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Cutting Edge: Inhibition of T Cell Activation by TIM-2

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T cell Ig and mucin domain protein 2 (TIM-2) has been shown to regulate T cell activation in vitro and T cell-mediated disease in vivo. However, it is still not clear whether TIM-2 acts mainly to augment T cell function or to inhibit it. We have directly examined the function of TIM-2 in murine and human T cell lines. Our results indicate that expression of TIM-2 significantly impairs the induction of NFAT and AP-1 transcriptional reporters by not only TCR ligation but also by the pharmacological stimuli PMA and ionomycin. This does not appear to be due to a general effect on cell viability, and the block in NFAT activation can be bypassed by expression of activated alleles of Ras or calcineurin, or MEK kinase, in the case of AP-1. Thus, our data are consistent with a model whereby TIM-2 inhibits T cell activation. The Journal of Immunology, 2006, 177: 4966–4970.

Transmembrane proteins of the T cell Ig and mucin domain (TIM) family have recently been implicated in the regulation of T cell activation and differentiation (1, 2). Thus far, four of these proteins (TIM-1 through TIM-4) have been studied in mice. These genes are generally well conserved in humans, although the human genome does not contain a gene encoding TIM-2. Given its close sequence homology to TIM-1, it has been postulated that murine TIM-2 may share some of the functions conducted by human TIM-1 (3). Several reports now suggest that TIM-1 can function to augment TCR-dependent T cell activation (4–6). The activity of TIM-1 may be regulated in vivo by binding to another TIM protein—TIM-4—which is preferentially expressed by APCs (5).

TIM-2 has also been reported to increase the efficiency of T cell activation through the TCR, possibly through binding to one of its ligands—the semaphorin Sema4A (7). A recent study has identified another ligand for TIM-2, the H chain of ferritin (8). It is not yet clear how this ligand for TIM-2 might regulate its function. Another group has shown that TIM-2 is preferentially expressed by Th2 T cells and serves as a negative regulator of this cell type (9), in contrast to the initial description of TIM-2 as a positive regulator of T cell activation (7). Since we, and others, recently demonstrated that TIM-1 can provide a co-stimulatory signal, we wanted to determine whether TIM-2 behaves in a similar fashion. Also, as murine models are being used to address the function of TIMs, including TIM-1 (5, 6, 10), it is important to know whether mouse TIM-1 and TIM-2 are redundant, or whether they have distinct functions. Thus, we have transiently expressed TIM-2 in human and murine T cell lines and probed its effects on downstream signaling pathways with well characterized transcriptional reporter assays.

Materials and Methods

Cell lines, Abs, and reagents

Experiments were performed with the Jurkat human T cell leukemia cell line or a fast growing derivative of the murine D10 T cell clone (11). Abs for stimulation of Jurkat and D10 T cells were as described previously (4). PMA and ionomycin were obtained from EMD Biosciences and used at 25 ng/ml and 1 µM, respectively. Anti-Flag M2 and carbachol were obtained from Sigma-Aldrich; carbachol was used at a final concentration of 500 µM. Anti-Flag staining was coupled with anti-mouse-PE (Caltag) for flow cytometry.

DNA constructs

A full-length TIM-2 cDNA (clone no. 4158605) generated by the I.M.A.G.E. consortium was purchased from Open Biosystems. The open reading frame (excluding the signal sequence) was PCR amplified from this plasmid and cloned in-frame with the human CD8 signal sequence and a Flag epitope tag, as described previously (4). A TIM-2 fusion protein lacking a cytoplasmic tail was generated by PCR, using the same forward primer as for the full-length construct, and a reverse primer designed to terminate the protein two residues after the presumptive transmembrane domain (12). Constructs were verified by automated sequencing.

Transfection efficiency was monitored with pMax-GFP from Amaxa. Constitutively active Ras (V12) and calcineurin (regulatory domain-deleted) were obtained from Dr. A. Weiss (University of California, San Francisco, CA). Full-length (untagged) murine TIM-1 (from the BL/6 strain) was generated by PCR amplification of the full open reading frame, verified by automated sequencing, and cloned into pCDEF3.

Transient transfections, stimulations, and luciferase assays

Jurkat and D10 cells were transfected by electroporation, then stimulated the next day, followed by determination of luciferase activity, as all described previously (11, 13).

ERK phosphorylation

Jurkat T cells were transfected with empty vector or Flag-TIM-2 plus pMax-GFP. The next day, cells were stimulated with anti-TCR and -CD28 Abs. Cells
were then fixed (1.5% paraformaldehyde), permeabilized (MeOH) and stained with a PE-conjugated Ab to phospho-ERK (BD Biosciences). PE staining within the GFP-positive population was determined on a BD Biosciences LSRII flow cytometer.

