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Discoidin Domain Receptor 1 Expression in Activated T Cells Is Regulated by the ERK MAP Kinase Signaling Pathway

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ABSTRACT

The expression and function of discoidin domain receptor 1 (DDR1) in T cells are still poorly explored. We have recently shown that activation of primary human T cells via their T cell receptor leads to increased expression of DDR1, which promoted their migration in three-dimensional collagen. In the present study, we provide evidence that activated T cells bind collagen through DDR1. We found that the DDR1:Fc blocking molecule significantly reduced the ability of activated T cells to bind soluble biotinylated collagen. However, DDR1:Fc had no impact on the adhesion of activated T cells to collagen and overexpression of DDR1 in Jurkat T cells did not enhance their adhesion. Together, our results indicate that DDR1 can promote T cell migration without enhancing adhesion to collagen, suggesting that it can contribute to the previously described amoeboid movement of activated T cells in collagen matrices. Our results also show that CD28, in contrast to IL-2 expression, did not costimulate the expression of DDR1 in primary human T cells. Using specific inhibitors, we demonstrated that TCR-induced expression of DDR1 in T cells is regulated by the Ras/Raf/ERK MAP Kinase and PKC pathways but not by calcium/calcineurin signaling pathway or the JNK and P38 MAP Kinases. Thus, our study provides additional insights into the physiology of DDR1 in T cells and may therefore further our understanding of the regulatory mechanisms of T cell migration. J. Cell. Biochem. 112: 3666–3674, 2011.

KEY WORDS: T CELL; DISCOIDIN DOMAIN RECEPTOR; CELL SIGNALING; ERK MAPK; COLLAGEN I BINDING

Cell migration is essential for development, wound healing, and immune response and surveillance. After transendothelial migration, activated T lymphocytes reach target inflammatory sites by migrating through the extracellular matrix (ECM) of the basement membrane and the interstitial tissue that are rich in collagens. Beta1 (β1) integrins are major receptors in mediating cellular interactions with ECM components [von Andrian and Mackay, 2000; Barreiro et al., 2010]. Recent studies have shown that lymphocytes migrate in three-dimensional (3D) collagen using the amoeboid movement resembling that of amoeba Dictyostelium discoideum and zebrafish germ lines independently from β1 integrins and strong adhesive forces [Friedl et al., 1998; Nourshargh et al., 2010; Schmidt and Friedl, 2010]. This amoeboid movement also applies to other leukocytes as integrin blockade with antibodies or genetic ablation of integrin subunits did not affect migration of dendritic cells and monocytes in 3D collagen and in interstitial tissues [Lammermann et al., 2008]. In addition to β1 integrins, mammalian cells also express additional collagen receptors. Discoidin domain receptors (DDR1 and DDR2) are nonintegrin tyrosine kinase transmembrane receptors that bind different types of collagen [Vogel et al., 2006; Heino et al., 2009]. DDR1 is expressed as five isoforms (a–e) and is found in many epithelial and carcinoma cells, whereas DDR2 (no isoform) is found in cells with mesenchymal origin such as fibroblasts and smooth muscle cells. DDRs have been reported to control cell adhesion to collagen, ECM remodelling, proliferation, and development. With regard to cell migration, DDR1a/b have been shown to enhance the haptotactic response of vascular smooth muscle cells towards collagen and the motility of NIH3T3 and MCF-7 cells in 2D collagen [Hou et al., 2002; Huang et al., 2009; Lu et al., 2010]. The expression and function of DDRs are still poorly explored especially in leukocytes. In this regard, we have recently shown that DDR1 is induced in T cell receptor (TCR)-activated human T cells and that it participated in their migration in 3D collagen [Hachehouche et al., 2010]. Furthermore, a previous study also reported that overexpression of DDR1 in the monocytic cell line THP-1 enhanced their migration in 3D collagen [Kamohara

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et al., 2001]. Despite these findings, it remains unknown how DDR1 promotes T cell interactions with collagen. In addition, although TCR-engagement [Hachehouche et al., 2010] or phytohemagglutinin A [Kamohara et al., 2001; Dang et al., 2009] enhance DDR1 expression in human primary T cells, the signaling pathways that regulate DDR1 expression in T cells have not yet been identified.

The engagement of TCR/CD3 complex activates several proximal signaling events that culminate into the activation of intracellular pathways that regulate cytokine gene expression and T cell activation [Lin and Weiss, 2001]. TCR/CD3 engagement activates phospholipase Cγ1 (PLCγ1) which leads to the synthesis of second messengers such as diacylglycerol (DAG) and inositol 1,4,5-triphosphate (Ins(1,4,5)P_3). DAG activates various isoforms of Protein Kinase C (PKC), while Ins(1,4,5)P_3 contributes to the elevation of the intracellular calcium (Ca^{2+}) levels by releasing Ca^{2+} stores from the endoplasmic reticulum and by stimulating the opening of plasmic membrane Ca^{2+} pores leading to the extracellular calcium influx. Calcineurin, a phosphatase activated by intracellular Ca^{2+}, dephosphorylates the transcription factor NF-AT, which subsequently translocates to the nucleus where it stimulates the transcription of several genes implicated in the immune response. Both PKC and DAG activate the small GTPase Ras, which leads to the activation of ERK, JNK, and P38 MAP kinases, resulting in the activation of several transcriptional factors involved in gene expression [Lin and Weiss, 2001]. Optimal T cell activation also requires signals delivered by costimulatory molecules. CD28 is a crucial costimulatory molecule and its engagement enhances TCR signaling and activates the PI3 kinase/AKT pathway, which is important for the activation of the transcription factor NF-kappaB (NF-κB) [Lin and Weiss, 2001].

