting that lipid rafts, or the interaction of pERK with these structures, is defective under conditions of tolerance. Conversely, there was marked colocalization of Rap1 with lipid rafts in tolerized, but not primed, Ag-specific T cells after restimulation with Ag (Fig. 2*G*).

Role for Rap1 in the maintenance of tolerance in vivo

To investigate whether similar inverse patterns of Rap1 and pERK accumulation are found during tolerance and priming in vivo, we examined Rap1 expression in Ag-specific $CD4^+$ T cells taken from two models of Ag-specific priming and tolerance (37–40). Twenty-four hours after adoptive transfer of Tg Ag-specific T cells, recipient mice received $OVA_{323–339}$ peptide i.v., either alone or together with LPS to induce systemic tolerance or priming respectively. The efficacy of these regimens was confirmed by assessing the clonal expansion (Fig. 3*A*) and follicular migration (Fig. 3*B*) of Ag-specific $CD4^+$ T cells in the peripheral lymph nodes in response to the primary challenge with Ag ± LPS. In addition, we assessed the proliferation (Fig. 3*C*) and cytokine (Fig. 3*D*) recall responses of such Ag-specific $CD4^+$ T cells from peripheral lymph nodes ex vivo. As shown previously (40, 41), at all times after primary immunization there was significantly higher clonal expansion in the PLNs of primed mice than in those of tolerized mice, and significantly fewer Ag-specific $CD4^+$ T cells migrated to the follicular areas of PLN of tolerized compared with primed mice. Moreover, after re-stimulation with

$OVA_{323–339}$ in vitro, tolerized T cells from PLN also showed reduced proliferation (Fig. 3*C*) and IFN-γ production (Fig. 3*D*). PLN cells from naive mice proliferated significantly more than the tolerized group but less than the primed group. In parallel, tolerized mice showed considerably lower serum OVA-specific IgG1 Ab responses after challenge with Ag in vivo than primed mice (Fig. 3*E*). Collectively, these data show that i.v. administration of OVA with or without LPS as an adjuvant induced priming and tolerance, respectively, in vivo.

To investigate the role of pERK and Rap1 signaling in the maintenance phase of priming and tolerance in vivo, their expression was examined in Ag-specific Tg T cells in situ by LSC 24 h after secondary challenge (day 8 after adoptive transfer) with $OVA_{323–339}$ + LPS i.v. (Fig. 4). As we found in vitro, a significantly higher percentage of primed Ag-specific T cells expressed pERK relative to tolerized cells when challenged with Ag in vivo (Fig. 4, *A* and *B*). Conversely, a significantly higher proportion of Ag-specific tolerized T cells expressed Rap1 than did primed T cells (Fig. 4, *D* and *E*), and the tolerized T cells also expressed significantly higher levels of Rap1 (Fig. 4*F*).

We next examined the pattern of Rap1 and pERK expression in a more physiologically relevant model of oral tolerance (41, 42). Mice were fed soluble OVA to induce tolerance or immunized s.c. with OVA/CFA to induce priming. Ten days after immunization, mice were challenged with $OVA_{323–339}$ i.v., and 1 h later the expression of pERK and Rap1 in Ag-specific Tg T cells was examined in the inguinal PLNs. As before, significantly more primed Ag-specific T cells expressed pERK than did tolerized cells after challenge (Fig. 5, *A* and *B*), and significantly more tolerized Ag-specific T cells expressed Rap1 than did primed cells (Fig. 5, *D* and *E*). In addition, the tolerized Ag-specific T cells expressed Rap1 at significantly higher levels than the primed Ag-specific T cells (Fig. 5*F*). Collectively, these data demonstrate that Rap1 and pERK exhibited inverse patterns of expression in both systemic and oral models of priming and tolerance.