**Calcium mobilization**

Jurkat T cells were transfected with empty vector or Flag-TIM-2. The next day, cells were stained with an anti-Flag Ab, then loaded with Calcium Green AM ester (Invitrogen Life Technologies). Intracellular calcium was monitored in the FITC channel before and after addition of anti-TCR/CD28 Abs. Samples were maintained at 37°C for the duration of analysis.

**Results and Discussion**

To easily track expression and possibly manipulate dimerization of TIM-2, we constructed a version of the molecule that contains an extracellular Flag tag. This construct is expressed at the surface of transfected Jurkat and D10 T cells, as shown in Fig. 1. Our previous studies had shown that ectopic expression of TIM-1 in these cell lines leads to increased basal activation of an NFAT/AP-1 reporter, as well as augmented TCR/CD28-stimulated NFAT/AP-1 activity (4). As shown in Fig. 1, we observed that ectopic expression of Flag-TIM-2 in Jurkat (B) or D10 (C) T cells did not result in an increase in NFAT/AP-1-dependent transcription, but rather inhibited activation of this reporter, whether stimulation was in the form of TCR/CD3 plus CD28 Abs or the pharmacological stimuli PMA plus ionomycin. The inhibitory activity of TIM-2 appears to be mediated mainly by its cytoplasmic tail, since expression of a Flag-TIM-2 construct lacking the cytoplasmic tail led to little if any inhibition of NFAT/AP-1 reporter activity (Fig. 1D), although this construct is expressed at the cell surface equivalently to full-length TIM-2 (data not shown).

Since we observed inhibition of antibody-induced reporter activity in D10 cells, we also investigated whether stimulation of these cells with Ag/APCs is affected by TIM-2. Indeed, we did observe significant inhibition of Ag-dependent NFAT/AP-1 responses when Flag-TIM-2 was expressed in D10 cells (Fig. 2A). To further explore how general TIM-2 inhibition of signaling might be, we transfected Flag-TIM-2 into the Jurkat derivative JH-M1, which expresses the human type 1 muscarinic receptor (14). Stimulation of this cell line with the agonist carbachol results in G-protein-dependent activation of NFAT/AP-1, downstream of PLC-β. As shown in Fig. 2B, expression of Flag-TIM-2 significantly impaired the ability of carbachol to stimulate the activity of a co-expressed NFAT/AP-1 luciferase reporter, in addition to its inhibition of the effects of TCR/CD28 cross-linking and PMA/Iono. This result suggests that the inhibitory effect of TIM-2 occurs downstream of the proximal tyrosine kinases and adaptor proteins that result in PLC-γ1 activation. Inhibition by TIM-2 may therefore occur at the level of PLC itself (although carbachol stimulation requires PLC-β), or further downstream. We recently reported that transfection of TIM-1 into Jurkat T cells leads to a significant increase in basal levels of NFAT/AP-1 reporter activity (4). As shown in Fig. 2C, co-expression of Flag-TIM-2 with an untagged form of TIM-1 resulted in suppression of TIM-1-mediated NFAT/AP-1 activation, to a similar degree as its inhibition of signals from the TCR, PMA/Iono or carbachol. Importantly, expression of TIM-1 was not affected by co-transfection of Flag-TIM-2 (data not shown).

We were concerned that the inhibition of T cell activation by TIM-2 was the result of a general effect on cell viability. Therefore, we co-transfected increasing amounts of Flag-TIM-2, along with a constitutively expressed GFP reporter. Cells were analyzed for GFP expression by flow cytometry the next day. Expression of the GFP marker remained relatively constant, regardless of the amount of co-transfected Flag-TIM-2 (Fig. 2D). Furthermore, the percentage of cells expressing the co-transfected GFP marker with either empty vector or Flag-TIM-2 did not diverge over the same time period in which luciferase activity was measured (i.e., 24 h after transfection), or even up to 48 h after transfection (data not shown). As an additional assay for possible effects on cell viability, we determined whether the expression of TIM-2 resulted in increased numbers of apoptotic cells. Cells were stained the day after transfection with Annexin V, which binds exposed phosphatidylserine, an early marker of apoptosis. The day after transfection with empty vector or Flag-TIM-2, there was no difference in the proportion of Annexin

![FIGURE 1.](http://www.jimmunol.org/)

Expression of Flag-TIM-2 on the surface of transfected T cells and suppression of NFAT/AP-1 activation. A, Jurkat T cells were transfected with empty vector or Flag-TIM-2. Eighteen hours later, cells were stained with anti-Flag Ab M2 and PE-conjugated secondary Ab. B, Jurkat T cells were transfected with an NFAT/AP-1 luciferase reporter and either empty vector or Flag-TIM-2. The next day, cells were treated with the indicated stimuli for 6 h, followed by determination of luciferase activity. Results are the average (± SEM) of triplicate determinations from a single experiment, representative of over ten that were performed. C, D10 T cells were transfected and stimulated as shown in B. Results are representative of four experiments. D, The cytoplasmic tail of TIM-2 is required for optimal inhibition of NFAT/AP-1. Jurkat T cells were transfected as shown in B, with the indicated constructs. Results are representative of six that were performed.
V-positive cells (data not shown). We conclude that TIM-2 inhibits signaling pathways that lead to NFAT/AP-1-dependent transcription, but not general cell viability, at least in these relatively short-term assays.