In the present study, we demonstrate that activated T cells bind collagen type I (collagen I) in a DDR1-dependent fashion. Cell adhesion assays indicate that DDR1 does not promote T cell adhesion to collagen I suggesting that DDR1 regulates T cell migration by promoting the amoeboid movement. We also show that TCR-induced DDR1 expression in activated T cells depends on the ERK/MAP Kinase signaling pathway and requires the activation of PKC. Other signaling pathways such as JNK and P38 MAP kinases, or Ca^{2+}/Calcineurin pathway seem not to be essential. Together, these results provide new insights into the expression and function of DDR1 in activated T cells and may therefore further our understanding of T cell migration.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

The anti-CD3 Antibody (Ab) (OKT3), anti-CD28 (clone CD28.2), and control immunoglobulin (IgG) were from BD Biosciences (San Diego, CA). The anti-DDR1 (c-20) and anti-actin (C-2) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). The MEK1/2 inhibitor U0126, the p38 inhibitor SB 203580, the JNK inhibitor SP600125, the PKC broad range inhibitor GF109203X (Bisindolylmaleimide I) and the calcineurin specific inhibitor cyclosporine-A were from EMD biosciences (San Diego, CA). Collagen type I was purchased from Sigma (St. Louis, MO). The blocking DDR1:Fc recombinant protein was purchased from R&D systems (Minneapolis, MN).

T CELL ISOLATION AND ACTIVATION

Peripheral blood mononuclear cells (PBMC) from healthy adult volunteers were isolated on Ficoll gradients. T cells were then purified using T cell enrichment columns (R&D systems) according to the manufacturer's instructions. The participating volunteers signed a consent form and the ethical committee of Laval University approved the study. Staining with anti-CD3 and flow cytometry analysis indicated that more than 97% of the isolated cells were CD3-positive T cells. Primary T cells (10^6/ml) in RPMI containing 2.5% FBS were stimulated in six-well-plates with 5 μg/ml of coated anti-CD3 Ab or with a combination of 5 μg/ml of anti-CD3 and 10 μg/ml of soluble anti-CD28 Abs.

COLLAGEN I BINDING ANALYSIS

Collagen I was biotinylated as previously described [Holleran et al., 2003]. T cells (5×10^5) were cultured with 20 μg/ml of soluble biotinylated collagen I at 37°C. Where indicated, biotinylated collagen I was preincubated with 20 μg/ml of DDR1:Fc recombinant protein (R&D systems) or with control IgG for 4 h at 4°C before adding it to the cells. After 1 h of incubation with collagen I, the cells were pelleted, washed three times with PBS, and incubated for 40 min at room temperature with phycoerythrin (PE)-conjugated streptavidin. After washing with PBS, labeled cells were analyzed by flow cytometry using FACScan (BD Biosciences).

CELL ADHESION ASSAY

Cell adhesion assays were performed in 96-well plates as we previously described [Boisvert et al., 2010]. A 96-well microtiter plate (TC plate, flat bottom, Falcon) was coated with 20 μg/ml of collagen I in PBS overnight at 4°C. The wells were then washed with PBS and nonspecific sites were blocked for 1 h at 37°C with 1% BSA. Where indicated, collagen I was incubated for 4 h with DDR1:Fc or control IgG at 4°C before being coated in wells. Cells (10^5) in 100 μl of RPMI were added to the wells and were incubated for 1 h at 37°C. After their attachment, the cells were washed with PBS and fixed in a PBS solution containing 1% formaldehyde for 1 h at room temperature. The cells were then washed with PBS and stained with a methanol solution containing 0.5% crystal violet. After several washes with PBS, the cells were lysed in a 1% SDS solution and the absorbance at 650 nm determined by an ELISA plate reader.

PLASMIDS AND JURKAT T CELL TRANSFECTION

The human Jurkat T-cell line E6.1 was obtained from ATCC (Manhasset, VA) and maintained in complete RPMI 1640 medium containing 10% FBS, 2 mM glutamine, and 100 U/ml penicillin and streptomycin.

The plasmid encoding the kinase dead form of DDR1a in which a lysine was mutated to alanine in the catalytic domain (K618A plasmid) was a gift from Dr. Wolfgang Vogel (Toronto University) [Vogel et al., 2000]. DDR1-K618A plasmid or empty control plasmid were transfected in Jurkat T cells by electroporation at 250 V and 960 μF settings using a Bio-Rad electroporator as we previously described [Gendron et al., 2003]. One day after transfection, dead cells were removed by a ficoll/hypaque gradient and the remaining cells cultured for another day before being used in subsequent experiments.

IMMUNOBLOT ANALYSIS

After stimulation, the cells were washed in cold PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors. Cell lysates were subjected to SDS–PAGE and analyzed by immunoblot using specific antibodies. The blots were stripped and reprobed with control anti-actin Ab to ensure equal loading. In all experiments, immunoblots were visualized using an HRP-conjugated secondary antibody followed by enhanced chemiluminescence's detection (Pierce, Rockford, IL).

RT-PCR ANALYSIS

Total RNA was extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). First strand cDNA was prepared from 1 μg of total RNA using the Thermoscript RT-PCR system from Invitrogen. IL-2 and β-actin transcripts were amplified by PCR using specific primers previously described [Gendron et al., 2005]. PCR reactions were done with 1 U Taq polymerase in a total volume of 50 μl, and amplifications were carried out in a Peltier Thermal Cycler from MJ Research. From the reaction mixture 10 μl was size-separated on a 2% agarose gel, and specifically amplified products were detected by ethidium bromide and UV illumination.

