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Abstract

T CELL INTEGRIN DEPENDENT, MKK3/p38 MAPK-MEDIATED STABILIZATION OF LABILE CYTOKINE mRNAs.

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The goal of this project is to elucidate the signaling pathway involved in the T cell integrin lymphocyte function-associated antigen 1 (LFA-1) engagement-mediated, HuR nuclear-to-cytoplasmic translocation and subsequent mRNA stabilization.

To examine the pathway transducing LFA-1 engagement-mediated mRNA stabilization, we employed quantitative, real time RT-PCR in the presence of a transcriptional inhibitor to examine the half-life of interferon gamma (IFN-γ) mRNA, a labile cytokine transcript. We used immunofluorescence to observe the nuclear-to-cytoplasmic tranlocation of HuR, an mRNA-binding and –stabilizing protein. We also used Western blot analysis to analyze the activity levels of specific mitogen activated protein kinases (MAPKs) upon LFA-1 engagement. These techniques were performed using murine splenocyte-derived T cells from wild type as well as transgenic mice lacking one or both copies of the MAPK kinase MKK3 gene. They were also performed in the presence of MAPK pharmacologic inhibitors, RNA interference for HuR, and transfection vectors for constitutively active Rho-GTPases and MAPKs.

Our data show that, in murine wild type T cells, LFA-1 engagement by its ligand, intercellular adhesion molecule-1 (ICAM-1), causes both HuR translocation and an increased half-life of IFN-γ mRNA transcripts. This mRNA stabilization is HuR-dependent: a decrease in HuR expression in T cells by RNA interference abrogates the LFA-1 induced IFN-γ mRNA stabilization.

To examine the pathway responsible for LFA-1 engagement-mediated mRNA stabilization, we looked at MAPKs since T cell integrin engagement triggers mitogenesis. LFA-1 engagement activates both c-jun NH_2 terminal protein kinase (JNK), and stress activated protein kinase (SAPK) (also known as the p38 MAPK). Pharmacologic inhibition of JNK, however, causes no decrease in HuR translocation or IFN-γ mRNA stabilization upon T cell integrin engagement, while pharmacologic inhibition of p38 MAPK inhibits both events. Interruption of the p38 MAPK pathway by using T cells lacking the p38 MAPK kinase MKK3 also causes a loss of HuR translocation and subsequent IFN-γ mRNA stabilization upon LFA-1 engagement. Activation of the p38 MAPK pathway alone via a constitutively active p38 MAPK kinase MKK6, however, was not sufficient to obtain IFN-γ mRNA stabilization.

From these data, we conclude that T cells utilize LFA-1 engagement by ICAM-1 to modulate the stability of labile mRNA transcripts encoding proinflammatory cytokines. Modulation of the RNA binding molecule HuR is required for this mRNA stabilization. Furthermore, the MKK3/p38 MAPK pathway is necessary for LFA-1 engagement-mediated mRNA stabilization. Activation of the p38 MAPK pathway alone, however, is not sufficient to generate the stabilization of labile, proinflammatory cytokine transcripts.

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Introduction

Leukocytes are specialized immune cells that continually migrate throughout the bloodstream, into peripheral tissues and peripheral lymphoid organs (1). The functions of leukocytes can be divided into those subserving innate and adaptive immunity. Innate immunity utilizes granulocytes and macrophages in a non-adaptive mechanism that provides a first line of defense against infection. The adaptive immune system, on the other hand, is a delayed, pathogen-specific response mediated by lymphocytes. The lymphocyte population has an enormous range of receptors that recognize various components of pathogens which are called antigens (2). Lymphocytes are divided into B lymphocytes and T lymphocytes based on their receptors as well as their response to antigens. B lymphocytes have antibodies (produced by the cells themselves) as their antigen receptor. T lymphocytes, on the other hand, have a T cell receptor (TCR) that only recognizes peptide antigen bound to a major histocompatability complex (MHC) molecule on the cell membrane.

Because T cells only recognize antigen bound to MHC proteins expressed on a cell's plasma membrane, T cells require adhesive interactions with other cells for their functional activation. In the thymus, T cells adhere to thymic epithelial cells for positive or negative selection where, depending on their reaction to specific MHC-bound proteins, they will receive mitogenic or apoptotic signals. Upon entering the peripheral circulation, T cells will adhere to various antigen presenting cells (APCs) and receive mitogenic or lineage-specific differentiation signals depending on the environment within which the T cell binds

to the APC. Once T cells have differentiated, strong adhesion with APCs is a requirement for activation of T cell effector functions (3).