Discussion

We have shown previously that there are marked differences in the kinetics, amplitude, and localization of pERK signals in individual naive, primed, and tolerized primary Ag-specific T cells responding to Ag in vitro (23). In particular, we showed that primed T cells display more rapid kinetics of phosphorylation and activation of ERK than naive T cells, whereas tolerized T cells display a reduced ability to activate ERK upon challenge. In addition, the low levels of pERK found in tolerized T cells were distributed diffusely throughout the cell, whereas in primed T cells, pERK appeared to be targeted to the same regions of the cell as the TCR (23). We have now extended these studies to in vivo models of Ag-specific priming and tolerance and show that a higher percentage of primed Ag-specific T cells express pERK in situ on rechallenge with Ag. These results indicate that sustained ERK signaling in vivo correlates with productive T cell responses and that defective ERK signaling is associated with the maintenance of tolerance.

The precise mechanisms underlying such differential ERK signaling remain unclear. We show here that the GTPase Rap1, which has previously been reported to accumulate and antagonize signals upstream of ERK activation under tolerogenic conditions in vitro (16, 17), is expressed in a significantly higher percentage of tolerized Ag-specific T cells both in vitro and in vivo, and at significantly higher levels, after challenge with Ag in vivo. Our finding that Rap1 expression is down-regulated in naive and primed T cells after activation in the presence of costimulation is consistent with previous reports that CD28 signaling down-regulates Rap1. Moreover, the pattern of expression of Rap1 in such cells is inverse to that of pERK. In tolerized cells, Rap1 shows a highly focused, peripheral expression that mirrors that of the TCR. Conversely, Rap1 is less polarized to the periphery of primed T cells and is found more diffusely throughout the cell. However, the finding of foci of Rap1 expression at the periphery of primed cells suggests that such localization alone is not itself sufficient to disrupt the peripheral recruitment and activation of pERK necessary for priming and that Rap1 activation and/or complex formation with other signal transducers may be required. In this respect, the association between pERK and Rap1 and lipid rafts in restimulated, primed, and tolerized cells also revealed an inverse relationship, with pERK being localized within such membrane microdomains in primed but not tolerized cells and Rap1 being targeted to lipid rafts in tolerized Ag-specific T cells. Collectively, these data suggest that Rap1 may be up-regulated and recruited to the immune synapse upon stimulation of tolerized T cells with Ag and that this may result in down-regulation of ERK recruitment and activation, possibly as a result of Raf-1 sequestration. This would lead to the uncoupling of the Ras-ERK-MAPK pathway from the TCR as has been observed in tolerant cells (43–45).

Rap1 has long been implicated in the desensitization of ERK and the consequent defective IL-2 production found in tolerized T cells (17, 20, 46, 47). Recent studies by Boussiotis and coworkers (48) have directly demonstrated that $CD4^+$ T cells from Tg mice expressing a constitutively active Rap1 mutant, *Rap1E63*, exhibit defective ERK activation, IL-2 production, and proliferation when primed in vivo and restimulated with specific Ag in vitro. These results were interpreted as showing that the expression of *Rap1E63* may be responsible for maintaining the anergic state. *Rap1E63*-Tg mice also have increased numbers of $CD4^+CD103^+$ T cells with regulatory function (48), and other recent work shows that $CD4^+CD25^+$ T regulatory cells from human cord blood display sustained Rap1, but impaired ERK, signaling in response to challenge with Ag, resulting in defective IL-2 production, cell cycle arrest, and apoptosis (49). Together these results suggest a central role for Rap1 in T cell hyporesponsiveness.

The mechanisms responsible for the tolerance observed in the present studies have not been fully elucidated. Indeed, this is an area of considerable uncertainty in the field in general and with tolerant T cells in vivo in particular. The anti-CD3 treatment regimen has classically been used to induce anergy in T cells in vitro (26, 50, 51); in our studies, the behavior of such treated cells was entirely consistent with this definition, because they do not proliferate or produce IL-2 when restimulated with Ag (31). Similarly, the T cell proliferation that we observed during the induction phase of tolerance in vivo, followed by their failure to proliferate or make effector cytokines on restimulation, is entirely consistent with this form of tolerance (13–18). Nevertheless, in the absence of specific markers of anergy, it is difficult to prove that the T cells we studied in vivo are truly anergic. Moreover, such T cell proliferation during the induction phase of tolerance with consequent failure to expand in response to a secondary antigenic challenge is an almost universal feature of all models of peripheral tolerance. We have therefore simply described these cells as tolerized because it is possible that more than one form of T cell unresponsiveness could be present (31, 50).