STATISTICAL ANALYSIS

Statistical analysis was performed using paired Student's *t*-test. Results with $P < 0.05$ were considered significant.

RESULTS

DDR1 EXPRESSED BY ACTIVATED T CELLS BINDS COLLAGEN I

We have recently shown that activation of human primary T cells through T cell receptor (TCR/CD3) induced the expression of DDR1, which enhanced their migration in 3D collagen [Hachehouche et al., 2010]. As cell contact with ECM is necessary for cell movement, we examined if activated T cells bind collagen I through DDR1. To this end, we assessed the role of DDR1 in the ability of T cells to bind biotinylated collagen I. Our preliminary studies showed that maximal collagen binding is observed after 48 h activation with anti-CD3 and therefore, analysis of collagen binding to activated T cells was examined at this time. Flow cytometry analysis (Fig. 1A, left panel) revealed that activated T cells, compared to nonactivated cells binds biotinylated collagen I. Quantification of collagen binding indicates that between 20% and 25% of anti-CD3-activated T cells bind collagen I whereas less than 5% of nonactivated T cells display collagen I binding (Fig. 1A, right panel).

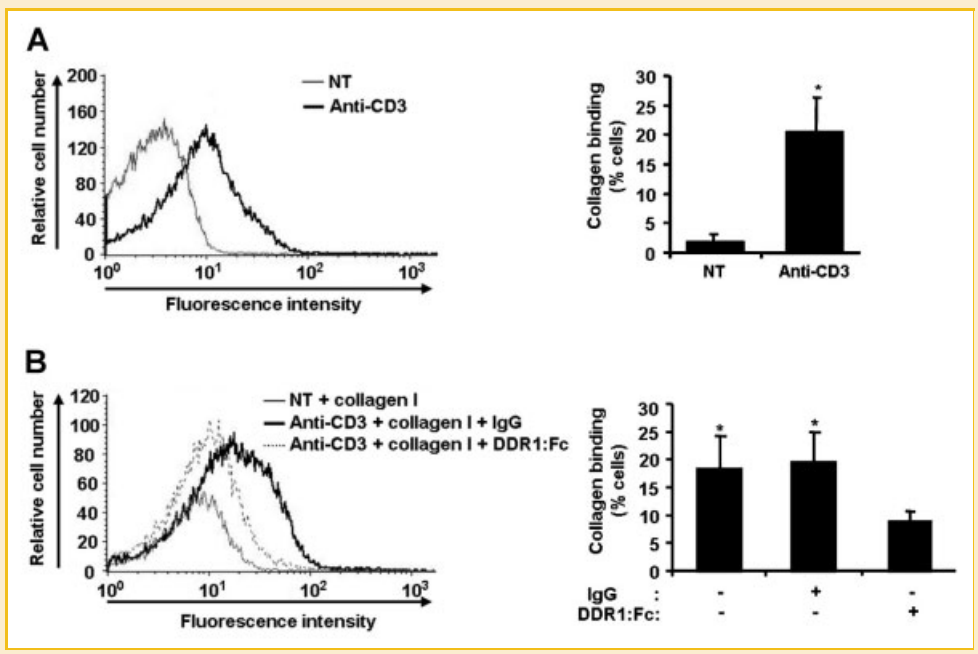

Fig. 1. DDR1 expressed by activated T cells binds to collagen I. A: Peripheral blood T cells were treated or not (NT) with anti-CD3 (10 μg/ml) for 48 h. The cells were then incubated in the presence of biotinylated collagen I (25 μg/ml) for 1 h at 37°C and bound collagen I was revealed with phycoerythrin-conjugated streptavidin and detected by flow cytometry. A representative flow cytometric profile of collagen I binding is shown (left panel). Quantification of collagen I-binding cells and comparison between activated (anti-CD3) and non-activated (NT) cells is shown (right panel). The results represent mean values (±SD) from five independent experiments performed with T cells isolated from different blood donors and are expressed as the percentage of cells that attached biotinylated collagen I. $^*P < 0.01$ between anti-CD3-and nontreated (NT) samples. B: DDR1:Fc chimera impaired the attachment of biotinylated collagen I to activated T cells. Anti-CD3-activated T cells or nontreated (NT) cells were cultured for 1h with biotinylated collagen I. Activated T cells were incubated with biotinylated collagen I that had been preincubated with DDR1:Fc recombinant protein or with control IgG. Collagen I bound to T cells was revealed with phycoerythrin-conjugated streptavidin and detected by flow cytometry. A representative flow cytometric profile of collagen I binding in the presence or absence of DDR1:Fc is shown (left panel). Quantification of collagen I bound to activated T cells in the presence or absence of DDR1:Fc or control IgG (right panel). The results represent mean values (±SD) from five independent experiments performed with T cells isolated from different blood donors and are expressed as the percentage of cells that attached biotinylated collagen I. $^*P = 0.025$ between control (−) or IgG-treated samples and DDR1:Fc-treated samples.

Pretreatment of biotinylated collagen I with nonlabeled DDR1:Fc chimera protein, which has been shown to block DDR1-collagen interactions [Bhatt et al., 2000; Kim et al., 2007] and to reduce T cell migration in 3D collagen I [Hachehouche et al., 2010], reduced the capacity of collagen I to bind to activated T cells compared to collagen I pretreated with control IgG (Fig. 1B, left panel). Quantification analysis indicates that DDR1:Fc reduced by half the binding of collagen I to activated T cells (Fig. 1B, right panel). DDR1:Fc molecule did not block collagen binding to nonactivated T cells (data not shown), consistent with the fact that these cells do not express significant levels of DDR1 and also with the fact that DDR1:Fc did not block basal migration of nonactivated T cells in 3D collagen [Hachehouche et al., 2010]. This suggests that the observed blocking effect of DDR1:Fc on collagen binding to activated T cells occurs at the level of DDR1–collagen interaction. Together these results indicate that DDR1 contributes to the ligation of collagen I by activated T cells.