The T cell receptors for adhesive interactions are integrins, a ubiquitously expressed, highly conserved family of receptors involved in cell-extracellular matrix and cell-cell adhesion (1, 3, 4). Integrins are heterodimeric proteins consisting of two subunits. The ligands for the β_2 subset (leukocyte-specific) integrin receptors are the intercellular adhesion molecules (ICAMs), a group of proteins within the immunoglobulin superfamily that are expressed by APCs as well as vascular endothelium (3, 4).

Specifically, T cells express the $\alpha_L\beta_2$ intregrin lymphocyte function-associated antigen 1 (LFA-1), which is composed of the subunits CD11a and CD18 (1). Within the thymus, LFA-1 appears to be important in the process of T cell selection. Research suggests that LFA-1 is required for the maturation of $CD8^+$ T cells (4, 5). LFA-1 also appears to play an integral role in T cell mitogenesis. Simultaneous engagement of both the TCR and LFA-1 causes a more robust cell cycle transition from G1 to S phase than engagement of the TCR alone (6). In the periphery, LFA-1 appears to play a key role in T cell differentiation, especially in the differentiation of naïve T cells into T_H1 cells. Inhibition of naïve T cell LFA-1 interaction with ICAM-1 on the dendritic cell membrane leads to a predominance of T_H2 cell development (7). Furthermore, addition of ICAM-1 to anti-CD3/CD28-stimulated naïve T cells (anti-CD3/CD28 is used to stimulate the TCR) causes a Th1 phenotype which is blocked by the addition or anti-ICAM-1 or anti-LFA-1 blocking antibodies (8).

One of the important effector functions of differentiated T cells is the production of pro-inflammatory cytokines, which have tightly controlled expression profiles. LFA-1 engagement in the presence of CD3-activated T cells causes an amplification of the steady state IFN-γ mRNA levels. Furthermore, IFN-γ is a labile transcript with a destabilizing AU-rich element (ARE) in its 3' untranslated region (3'-UTR). Measurement of IFN-γ mRNA half-life using a mRNA polymerase II inhibitor reveals a profound stabilization of the transcript when LFA-1 is engaged in CD3-stimulated T cells (3, 9). Thus, LFA-1 engagement leads to an increase in IFN-γ mRNA transcripts, as well as increased stability of those transcripts, within T cells (10).

Another important effector function of differentiated T cells is migration of T cells across vascular endothelium. T cell migration is essential to the pathogenesis of many inflammatory diseases, as well as to the ultimate localization of cells homing to a particular tissue or site of inflammation. Endothelial migration requires interaction between the T cell and the endothelial cell and its underlying basement membrane. The process begins with a process called rolling, followed by arrest and firm adhesion. Rolling is mediated by selectins, while firm adhesion requires the integrins LFA-1 and VLA-4, which bind ICAM-1 and VCAM-1, respectively, on the endothelial cell (1, 3). Blockade of ICAM-1, VCAM-1, or both on endothelial cells prevents the formation of pseudopodia and lamellipodia by leukocytes, which are phenotypic indicators of cell arrest and are required for transendothelial migration (11).

To migrate across the endothelium successfully, T cells must be able to degrade proteins in the basement membrane and extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are a family of endopeptidases used by T cells to break down ECM. T cells constitutively express small quantities of MMPs, and engagement of the integrin VLA-4 can both increase the expression of MMPs by T cells as well as increase enzyme activity (12). While MMPs are vital to T cell migration through the basement membrane and ECM, the plasminogen activation system plays a crucial role in cell migration into surrounding tissue. Plasmin is a serine protease that breaks down ECM proteins outside of the endothelium. It circulates through the body in the inactive, pro-form plasminogen and is converted to plasmin by urokinase plasminogen activator (uPA). uPA binding to its receptor, uPAR, is required for its activation. uPAR is inducibly expressed by T cells. Engagement of LFA-1 or VLA-4 in CD3-stimulated T cells potently induces T cell expression of uPAR (13). Like IFN-γ mRNA, the uPAR transcript contains a destabilizing ARE in its 3'-UTR. Furthermore, CD3/LFA-1 co-stimulation-induced T cell transmigration across a matrigel-coated filter is enhanced by the addition of plasminogen and inhibited by amiloride (uPAR inhibitor) as well as by an antibody that blocks the uPA binding site on uPAR (3). Thus, induced T cell uPAR is functional to promote migration.