To determine whether we could bypass the block imposed by TIM-2, we expressed active forms of signaling molecules located further downstream from where PMA and ionomycin act. First, we expressed an oncogenic, constitutively active, form of Ras (Ras V12). As shown in Fig. 3A, expression of Ras V12 reversed the TIM-2 inhibition of TCR/CD28-induced NFAT/AP-1 reporter activity (gray bars). Ras V12 could also synergize with ionomycin to activate this reporter (15), and this response was not inhibited by co-transfection of TIM-2 (black bars). Conversely, expression of a constitutively active form of calcineurin (Fig. 3B) allows for much greater NFAT/AP-1 activation with PMA alone, which was also not inhibited by TIM-2 expression. TIM-2 inhibition of CD3/CD28-stimulated NFAT/AP-1 was also reversed by active calcineurin (data not shown). A curious finding in these experiments was increased NFAT/AP-1 activation (40%) when TIM-2 was co-expressed with activated Ras or calcineurin, compared with either of these signaling proteins alone. We have not yet explored this observation further, given the admittedly artificial nature of the experiment and the consistent inhibition of NFAT/AP-1 by TIM-2 under most other conditions.

Given the ability of TIM-2 to inhibit activation of an NFAT/AP-1 reporter, we wanted to know whether TIM-2 expression affects calcium mobilization downstream of the TCR. Jurkat T cells were transfected with empty vector or TIM-2 and analyzed by flow cytometry for intracellular calcium concentration before and after stimulation. As shown in Fig. 4A, cells transfected with TIM-2 responded with a lower overall level of calcium mobilization after addition of anti-TCR/CD28 Abs, and also maintained cytoplasmic free calcium above basal levels for a

FIGURE 2. Scope of NFAT inhibition by TIM-2. A, D10 T cells were transfected with NFAT/AP-1-luciferase and either empty vector or TIM-2. Cells were stimulated for 8 h with CH27 B cells, with or without conalbumin, either in an equal proportion to or at one quarter the number of D10 cells. B, J-HM1 Jurkat cells, which express the type I human muscarinic receptor, were transfected with NFAT/AP-1-luciferase plus empty vector or Flag-TIM-2. The next day cells were stimulated as indicated and assayed for luciferase activity. C, Jurkat T cells were transfected with NFAT/AP-1-luciferase and the indicated plasmids. Cells were analyzed for luciferase activity 24 h later. Results are the average (± SEM) of triplicate determinations from a single experiment, representative of two that were performed in each case. D, TIM-2 expression does not cause nonspecific death of transfected cells. Jurkat T cells were transfected with pMax-GFP and the indicated amounts of Flag-TIM-2. Total amounts of DNA were equalized with empty vector (pCDEF3). Cells were analyzed after 24 h for expression of GFP (white bars) and Flag-TIM-2 (black bars).

FIGURE 3. Constitutively active Ras or calcineurin can bypass TIM-2-mediated suppression of NFAT/AP-1 reporter activity. Jurkat (A) or D10 (B) T cells were transfected with NFAT/AP-1-luciferase and the specified plasmids, then stimulated the next day as indicated. “Ras” denotes the oncogenic V12 form of Ras, whereas “CaN” denotes a truncated, active, form of calcineurin. Results shown are the average (± SD) of triplicate samples from a single experiment, representative of two experiments in each case.
shorter amount of time. Activation of an NFAT/AP-1 reporter requires not only calcium mobilization but also Ras/MAP kinase activation (15), so we also examined the effect of TIM-2 on activation of the MAP kinase ERK. As shown in Fig. 4B, induction of ERK phosphorylation by anti-TCR/CD28 Abs was also impaired by expression of TIM-2. Thus, both the calcium and Ras/MAP kinase pathways appear to be inhibited by TIM-2.