DDR1 DOES NOT ENHANCE T CELL ADHESION TO COLLAGEN I

To determine if DDR1 on activated T cells promotes their adhesion to collagen I, we studied the effect of DDR1:Fc. To this end, primary human T cells were first activated with anti-CD3 Ab for 48 h to induce DDR1 expression and were subsequently examined for their ability to attach to collagen I. As shown in Figure 2A, anti-CD3-activated T cells show weak adhesion to collagen I, which is not affected by DDR1:Fc. Since adhesion of T cells can be enhanced upon PMA stimulation, which activates β1 integrins thereby mediating stronger T cell interactions with collagens, we also assessed the role of DDR1 in this setting. As expected, PMA increased adhesion of anti-CD3-activated T cells to collagen I but the presence of DDR1:Fc did not inhibit the observed increased cell adhesion (Fig. 2A). The effect of DDR1:Fc on cell adhesion is similar to that of the IgG, which was used as a control.

The form of DDR1 that was detected in activated T cells is likely to correspond to the DDR1e isoform, which represents a kinase-dead form of DDR1 [Hachehouche et al., 2010]. Thus, to further study the role of DDR1 in T cell adhesion to collagen I, we tested the effect of over-expressing a kinase-dead form of DDR1 (K618A) on Jurkat T cell adhesion to collagen I. Upon transfection, Jurkat T cells express the K618A form of DDR1 (Fig. 2B, left panel). Interestingly, these cells activated or not with PMA did not show an enhancement of adhesion in comparison to control cells (Fig. 2B right panel). Together, our results demonstrate that while

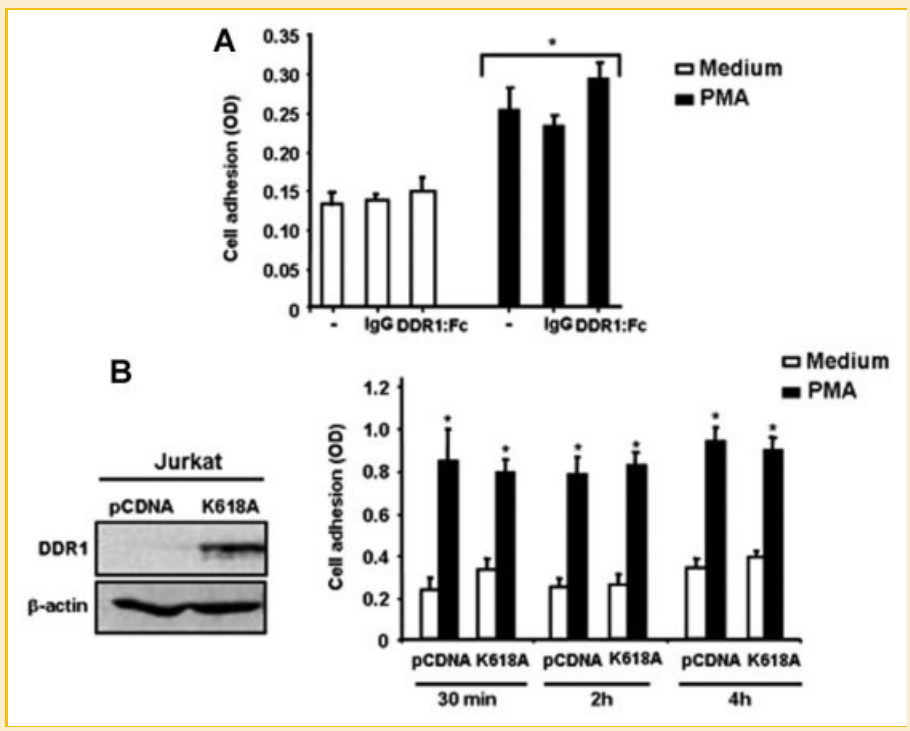

Fig. 2. DDR1 does not enhance T cell adhesion to collagen I. A: DDR1:Fc chimera does not reduce adhesion of activated T cells to collagen I. Human peripheral blood T cells were activated with anti-CD3 Ab for 48 h, then activated or not with PMA and allowed to attach to collagen I-coated wells for 2 h at 37°C in the presence or absence of DDR1:Fc or control IgG. Cell adhesion was quantified as described in "Materials and Methods" Section. The results are expressed as mean values (±SD) from three independent experiments performed with T cells isolated from three different blood donors. $^{*}P = 0.03$ between PMA-treated and nontreated samples (Medium). B: DDR1 kinase-dead over-expression in Jurkat T cells does not enhance cell adhesion to collagen I. A representative immunoblot analysis of DDR1 expression in Jurkat T cells transfected with control plasmid (pCDNA) or with DDR1-kinase dead plasmid (K618A) (left panel). Actin was used as a loading control. Control and K618A-DDR1-expressing Jurkat T cells activated or not with PMA were tested for adhesion to collagen (right panel). The results are presented as mean values (±SD) from three independent experiments. $^{*}P < 0.01$ between PMA-treated and nontreated samples (Medium).

DDR1 on activated T cells binds collagen I, it did not promote their adhesion to collagen I.