T cell integrin engagement is required for many of the T cell's most important functions, such as thymic selection and mitogenesis, T cell differentiation, cytokine expression, and migration across vascular endothelium into surrounding tissues. Data suggest that integrin engagement regulates T cell

post-transcriptional events, which in turn affect T cell function. As previously mentioned, many of the mRNA transcripts affected by integrin engagement contain a destabilizing ARE in the transcript's 3'-UTR. These transcripts include cyclin A and cyclin B1 (responsible for thymic mitogenesis) in addition to IFN-γ and uPAR (14). LFA-1 engagement in CD3-stimulated T cells causes a stabilization of each of these transcripts. To determine if the ARE in the 3'-UTR is important to LFA-1-mediated mRNA stabilization, the uPAR 3'-UTR was cloned into an otherwise stabile rabbit β-globin transcript. When the construct was expressed in T cells, the 3'-UTR caused a destabilization of the rabbit β-globin transcript, which was reversed by mutagenesis of the uPAR ARE within the 3'-UTR. Furthermore, LFA-1 engagement of the T cell containing the construct conferred stability to the rabbit β-globin transcript (9). These findings confirm that LFA-1 engagement on T cells generates at least one signal that inhibits mRNA instability caused by an ARE in the transcript's 3'-UTR.

One possible candidate for the mRNA stabilization signal signal generated by LFA-1 engagement is the molecule HuR. HuR, which is a member of the embryonic lethal, abnormal vision (ELAV) family of proteins, has been shown to bind AREs within the 3'-UTR of mRNA transcripts and have a stabilizing effect on these transcripts (15, 16). In a resting T cell, HuR resides in the nucleus. LFA-1 engagement causes a nuclear-to-cytoplasmic translocation of HuR, which is required for stabilization of mRNA transcripts containing AREs (9, 17, 18). This mRNA stabilization facilitates enhanced expression of critical T cell gene products in response to integrin engagement.

The molecular signaling pathway responsible for transducing the signal between LFA-1 engagement and HuR nuclear-to-cytoplasmic translocation is unknown. Previous work done in the lab demonstrates that LFA-1 activates Vav-1 in a PI-3 Kinase dependent manner. Vav-1 is a guanine nucleotide exchange factor (GEF) for Rac-1 and Rac-2. Rac-1 and Rac-2 are Rho-GTPases, implicated in MAPK signaling in T cells, which exist in a non-active, GDP-bound state. Activation of Vav-1 causes Rac to exchange its GDP for GTP, activating Rac and thus its downstream effectors. Both Vav and Rac activation are required for LFA-1-mediated mRNA stabilization (Ramgolam et al, in preparation). The pathway distal to Rac-1/Rac-2 activation, however, remains unknown. Since two of the proteins stabilized by LFA-1 engagement are responsible for mitogenesis within T cells, the most likely candidates for the signaling pathway include the mitogen-activated protein kinases (MAPKs). In T cells, there are three major MAPK pathways: the extracellular signal regulated protein kinase (ERK) pathway, the c-jun NH_2 terminal protein kinase (JNK) pathway, and the stress activated protein kinase (SAPK) pathway (also known as the p38 MAPK pathway) (19). Of these three pathways, the JNK pathway and the p38 MAPK pathway are the most likely candidates. In the thymus, the MAPK kinase MKK6, which activates p38 MAPK, is responsible for T cell selection. Specifically, it is responsible for the deletion of $CD4^+CD8^+$ T cells within the thymus (20). The JNK and p38 pathways are both integral in T cell differentiation. Activation of p38 in naïve T cells causes the release of IL-12, while both p38 and the JNK isoform JNK2 stimulate differentiation of naïve T

cells into Th1 cells (21, 22). Furthermore, JNK1 inhibits Th2 differentiation of naïve T cells, promoting a Th1 phenotype (23). Thus, the JNK and p38 MAPK pathways both promote Th1 differentiation of naïve T cells, which induces the expression of IFN-γ and TNF-α, two cytokines whose mRNA transcripts contain AREs in their 3'-UTRs.

There is also evidence linking the p38 MAPK pathway to HuR nuclear-to-cytoplasm translocation and mRNA stabilization directly (24, 25). These findings were confirmed in a paper by Han et al which established that the Rac-1/MKK3/p38MAPK/MK2 pathway is involved in ARE-mediated mRNA stabilization in breast cancer cells (26). This study is important because it not only confirms the possible role of p38 MAPK in HuR-mediated mRNA stabilization, it also established the Rho-GTPase Rac-1 as being upstream of p38 MAPK, as well as MAPK-activated protein kinase 2 (MK2) as being a downstream effector of p38. MKK3, which is directly downstream of Rac-1 in this study, is the MAPK kinase that activates p38 MAPK within T cells outside of the thymus (27). Another study linked MK2 to HuR mediated mRNA stabilization of uPAR transcripts, providing indirect evidence that LFA-1 engagement may mediate HuR translocation and subsequent mRNA stabilization through the p38 MAPK pathway (17). However, no study up to this point has directly investigated and defined the intermediaries between LFA-1 and HuR.