Despite that fact that Rap1 up-regulation is widely associated with tolerance, the precise role(s) of Rap1 in T cell biology has become increasingly controversial. Indeed, mice transgenic for a different constitutively active mutant of Rap1, *Rap1V12*, showed normal T cell proliferation and ERK activation in response to anti-CD3 stimulation (52). Other studies have also implicated a positive role for Rap1 in T cell signaling (21, 53), possibly mediated by the adhesion- and degranulation-promoting adaptor protein (54, 55) which plays a key role in cytoskeletal rearrangement and in the

regulation of synapse formation following TCR activation (56). It is not known how the TCR and Rap1 might connect to adhesion- and degranulation-promoting adaptor protein signaling, but it is likely to involve Fyn and SLP-76 (54, 55) activating Rap1 via PLCε, which contains a Ras-Rap-binding domain and can act as a guanine nucleotide exchange factor for Rap1 (57, 58). Consistent with this, there is evidence that Rap1 activation after Ag-specific triggering of lymphocytes requires PLC activation and consequent calcium mobilization and 1,2-diacylglycerol generation (52, 59, 60).

At first sight, it might appear difficult to reconcile such opposing models of Rap1 signaling. However, it is possible that Rap1 may play distinct roles depending on the context of the signal and/or on stage of priming and tolerance. Indeed, our data suggest that Rap1 expression is up-regulated during the induction phases of both priming and tolerance, relative to the levels seen in naive cells. Thus, in the early stages of T cell activation common to the induction of both priming and tolerance, Rap1 may promote TCR signaling via integrin-mediated inside-out signaling. However, once priming or tolerance is established, it is possible that rewiring of Rap1 signaling occurs, reflecting differential levels/kinetics of Rap1 expression. Consistent with a role for signal strength in directing these processes, Boussiotis and associates (48) suggested that the differential responses of the *Rap1V12*- and *Rap1E63*-Tg mice might reflect the finding that the *Rap1E63* mutant exhibits five times the biological activity of *Rap1V12* (61) being insensitive to the negative regulator of Rap1, *Rap1GAP* (62). This proposal is supported by recent studies showing that mice that are deficient in the *Rap1GAP*, SPA-1, exhibit defective ERK activation and progressive unresponsiveness or tolerance of T cells (63).

Alternatively, rewiring of Rap1 signaling may reflect compartmentalization mediated by the Fyn-Cbl-CrkL-C3G-Rap1 complex which is generated selectively in tolerant T cells (17). Consistent with this latter idea, we found that the preferential expression of Rap1 in tolerized cells showed an inverse pattern of expression to that of pERK, with Rap1 expression being localized within lipid rafts at the plasma membrane of tolerized cells (23). The mechanisms involved in such temporal and spatial segregation are not clear but may reflect the functional sequestration of Fyn (within lipid rafts) from Lck in the absence of productive priming (64). Defective partitioning of Lck and Fyn has been postulated to be an early negative signal in the induction of T cell anergy (65) and may contribute to the reduced phosphorylation and lipid raft recruitment of linker for activation of T cells (66), with the consequent uncoupling of downstream signals such as PLCγ, protein kinase C (PKC), and ERK (67, 68) observed in tolerized T cells. Such decreased PLC-γ1 and PKC-θ signaling, and the subsequent decrease in LFA-1 function, have in turn been suggested as being responsible for the defective translocation of TCR, PKC-θ, and lipid rafts into the immunological synapse in tolerized T cells (34, 35). However, the inverse localization of pERK staining within plasma membrane lipid rafts in primed but not tolerized cells might suggest that Rap1 antagonism of ERK activation could also reflect disruption and termination of productive synapse formation and signaling. Consistent with this, recent reports have indicated that ERK is an intermediate signal in the Vav/Rac2-mediated pathway (69), leading to nucleation of actin filaments and cytoskeleton remodeling at the immunological synapse (70). Thus, partitioning of Fyn and Lck and the consequent generation of the negative regulatory complex comprising Fyn, PAG, and Csk (71, 72), might be required for compartmentalization and rewiring of Rap1 signaling via the assembly of the Fyn-Cbl-CrkL-C3G-Rap1 complex (73–75), that is found to be selectively expressed in tolerized cells (17).