CD28 COSTIMULATION DOES NOT UPREGULATE DDR1 EXPRESSION

To get insights into the regulation of DDR1 expression in T cells, we have first assessed if CD28 costimulation enhances TCR-induced DDR1 expression. As shown in Figure 3A, co-engagement of CD28 and CD3 molecules with antibodies led to similar expression levels of DDR1 after 24–72 h of stimulation as did engagement of CD3 alone. However and as expected, CD28 costimulation significantly enhanced anti-CD3-induced IL-2 gene expression (Fig. 3B). CD28 engagement alone had no effect on DDR1 or IL-2 expression.

TCR-INDUCED DDR1 EXPRESSION IS NOT REGULATED BY THE Ca^{2+} SIGNALING CASCADE

TCR/CD3 engagement leads to T cell activation by inducing several signaling cascades, among which is the Ca^{2+}/calcineurin pathway [Lin and Weiss, 2001]. We thus investigated whether the inhibition of Ca^{2+}-dependent signaling using cyclosporin-A (cyc-A), a specific inhibitor of calcineurin, could affect anti-CD3-induced DDR1 expression in activated T cells. The results show that cyc-A at 100 and 200 nM did not affect the expression levels of DDR1 (Fig. 4A). As a control, cyc-A significantly inhibited anti-CD3-induced IL-2 mRNA expression by T cells (Fig. 4B). Our results thus exclude a significant role for the Ca^{2+}/calcineurin signaling pathway in TCR-induced DDR1 expression.

TCR-INDUCED DDR1 EXPRESSION IS REGULATED BY THE Ras/Raf/ERK MAPK SIGNALING PATHWAY

ERK1/2, p38 and JNK MAP Kinases are activated by TCR engagement and play a central role in T cell activation [Lin and Weiss, 2001]. To determine whether DDR1 expression depends on the activation of MAPK signals, freshly isolated T cells were activated with anti-CD3 in the presence of specific MAPK inhibitors; U0126 for the ERK1/2 pathway, SB203580 for the p38 pathway, or SP600125 for JNK pathway and the expression of DDR1 was evaluated. As shown in Figure 5A (left panel), anti-CD3-induced DDR1 expression is strongly inhibited by the ERK MAPK signaling inhibitor U0126. However, the p38 (SB203580) or the JNK (SP600125) inhibitors had no effects. These two inhibitors, used at the same concentration, impaired IL-2 mRNA expression by activated T cells (Data not shown). Quantification analysis shows that the U0126 inhibitor almost completely abrogates anti-CD3-induced expression of DDR1 (Fig. 5A, right panel). Together these results indicate that the expression of DDR1 is regulated by the ERK MAPK pathway.

To further characterize the upstream signaling pathway leading to ERK-dependent DDR1 expression upon TCR engagement, we studied the implication of Ras and c-Raf, which are upstream of MEK-1 and ERK. To this end, we used the specific pharmacological inhibitors for Raf (GW5074) and Ras (Manumycin-A). We found that manumycin and GW5074 drastically reduced anti-CD3-induced DDR1 expression (Fig. 5B, left panel). Quantification analysis indicates that GW5074 and manumycin have the same inhibitory potential on DDR1 expression as did the U0126 inhibitor (Fig. 5B, right panel). Taken together, our results demonstrate that expression of DDR1 in activated T cells is regulated by the Ras/Raf/ERK signaling pathway but not by the P38 and JNK MAPKs.

TCR-INDUCED DDR1 EXPRESSION IS DEPENDENT ON PKC PATHWAY

TCR engagement in T cells leads also to PKC activation, which has been involved in TCR-mediated activation of ERK MAPK pathway [Lin and Weiss, 2001]. Therefore, we investigated the potential

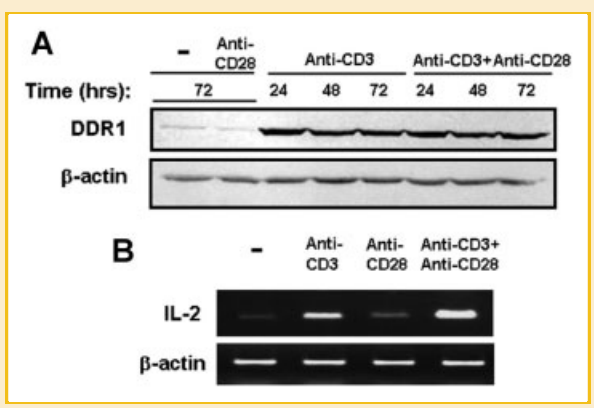

Fig. 3. CD28 costimulation does not upregulate DDR1 expression. A: T cells were activated or not (–) with coated anti-CD3 in the presence or absence of soluble anti-CD28 mAb as indicated. DDR1 and β-actin expression levels were detected by immunoblot analysis. B: CD28 upregulates IL-2 mRNA levels. T cells were activated or not (–) for 6 h with anti-CD3, anti-CD28 or with anti-CD3 + anti-CD28. Expression of IL-2 and β-actin mRNA were determined by RT-PCR analysis. The results (A,B) are representative of three different experiments performed with T cells isolated from different blood donors.

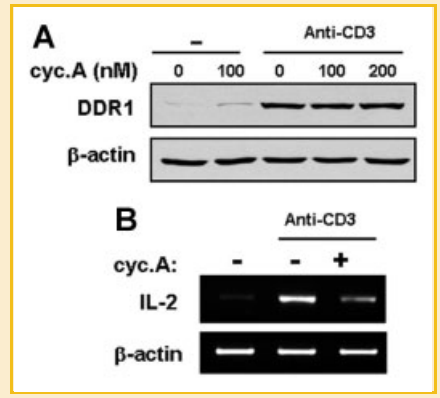

Fig. 4. The Ca^{2+}/calcineurin-signaling pathway is not involved in DDR1 expression. A: T cells were pretreated or not for 1 h with the calcineurin inhibitor cyclosporine-A (cyc.A) at the indicated concentration and then activated or not (–) with immobilized anti-CD3 Ab (10 μg/ml) for 48 h. DDR1 and β-actin expression levels were detected by immunoblot analysis. B: T cells were pretreated or not with cyc-A (100 nM) for 1 h and then activated or not with anti-CD3 Ab for 6 h. The mRNA expression levels of IL-2 and β-actin were then determined by RT-PCR analysis. The results (A,B) are representative of three different experiments performed with T cells isolated from different blood donors.