Statement of Purpose

The goal of this project is to elucidate the signaling pathway involved in T cell LFA-1 engagement-mediated HuR nuclear-to-cytoplasmic translocation and subsequent mRNA stabilization. *We hypothesize that the p38 MAPK pathway, acting through Rac and MKK3, is the pathway linking LFA-1 with HuR translocation and Th1 cell effector function.*

Methods

(All work was performed by Scott D. DeGregorio unless stated otherwise).

Antibodies and Reagents

The following antibodies (Ab) were used: anti-p38MAPK, phospho-p38MAPK, JNK Abs were purchased from Cell Signaling (Beverly, MA), anti-phospho-c-Jun, anti-GFP mouse mAbs were purchased from Santa Cruz (CA). Anti-HuR (3A2) mouse monoclonal Ab was a kind gift from Dr. Joan Steitz (Yale University, New Haven, CT). Murine anti-CD3 FITC conjugated Abs and NK1.1 PE were purchased from BD Biosciences (San Jose, CA). Phorbol Myristate Acetate (PMA), poly-L-lysine, 5.6-dichlorobenzimidazole (DRB), and DAPI were purchased from Sigma (St. Louis, MO). The pharmacological inhibitors SB203580 and SP600125 were purchased from Calbiochem (San Diego, CA). The murine soluble ICAM-1 producing cell line, NS-1, was a generous gift from Dr. F. Takei (University of British Columbia, Vancouver, Canada) and murine soluble ICAM-1 was purified as described (Welder et al. 1993). The anti-murine ICAM-1 mAb YN1/1.74 has been described (Takei et al. 1985).

Cell preparation, Cell culture, and Transient Transfection

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). MKK3-/- deficient mice were a gift from Dr. R. Flavell and have been described before (28). Murine spleens were harvested from wild type C57BL/6, C57BL/6-backcrossed MKK3+/-, or C57BL/6-backcrossed MKK3-/- mice. Murine splenic T cells were isolated by negative selection as described before (29). Briefly, murine spleens were homogenized, centrifuged, and the supernatant discarded. The cell pellet was rinsed with sterile water to lyse red blood cells. The cells then passed over a nylon wool column to remove B cells and macrophages. NK cells were removed using anti-NK1.1 antibodies conjugated to magnetic beads. T cells were then stained with murine anti-CD3 FITC-conjugated Abs and anti-NK1.1 PE-conjugated Abs, of which greater than 95% were CD3+ positive (Figure 1). Jurkat cells were obtained from the American Type Culture Collection and maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 U/ml each of penicillin G and streptomycin.

Plasmids and siRNA sequence

The Rac-2 wild type and constitutively active Rac-2 constructs were a gift from Dr. Honghbo Chi (Yale University, CT, USA). The pGEF-BOS-CAT and pBBB-3'UPAR were cloned in our laboratory, by Vinod Ramgolam, as previously described (9). The constitutively active $MKK6_{2E}$ constuct was a generous gift

from Dr. Holtmann (Medical School of Hanover, Germany). Jurkat cells were transfected by electroporation. Briefly, cells were washed twice with Optimem (Invitrogen) and resuspended in Optimem at a concentration of 50 x 10^6 cells/ml. Approximately, 20 x 10^6 cells (400 µl) were transferred in a 0.4 cm electroporation cuvette (Bio-Rad, CA) with 25 µg of each pDNA (total 75 µg of pDNA/20 x 10^6 cells). Transfections were conducted at 960 µF/250V (Gene Pulser, Bio-Rad, CA). Cells were cultured overnight in 10 ml media containing 0.5% FBS and 2 mM L-glutamine. Transfection was confirmed by SDS-PAGE of lysates from transfected cells, or by exogenous mRNA expression by quantitative real-time PCR.

HuR siRNA primers were designed by Mark Collinge and synthesized by the Keck Biotechnology resource facility at Yale. The duplex used to specifically target HuR was: AAGAGUGAAGGAGUUGAAAU, (HuRsiRNA) corresponding to nucleotides 1187-1205 of human HuR mRNA and is located in the 3'UTR: The scrambled siRNA sequence was AGCCAAUUCAUCAGCAAUGG (SCsiRNA). The control duplex was a scrambled version of HuRsiRNA. The duplexes HuRsiRNA and SCsiRNA were prepared by Qiagen (Valencia, CA).