In summary, our data show that the defective ERK signaling observed in tolerized Ag-specific $CD4^+$ T cells (23) correlates with up-regulation of Rap1 in tolerized, relative to primed, cells after subsequent stimulation with Ag in vitro and in both systemic and oral tolerance models in vivo. Importantly, we have for the first time demonstrated in situ a physiologically relevant, inverse relationship between endogenous Rap1 and pERK expression and signaling in individual Ag-specific $CD4^+$ T cells that have been primed or tolerized in vivo. Because this occurs after the induction of both systemic and oral routes of tolerance, these data suggest that Rap1 antagonism of pERK signaling plays an important and general role in the maintenance of Ag-specific $CD4^+$ T cell tolerance. By advancing our knowledge of these key signals in regulating tolerance and priming at the single cell level in vitro and in vivo, we will increase our understanding of an important physiological process at the molecular level, ultimately leading to identification of potential targets for enhancing or inhibiting immunity and tolerance.

Disclosures

The authors have no financial conflict of interest.

References

1. Mowat, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat. Rev. Immunol.* 3: 331–341.
2. Garside, P., and A. M. Mowat. 2001. Oral tolerance. *Semin. Immunol.* 13: 177–185.
3. Alegre, M. L., K. A. Frauwirth, and C. B. Thompson. 2001. T-cell regulation by CD28 and CTLA-4. *Nat. Rev. Immunol.* 1: 220–228.
4. Lechler, R., J. G. Chai, F. Marelli-Berg, and G. Lombardi. 2001. T-cell anergy and peripheral T-cell tolerance. *Philos. Trans. R. Soc. Lond. B* 356: 625–637.
5. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* 356: 607–609.
6. DeSilva, D. R., W. S. Feeser, E. J. Tancula, and P. A. Scherle. 1996. Anergic T cells are defective in both jun NH2-terminal kinase and mitogen-activated protein kinase signaling pathways. *J. Exp. Med.* 183: 2017–2023.
7. Kang, S. M., B. Beverly, A. C. Tran, K. Brorson, R. H. Schwartz, and M. J. Lenardo. 1992. Transactivation by AP-1 is a molecular target of T cell clonal anergy. *Science* 257: 1134–1138.
8. Fields, P. E., T. F. Gajewski, and F. W. Fitch. 1996. Blocked Ras activation in anergic $CD4^+$ T cells. *Science* 271: 1276–1278.
9. Li, W., C. D. Whaley, A. Mondino, and D. L. Mueller. 1996. Blocked signal transduction to the ERK and JNK protein kinases in anergic $CD4^+$ T cells. *Science* 271: 1272–1276.
10. Li, W., C. D. Whaley, J. L. Bonnevier, A. Mondino, M. E. Martin, K. M. Aagaard-Tillery, and D. L. Mueller. 2001. CD28 signaling augments Elk-1-dependent transcription at the c-*fos* gene during antigen stimulation. *J. Immunol.* 167: 827–835.
11. Mondino, A., C. D. Whaley, D. R. DeSilva, W. Li, M. K. Jenkins, and D. L. Mueller. 1996. Defective transcription of the IL-2 gene is associated with impaired expression of c-Fos, FosB, and JunB in anergic T helper 1 cells. *J. Immunol.* 157: 2048–2057.
12. Sundstedt, A., and M. Dohlsten. 1998. In vivo anergized $CD4^+$ T cells have defective expression and function of the activating protein-1 transcription factor. *J. Immunol.* 161: 5930–5936.
13. Appleman, L. J., and V. A. Boussiotis. 2003. T cell anergy and costimulation. *Immunol. Rev.* 192: 161–180.
14. Schwartz, R. H. 2003. T cell anergy. *Annu. Rev. Immunol.* 21: 305–334.
15. Mueller, D. L. 2004. E3 ubiquitin ligases as T cell anergy factors. *Nat. Immunol.* 5: 883–890.
16. Abraham, R. T. 2002. Antigen receptors rap to integrin receptors. *Nat. Immunol.* 3: 212–213.
17. Boussiotis, V. A., G. J. Freeman, A. Berezovskaya, D. L. Barber, and L. M. Nadler. 1997. Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science* 278: 124–128.
18. Czyzyk, J., D. Leitenberg, T. Taylor, and K. Bottomly. 2000. Combinatorial effect of T-cell receptor ligation and CD45 isoform expression on the signaling contribution of the small GTPases Ras and Rap1. *Mol. Cell Biol.* 20: 8470–8474.
19. Reedquist, K. A., and J. L. Bos. 1998. Costimulation through CD28 suppresses T cell receptor-dependent activation of the Ras-like small GTPase Rap1 in human T lymphocytes. *J. Biol. Chem.* 273: 4944–4949.
20. Carey, K. D., T. J. Dillon, J. M. Schmitt, A. M. Baird, A. D. Holdorf, D. B. Straus, A. S. Shaw, and P. J. Stork. 2000. CD28 and the tyrosine kinase lck stimulate mitogen-activated protein kinase activity in T cells via inhibition of the small G protein Rap1. *Mol. Cell Biol.* 20: 8409–8419.
21. Bos, J. L., J. de Rooij, and K. A. Reedquist. 2001. Rap1 signalling: adhering to new models. *Nat. Rev. Mol. Cell Biol.* 2: 369–377.
22. Stork, P. J., and T. J. Dillon. 2005. Multiple roles of Rap1 in hematopoietic cells: complementary versus antagonistic functions. *Blood* 106: 2952–2961.
23. Adams, C. L., A. M. Grierson, A. M. Mowat, M. M. Harnett, and P. Garside. 2004. Differences in the kinetics, amplitude, and localization of ERK activation