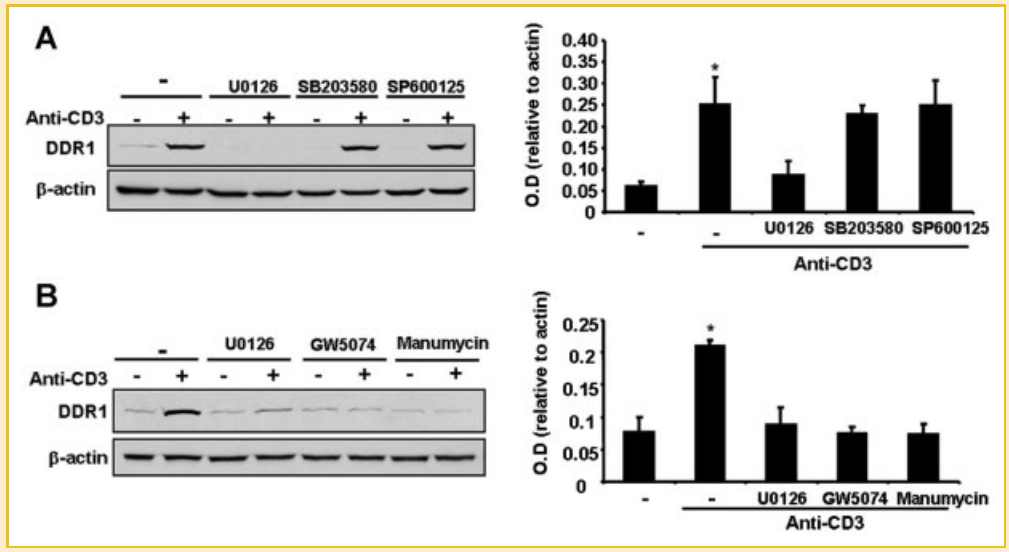

Fig. 5. TCR-induced DDR1 expression is regulated by the Ras/Raf/ERK pathway but not by the p38 or the JNK MAP Kinases. A: T cells were pretreated or not for 1 h with the MEK1 inhibitor U0126 (20 μM), the p38 inhibitor SB203580 (20 μM), or with the JNK inhibitor SP600125 (10 μM). The cells were then activated or not for 48 h with anti-CD3 Ab. The levels of DDR1 and β-actin proteins were determined by immunoblot analysis (left panel). B: Peripheral T cells were pretreated or not for 1h either with the MEK1-inhibitor U0126 (20 μM), the Raf inhibitor GW5074 (5 μM), or with the Ras inhibitor Manumycin (50 nM). The cells were then activated or not for 48 h with anti-CD3 Ab. The levels of DDR1 and β-actin proteins were determined by immunoblot analysis (left panel). The immunoblot data shown in (A) and (B) are representative of at least three different experiments performed with T cells isolated from different blood donors. The densitometric quantification in (A) and (B) represents mean values (±SD) from at least three different experiments performed with T cells isolated from different blood donors and are expressed as the ratio between DDR1 values and β-actin values. $^{*}P < 0.01$ between anti-CD3-treated samples and anti-CD3+U0126 (A) or between anti-CD3 and other samples (B) as indicated.

implication of the PKC pathway in the anti-CD3-induced DDR1 expression using the broad range PKC inhibitor GF109203x (GFx). Similar to U0126, treatment with GFx also inhibits anti-CD3-induced DDR1 expression (Fig. 6A). Since both ERK MAPK and PKC are major pathways activated by PMA, we examined if PMA could induce the expression of DDR1 in T cells. We found that PMA also induces DDR1 expression and both U0126 and GFx abrogated this expression. Quantification analysis indicates that GFx has similar inhibitory potential as U0126 (Fig. 6B). These results further support the implication of ERK MAPK and PKC in TCR-induced DDR1 expression in human T cells.

INHIBITION OF ERK MAPK AND PKC REDUCES THE ABILITY OF ACTIVATED T CELLS TO BIND COLLAGEN I

The results above indicate that ERK MAPK and PKC are critical signaling molecules in the expression of DDR1. To further support these results, we have evaluated the effects of the MEK-1 and PKC inhibitors (U0126 and Gfx) on the ability of activated T cells to bind collagen I. As shown in Figure 7, both inhibitors significantly reduce the ability of activated T cells to bind biotinylated collagen. However, the JNK (SP600125) and p38 (SB203580) inhibitors had no effect; consistent with the fact that these MAPKs do not upregulate DDR1 expression.

DISCUSSION

We have recently shown that DDR1 is implicated in the migration of activated T cells in 3D collagen [Hachehouche et al., 2010]. In this study, we further show that DDR1 binds collagen I but does not promote adhesion of activated T cells to collagen I. We further show that TCR-induced DDR1 expression is regulated by PKC and ERK signaling pathways.