Since we observed inhibition of MAP kinase activation in the presence of TIM-2, we also examined its effects on a pure AP-1 reporter. As shown in Fig. 5A, expression of TIM-2 with an AP-1 reporter resulted in significant impairment of its activation by TCR/CD28 or PMA, similar to the results with the NFAT/AP-1 reporter. We wanted to determine whether we could bypass this block by co-transfection of activated signaling molecules, as shown above for NFAT/AP-1 (Fig. 4). We first employed a truncated, constitutively active, form of the MAPK kinase MEKK1, which is known to activate AP-1-dependent transcription through the stress-activated protein kinases JNK and p38 (16, 17). Expression of activated MEKK1 resulted in potent activation of the AP-1 reporter activity, which was completely insensitive to co-expression of TIM-2 (Fig. 5B). We next moved further upstream in the AP-1 pathway, transfecting this time a partially activated allele of protein kinase C (PKC) θ (PKC θ A/E) to activate the reporter (18). Thus, as shown in Fig. 5C, PKC θ A/E expression led to robust AP-1 activation. When TIM-2 was also expressed, it led to a modest (~20%), although significant (p < 0.01) decrease in the PKC θ-mediated AP-1 activity, which was not due to an effect on levels of PKC θ protein (data not shown).

Taken together, our data suggest that TIM-2 acts to inhibit T cell activation by interfering with the TCR signaling cascade just downstream of PLC activation, but upstream of the NFAT and AP-1 transcription factors. Our results are consistent with the phenotype of TIM-2 knockout mice, which display increased inflammation and Th2 cytokine production in a murine model of asthma, apparently due to an increased sensitivity of TIM-2-deficient T cells to activation (19). The fact that either Ras or calcineurin could rescue NFAT induction is difficult to reconcile with the fact that stimulation with PMA and ionomycin was compromised by TIM-2. However, the PKC/Ras and calcium signaling modules do not function in a completely separate manner. Intriguingly, recent evidence points to

**FIGURE 4.** TCR-induced activation of ERK and calcium mobilization are impaired in TIM-2-expressing cells. A, Jurkat T cells were transfected with empty vector or Flag-TIM-2. Calcium mobilization was assessed the next day, as described in Materials and Methods. Results are presented as the mean fluorescence intensity (MFI) of all cells (vector) or Flag-positive cells (TIM-2), and are representative of two experiments. B, Jurkat T cells were transfected with empty vector or Flag-TIM-2, plus pMax-GFP, then stimulated the next day with anti-TCR/CD28 Abs. Intracellular staining was performed to analyze ERK phosphorylation and is represented as the PE channel MFI within the GFP+ gate. Results are representative of two experiments.

**FIGURE 5.** TIM-2 blockade of AP-1 reporter activity and reversal by expression of active signaling intermediates. A, Jurkat T cells were transfected with an AP-1 reporter plus empty vector or TIM-2. Cells were stimulated as indicated the next day. Results are the average light units (± SD) from triplicate determinations of a single experiment, representative of four. B and C, Jurkat T cells were transfected as above, with or without activated alleles of MEKK1 (B) or PKC θ (C). Luciferase activity in B and C was measured 24 h later, without any additional stimulation. Results shown are the average of duplicate points from a single experiment, representative of two performed (B) or the average triplicate points (± SD) from a single experiment, representative of four.
a role for PKC θ in the regulation of PLC-γ1 (20, 21). Also, classical PKC isoforms (like α and β) are known to require calcium as a co-factor for optimal activation, in addition to diacylglycerol.

We note that transient expression of TIM-2 was itself sufficient to inhibit T cell activation. We did not observe any augmentation or reversal of the inhibitory effect by actively cross-linking Flag-TIM-2 with anti-Flag Ab (data not shown). Furthermore, we could not detect the presence of any putative TIM-2 ligands on either the Jurkat or D10 T cells used in our experiments, because neither cell type exhibited positive staining with TIM-2-Ig (data not shown). Given its recent identification as a ligand for TIM-2 (8), we considered the possibility that ferritin contained in the FCS in our culture medium was modulating TIM-2 function. We conducted several experiments in the presence of 0.5% serum (including the postransfection overnight culture), rather than the usual 5%, but still observed the same degree of inhibition of NFAT/AP-1 by TIM-2 (data not shown). This is reminiscent of our previous findings on TIM-1, ectopic expression of which provides a costimulatory signal in the absence of active cross-linking (4). The apparent ligand-independent function of TIM-2 in our system could be explained by its homo-dimerization under conditions of transient expression, possibly through its heavily glycosylated mucin domain. CD45, which is heavily glycosylated and expressed at high levels on leukocytes, has been reported to homodimerize in a manner dependent on the level of glycosylation (22). Nonetheless, ligand-independent signaling is not a common phenomenon of transiently expressed transmembrane proteins because CD28 (L. P. Kane, unpublished observations) and DAP10 (4), for example, still require active cross-linking to transmit signals when transiently expressed.

We are currently attempting to understand better the role of dimerization in the regulation of TIM-2 function, as well as the precise biochemical mechanism by which it inhibits the signaling pathways that activate NFAT/AP-1-dependent transcription. Such knowledge should contribute to our understanding of how molecules of the TIM family regulate immune responses.

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