in anergy and priming revealed at the level of individual primary T cells by laser scanning cytometry. *J. Immunol.* 173: 1579–1586.
24. Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack. 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J. Exp. Med.* 157: 1149–1169.
25. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223: 77–92.
26. Chai, J. G., and R. I. Lechler. 1997. Immobilized anti-CD3 mAb induces anergy in murine naive and memory $CD4^+$ T cells in vitro. *Int. Immunol.* 9: 935–944.
27. Jenkins, M. K., C. A. Chen, G. Jung, D. L. Mueller, and R. H. Schwartz. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J. Immunol.* 144: 16–22.
28. Gupta, N., and A. L. DeFranco. 2003. Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated B-lymphocyte activation. *Mol. Biol. Cell* 14: 432–444.
29. Dietrich, C., Z. N. Volovyk, M. Levi, N. L. Thompson, and K. Jacobson. 2001. Partitioning of Thy-1, GM1, and cross-linked phospholipid analogs into lipid rafts reconstituted in supported model membrane monolayers. *Proc. Natl. Acad. Sci. USA* 98: 10642–10647.
30. Grierson, A. M., P. Mitchell, C. L. Adams, A. M. Mowat, J. M. Brewer, M. M. Harnett, and P. Garside. 2005. Direct quantitation of T cell signaling by laser scanning cytometry. *J. Immunol. Methods* 301: 140–153.
31. Adams, C. L., A. M. Grierson, A. M. Mowat, M. M. Harnett, and P. Garside. 2004. Differences in the kinetics, amplitude, and localization of ERK activation in anergy and priming revealed at the level of individual primary T cells by laser scanning cytometry. *J. Immunol.* 173: 1579–1586.
32. Grierson, A. M., P. Mitchell, C. L. Adams, A. M. Mowat, J. M. Brewer, M. M. Harnett, and P. Garside. 2005. Direct quantitation of T cell signaling by laser scanning cytometry. *J. Immunol. Methods* 301: 140–153.
33. Delon, J., N. Bercovici, R. Liblau, and A. Trautmann. 1998. Imaging antigen recognition by naive $CD4^+$ T cells: compulsory cytoskeletal alterations for the triggering of an intracellular calcium response. *Eur. J. Immunol.* 28: 716–729.
34. Heissmeyer, V., F. Macian, S.-H. Im, R. Varma, S. Feske, K. Venuprasad, H. Gu, Y.-C. Liu, M. L. Dustin, and A. Rao. 2004. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat. Immunol.* 5: 255–265.
35. Ise, W., K. Nakamura, N. Shimizu, H. Goto, K. Fujimoto, S. Kaminogawa, and S. Hachimura. 2005. Orally tolerized T cells can form conjugates with APCs but are defective in immunological synapse formation. *J. Immunol.* 175: 829–838.
36. Nel, A. E., and N. Slaughter. 2002. T-cell activation through the antigen receptor, part 2: role of signaling cascades in T-cell differentiation, anergy, immune senescence, and development of immunotherapy. *J. Allergy Clin. Immunol.* 109: 901–915.
37. Reinhardt, R. L., A. Khoruts, R. Merica, T. Zell, and M. K. Jenkins. 2001. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* 410: 101–105.