β1 integrins are the major high affinity receptors for collagen. Activated human T cells are known to express two collagen-binding integrin namely α1β1 and α2β1, which promote T cell adhesion to collagens [Pribila et al., 2004]. In addition to collagen-binding integrins, we and others have reported that activated T cells can also express DDR1 [Kamohara et al., 2001; Dang et al., 2009; Hachehouche et al., 2010], a nonintegrin collagen receptor [Vogel et al., 2006; Heino et al., 2009]. In this study, we show that activated T cells bind collagen I in a DDR1-dependent manner but that DDR1 did not enhance their adhesion to collagen I, which is dependent on β1 integrins [Boisvert et al., 2007] and (data not shown). This is in contrast to the reports showing that DDR1 promotes attachment of adherent cells to collagen [Hou et al., 2001, 2002; Curat and Vogel, 2002; Song et al., 2011]. One possible explanation for this discrepancy could be the nature of the cells. In contrast to adherent cells, activated T cells do not attach strongly to ECM especially to collagen but are very motile cells compared to adherent cells [Lammermann and Sixt, 2009; Friedl and Wolf, 2010]. In addition, DDR1 a/b are the major isoforms that have been shown to promote adhesion of adherent cells to collagen I. However, based on the molecular weight, the DDR1 isoform that we detected in activated T cells corresponds to DDR1e [Hachehouche et al., 2010], which is a kinase-dead isoform of DDR1. Along these lines, we also show that overexpression of a kinase-dead form of DDR1 did not enhance adhesion of Jurkat T cells to collagen. Together these results suggest

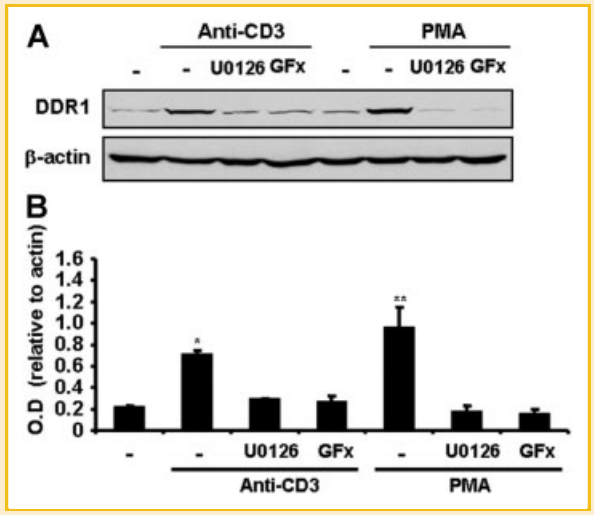

Fig. 6. PKC is involved in TCR- and PMA-induced DDR1 expression in T cells. A: T cells were pretreated or not with the broad range PKC inhibitor GFx (5 μM) or with the MEK1 inhibitor U0126 (20 μM) for 1 h. The cells were then activated or not (−) with immobilized anti-CD3 Ab (10 μg/ml) or with PMA (50 ng/ml) for 48 h. DDR1 and β-actin expression levels were detected by immunoblot analysis. The immunoblot data shown are representative of three different experiments performed with T cells isolated from different blood donors. B: Densitometric quantification of relative increase of DDR1 proteins in activated T cells in the presence of different inhibitors as indicated. The results represent mean values (±SD) from three independent experiments performed with T cells isolated from different blood donors and are expressed as the ratio between DDR1 values and β-actin values. $^{*}P < 0.01$ between anti-CD3-treated samples and anti-CD3 + U0126 or anti-CD3 + GFx-treated samples. $^{**}P < 0.01$ between PMA-treated samples and PMA + U0126 or PMA + GFx-treated samples.

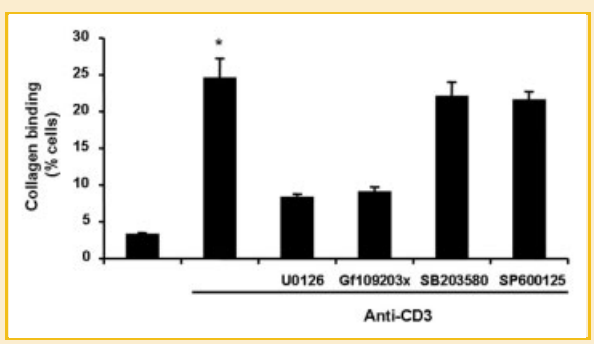

Fig. 7. Inhbition of ERK MAPK and PKC reduces the ability of activated T cells to bind collagen I. T cells were preincubated or not for 1 h with the MEK-1 inhibitor (U0126), the PKC inhibitor (Gfx), the JNK inhibitor (SP600125), or with the p38 inhibitor (SB203580) before being activated with anti-CD3 mAb. After 48 h of activation, the cells were washed and tested for their capacity to bind biotinylated collagen I as described under the "Materials and Methods" Section. The results represent mean values (±SD) from two independent experiments performed with T cells isolated from different blood donors and are expressed as the percentage of cells that attached biotinylated collagen I. $^{*}P < 0.01$ between anti-CD3- and anti-CD3 + U0126 or anti-CD3 + Gfx samples.

that DDR1 can regulate cell adhesion in an isoform-dependent manner. The fact that DDR1 expressed on activated T cells binds collagen without promoting adhesion is in line with its implication in the migration of activated T cells in 3D collagen [Hachehouche et al., 2010] and with the reports showing that these cells migrate in 3D collagen independently from integrins, strong adhesive forces, and stress fibers [Friedl et al., 1998; Lammermann and Sixt, 2009; Friedl and Wolf, 2010]. These observations suggest that the contact between activated T cells with collagen I through DDR1 could be a pathway that contributes to the amoeboid movement of T cells in 3D collagen. Interestingly, recent reports indicated that kinase-dead forms of DDR1 regulate fibrillogenesis with similar capacity as did tyrosine kinase DDR1 [Agarwal et al., 2007; Flynn et al., 2010]. In addition, the tyrosine activity of DDR1 is not required in DDR1-mediated collective migration of cancer cells [Hidalgo-Carcedo et al., 2011]. These studies indicate that the tyrosine kinase activity of DDRs could be dispensable for DDR1 signaling. Thus, it is likely that DDR1 kinase-dead receptors can associate with membrane receptors and signaling molecules to induce cell signaling and thereby regulate cell migration.