38. Zell, T., A. Khoruts, E. Ingulli, J. L. Bonnevier, D. L. Mueller, and M. K. Jenkins. 2001. Single-cell analysis of signal transduction in CD4 T cells stimulated by antigen in vivo. *Proc. Natl. Acad. Sci. USA* 98: 10805–10810.
39. Thorstenson, K. M., and A. Khoruts. 2001. Generation of anergic and potentially immunoregulatory $CD25^+CD4$ T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J. Immunol.* 167: 188–195.
40. Kearney, E. R., K. A. Pape, D. Y. Loh, and M. K. Jenkins. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity* 1: 327–339.
41. Smith, K. M., F. McAskill, and P. Garside. 2002. Orally tolerized T cells are only able to enter B cell follicles following challenge with antigen in adjuvant, but they remain unable to provide B cell help. *J. Immunol.* 168: 4318–4325.
42. Smith, K. M., J. M. Davidson, and P. Garside. 2002. T-cell activation occurs simultaneously in local and peripheral lymphoid tissue following oral administration of a range of doses of immunogenic or tolerogenic antigen although tolerized T cells display a defect in cell division. *Immunology* 106: 144–158.
43. Carey, K. D., R. T. Watson, J. E. Pessin, and P. J. Stork. 2003. The requirement of specific membrane domains for Raf-1 phosphorylation and activation. *J. Biol. Chem.* 278: 3185–3196.
44. Cook, S. J., B. Rubinfeld, I. Albert, and F. McCormick. 1993. RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. *EMBO J.* 12: 3475–3485.
45. Schmitt, J. M., and P. J. Stork. 2001. Cyclic AMP-mediated inhibition of cell growth requires the small G protein Rap1. *Mol. Cell Biol.* 21: 3671–3683.
46. Katagiri, K., M. Hattori, N. Minato, and T. Kinashi. 2002. Rap1 functions as a key regulator of T-cell and antigen-presenting cell interactions and modulates T-cell responses. *Mol. Cell Biol.* 22: 1001–1015.
47. Dillon, T. J., V. Karpitski, S. A. Wetzel, D. C. Parker, A. S. Shaw, and P. J. S. Stork. 2003. Ectopic B-Raf expression enhances extracellular signal-regulated kinase (ERK) signaling in T cells and prevents antigen-presenting cell-induced anergy. *J. Biol. Chem.* 278: 35940–35949.
48. Li, L., R. J. Greenwald, E. M. Lafuente, D. Tzachanis, A. Berezovskaya, G. J. Freeman, A. H. Sharpe, and V. A. Boussiotis. 2005. Rap1-GTP is a negative regulator of Th cell function and promotes the generation of $CD4^+CD103^+$ regulatory T cells in vivo. *J. Immunol.* 175: 3133–3139.
49. Li, L., W. R. Godfrey, S. B. Porter, Y. Ge, C. H. June, B. R. Blazar, and V. A. Boussiotis. 2005. $CD4^+CD25^+$ regulatory T-cell lines from human cord blood have functional and molecular properties of T-cell anergy. *Blood* 106: 3068–3073.
50. Fathman, C. G., and N. B. Lineberry. 2007. Molecular mechanisms of $CD4^+$ T-cell anergy. *Nat. Rev. Immunol.* 7: 599–609.
51. Hayashi, R. J., D. Y. Loh, O. Kanagawa, and F. Wang. 1998. Differences between responses of naive and activated T cells to anergy induction. *J. Immunol.* 160: 33–38.
52. Sebzda, E., M. Bracke, T. Tugal, N. Hogg, and D. A. Cantrell. 2002. Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling. *Nat. Immunol.* 3: 251–258.
53. de Bruyn, K. M., S. Rangarajan, K. A. Reedquist, C. G. Figdor, and J. L. Bos. 2002. The small GTPase Rap1 is required for Mn^{2+}- and antibody-induced LFA-1- and VLA-4-mediated cell adhesion. *J. Biol. Chem.* 277: 29468–29476.
54. Peterson, E. J., M. L. Woods, S. A. Dmowski, G. Derimanov, M. S. Jordan, J. N. Wu, P. S. Myung, Q. H. Liu, J. T. Pribila, B. D. Freedman, Y. Shimizu, and G. A. Koretzky. 2001. Coupling of the TCR to integrin activation by Slap-130/Fyb. *Science* 293: 2263–2265.
55. Griffiths, E. K., C. Krawczyk, Y. Y. Kong, M. Raab, S. J. Hyduk, D. Bouchard, V. S. Chan, I. Kozieradzki, A. J. Oliveira-Dos-Santos, A. Wakeham, et al. 2001. Positive regulation of T cell activation and integrin adhesion by the adapter Fyb/Slap. *Science* 293: 2260–2263.
56. Wang, H., F. E. McCann, J. D. Gordan, X. Wu, M. Raab, T. H. Malik, D. M. Davis, and C. E. Rudd. 2004. ADAP-SLP-76 binding differentially regulates supramolecular activation cluster (SMAC) formation relative to T cell-APC conjugation. *J. Exp. Med.* 200: 1063–1074.
57. Jin, T. G., T. Satoh, Y. Liao, C. Song, X. Gao, K. Kariya, C. D. Hu, and T. Kataoka. 2001. Role of the CDC25 homology domain of phospholipase Cε in amplification of Rap1-dependent signaling. *J. Biol. Chem.* 276: 30301–30307.
58. Mochizuki, N., S. Yamashita, K. Kurokawa, Y. Ohba, T. Nagai, A. Miyawaki, and M. Matsuda. 2001. Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. *Nature* 411: 1065–1068.
59. McLeod, S. J., R. J. Ingham, J. L. Bos, T. Kurosaki, and M. R. Gold. 1998. Activation of the Rap1 GTPase by the B cell antigen receptor. *J. Biol. Chem.* 273: 29218–29223.
60. McLeod, S. J., and M. R. Gold. 2001. Activation and function of the Rap1 GTPase in B lymphocytes. *Int. Rev. Immunol.* 20: 763–789.
61. Kitayama, H., T. Matsuzaki, Y. Ikawa, and M. Noda. 1990. Genetic analysis of the *Kirsten-ras-revertant 1* gene: potentiation of its tumor suppressor activity by specific point mutations. *Proc. Natl. Acad. Sci. USA* 87: 4284–4288.
62. Brinkmann, T., O. Daumke, U. Herbrand, D. Kuhlmann, P. Stege, M. R. Ahmadian, and A. Wittinghofer. 2002. Rap-specific GTPase activating protein follows an alternative mechanism. *J. Biol. Chem.* 277: 12525–12531.
63. Kometani, K., D. Ishida, M. Hattori, and N. Minato. 2004. Rap1 and SPA-1 in hematologic malignancy. *Trends Mol. Med.* 10: 401–408.
64. Filipp, D., and M. Julius. 2004. Lipid rafts: resolution of the "fyn problem"? *Mol. Immunol.* 41: 645–656.