Our results show that induction of DDR1 expression upon T cell receptor activation does not require CD28 costimulation. It is not yet clear whether other costimulatory molecules such as CD2, LFA-1 or costimulatory molecules of the TNF family could regulate the expression of DDR1 in T cells. However, the fact that CD28 did not influence DDR1 expression suggests that DDR1 expression might be regulated differently than cytokine expression, which requires costimulation for optimal expression. The regulatory pathways involved in the expression of DDRs are largely unknown. We show here that TCR-induced expression of DDR1 is regulated by the ERK MAP Kinase and the PKC pathways, while the P38 and JNK MAP Kinases and the Ca^{2+}/calcineurin pathways were not involved. Using chemical inhibitors, we showed that DDR1 expression is regulated by the Ras/Raf/MEK/ERK signaling cascade and by PKC, which during T cell activation contributes to the activation of Ras and thereby MAPKs as well as it contributes to the activation of NF-κB pathway [Lin and Weiss, 2001]. The fact that CD28 did not upregulate DDR1 expression argues against the implication of PI3 kinase/AKT and NF-κB pathways, which are downstream of CD28. Indeed, we found that NF-κB inhibitors did not affect TCR-induced DDR1 expression (data not shown). Therefore, our results suggest that PKC regulates TCR-induced DDR1 expression likely by promoting activation of ERK MAPK pathway. These observations are also supported by our results showing that PMA, which in T cells activates ERK MAPK through PKC [Bradshaw et al., 1996; Morley, 1997], induces DDR1 expression by a mechanism involving both PKC and ERK MAPK pathways. Our findings are in concordance with a recent study demonstrating the involvement of ERK MAP Kinase in the expression of DDR1 by primary lung fibroblasts [Ruiz and Jarai, 2011]. In contrast to our results, p38 MAPK has recently been involved in the expression of DDR2 in vascular smooth muscle cells in response to hypoxia [Chen et al., 2008; Shyu et al., 2009]. Whether DDR1 isoforms and DDR2 are regulated in a cell specific-manner or by different signaling pathways remain to be determined.

Little is known about the potential transcriptional factors involved in DDR1/2 expression. However, some transcriptional

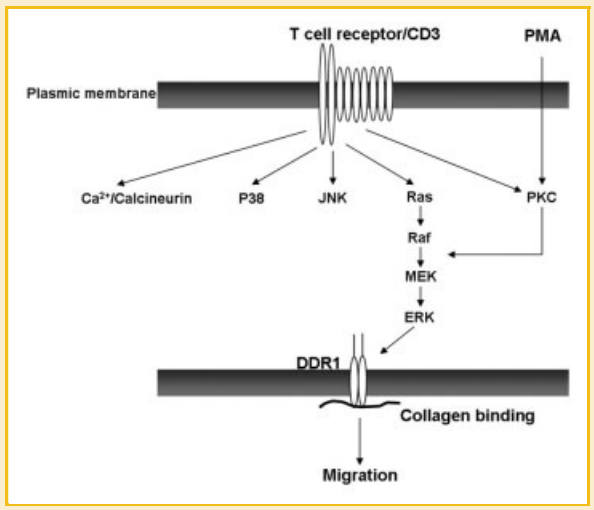

Fig. 8. A model by which TCR/CD3 signaling induces the expression of DDR1 in human T cells.

factors such as p53 [Ongusaha et al., 2003] and ATF-4 [Lin et al., 2010] were identified as regulators of DDR expression in different cell types. The promoter region of DDR1 gene contains potential binding sites for p53 [Sakuma et al., 1996], AP-1, PEA3 [Ruiz and Jarai, 2011], and NF-κB [Kamohara et al., 2001] transcriptional factors. The fact that our results showed that JNK is dispensable to TCR-induced DDR1 expression argues against the implication of AP-1 in the expression of DDR1 in activated T cells. AP-1 transcription factors are composed of jun/Fos or Jun/Jun heterodimers and Jun factors are activated by JNK. Activation of ERK can regulate the activation of several transcription factors in T cells including Fos, Elk-1 and members of the Ets family [Dong et al., 2002]. A recent study showed that in bronchial lung fibroblasts, ERK regulates DDR1 expression by activating the PEA3 transcription factor [Ruiz and Jarai, 2011]. Whether PEA3 or other transcription factors regulate DDR1 expression in activated T cells is not determined. The transcriptional regulation of DDR1 in activated T cells is currently underway in our laboratory.

A model by which DDR1 is regulated in T cells is presented in Figure 8. Based on our previous study showing the role of DDR1 in T cell migration [Hachehouche et al., 2010] and the findings herein, we propose that activation of PKC and ERK MAPK signaling pathways during T cell activation upregulate the expression of DDR1. Subsequently, T cells attach to collagen I through DDR1, which then promotes the migration of activated T cells in collagen-rich peripheral tissues. Thus, our study suggests that blocking the PKC/MAPK/ERK/DDR1 pathway could interfere with T cell migration in peripheral tissues and subsequently be beneficial for the treatment of inflammatory diseases.

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