65. Thomas, S., R. Kumar, A. Preda-Pais, S. Casares, and T. D. Brumeanu. 2003. A model for antigen-specific T-cell anergy: displacement of $CD4–p56^{lck}$ signalosome from the lipid rafts by a soluble, dimeric peptide-MHC class II chimera. *J. Immunol.* 170: 5981–5992.
66. Hundt, M., H. Tabata, M. S. Jeon, K. Hayashi, Y. Tanaka, R. Krishna, L. De Giorgio, Y. C. Liu, M. Fukata, and A. Altman. 2006. Impaired activation and localization of LAT in anergic T cells as a consequence of a selective palmitoylation defect. *Immunity* 24: 513–522.
67. Viola, A., S. Schroeder, Y. Sakakibara, and A. Lanzavecchia. 1999. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* 283: 680–682.
68. Bi, K., Y. Tanaka, N. Coudronniere, K. Sugie, S. Hong, M. J. van Stipdonk, and A. Altman. 2001. Antigen-induced translocation of PKC-θ to membrane rafts is required for T cell activation. *Nat. Immunol.* 2: 556–563.
69. Yu, H., D. Leitenberg, B. Li, and R. A. Flavell. 2001. Deficiency of small GTPase Rac2 affects T cell activation. *J. Exp. Med.* 194: 915–926.
70. Sechi, A. S., J. Buer, J. Wehland, and M. Probst-Kepper. 2002. Changes in actin dynamics at the T-cell/APC interface: implications for T-cell anergy? *Immunol. Rev.* 189: 98–110.
71. Yasuda, K., M. Nagafuku, T. Shima, M. Okada, T. Yagi, T. Yamada, Y. Minaki, A. Kato, S. Tani-Ichi, T. Hamaoka, and A. Kosugi. 2002. Cutting edge: Fyn is essential for tyrosine phosphorylation of Csk-binding protein/phosphoprotein associated with glycolipid-enriched microdomains in lipid rafts in resting T cells. *J. Immunol.* 169: 2813–2817.
72. Shimada, Y., M. Inomata, H. Suzuki, M. Hayashi, A. Abdul Waheed, and Y. Ohno-Iwashita. 2005. Separation of a cholesterol-enriched microdomain involved in T-cell signal transduction. *FEBS J.* 272: 5454–5463.
73. Gajewski, T. F., P. Fields, and F. W. Fitch. 1995. Induction of the increased Fyn kinase activity in anergic T helper type 1 clones requires calcium and protein synthesis and is sensitive to cyclosporin A. *Eur. J. Immunol.* 25: 1836–1842.
74. Gajewski, T. F., D. Qian, P. Fields, and F. W. Fitch. 1994. Anergic T-lymphocyte clones have altered inositol phosphate, calcium, and tyrosine kinase signaling pathways. *Proc. Natl. Acad. Sci. USA* 91: 38–42.
75. Quill, H., M. P. Riley, E. A. Cho, J. E. Casnellie, J. C. Reed, and T. Torigoe. 1992. Anergic Th1 cells express altered levels of the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$. *J. Immunol.* 149: 2887–2893.
76. Marshall, F. A., A. M. Grierson, P. Garside, W. Harnett, and M. M. Harnett. 2005. ES-62, an immunomodulator secreted by filarial nematodes, suppresses clonal expansion and modifies effector function of heterologous antigen-specific T cells in vivo. *J. Immunol.* 175: 5817–5826.
77. Smith, K. M., J. M. Brewer, C. M. Rush, J. Riley, and P. Garside. 2004. In vivo generated Th1 cells can migrate to B cell follicles to support B cell responses. *J. Immunol.* 173: 1640–1646.