Immunofluorescence

Glass coverslips were coated with 2.5 µg/ml murine sICAM-1 in PBS or 20 µg/ml poly-L-lysine at 4°C with in H_2O overnight at 4°C. Cells were pre-adhered to poly-L-lysine coated cover slips. LFA-1 conformational activation was achieved by treatment with LFA-1 activation buffer (100 mM Tris.HCl, pH 7.5,

0.9% NaCl, 2 mM $MnCl_2$, 2 mM MgCl, D-glucose 5mM 1.5% BSA). Mn^{2+} binds to the metal ion-dependent adhesion site (MIDAS) on the LFA-1α chain and promotes an allosteric transition to a high affinity state for its ligand(s), thereby inducing cell adhesion without cell activation. Cells were plated for 60 minutes on ICAM-1-coated cover slips or cells pre-adhered to poly-L-lysine were also treated with LFA-1 activation buffer for 60 minutes. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were blocked with 5% goat serum overnight at 4^{o}C, followed by staining with 5 μg/ml anti-HuR overnight at 4^{o}C, followed by cyanine 3-conjugated goat anti-mouse antibody (dilution 1:1000) immunostaining. Cells were co-stained with 0.005% DAPI for nuclear definition.

p38MAPK and c-Jun phosphorylation (Performed by Vinod Ramgolam, analyzed by Scott DeGregorio)

Murine T cells (10^7cells/ml), treated with LFA-1 activation buffer, were pre-adhered to poly-L-lysine-coated dishes or plated on 2.5 μg/ml sICAM-1 coated dishes for the indicated time points. Freshly isolated murine T cells were stimulated as described above, then lysed with 20 mM Tris.HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 20 mM NaH_2PO_4, 3mM β-glycerophosphate, 10mM NaF. Jurkat Cells were transfected as described above and stimulated with 10% serum for 4 hrs. p38MAPK and c-Jun phosphorylation was analyzed by SDS-PAGE and immunoblotted with anti-phospho-p38MAPK, anti-p38MAPK, phospho-c-Jun and JNK abs and probed with a secondary goat

anti- mouse or rabbit IRDye conjugated, species-specific antibody (Rockland, Gilbertsville, PA) and visualized using an Odyssey Imaging Systems (LI-COR, Lincoln, NE).

mRNA stabilization experiments and Quantitative Real Time-PCR

Petri dishes were coated with either 2.5 μg/ml murine ICAM-1 in PBS or 20 μg/ml poly-L-lysine in H_2O overnight at 4°C. Murine T cells (10^7/sample), stimulated with 2 ηg/ml phorbol 12-myristate 12-acetate (PMA), were pre-adhered to poly-L-lysine-coated dishes, or plated on ICAM-1-coated dishes. In the case of JNK inhibition with SP600125, murine T cells were pre-treated for 60 minutes before stimulation with 2 ηg/ml PMA. mRNA synthesis was inhibited after 3 hrs of incubation by the addition of 1.0 μM of the RNA polymerase II-specific inhibitor 5,6-dichloro-1-β-D-ribobenzimidazole (DRB), after which cells were lysed for RNA isolation. Transfected Jurkat cells were serum-stimulated for 4 hours to allow for rabbit β-globin gene transcription with 10% serum, after which cell samples were collected every hour up to 4 hours. In the case of p38MAPK or c-Jun phosphorylation inhibition, cells were treated with 1 μM SB203850 and 10 μM SP600125, respectively. RNA was isolated with the Qiagen RNeasy kit (Qiagen, Valencia, CA). cDNA was prepared from 1 μg of RNA with iScript (Bio-Rad, CA) and analyzed by quantitative real-time RT-PCR using the Opticon 2 System (MJ Research, Waltham, MA). SYBR Green PCR kit was purchased from Qiagen (Valencia, CA). Murine IFN-γ mRNA levels were normalized against Cyclophilin-α, with expression relative to time 0 when

transcription was inhibited. The sequence of the primers used were as followed: Cyclophilin-α Forward primers: ATTATTCCAGGATTCATGTGCCAG, Cyclophilin-α Reverse primers: TGAAGGGGAATGAGGAAAATATGG; IFN-γ Forward primers: AGCGGCTGACTGAACTCAGATTGTAG; IFN-γ Reverse primers: GTCACAGTTTTCAGCTGTATAGGG. Rabbit β-globin mRNA were normalized against CAT, with expression relative to time 0. The sequence of the primers were as follows: CAT Forward primers: GGATAGTTTCACCCTTGTT; CAT Reverse primers: GATTGGCTGAGACGAAAAAC; Rabbit β-globin Forward primers: TGGTTGTCTACCCATGGACC; Rabbit β-globin Reverse primers: ATCCACGTGCAGCTTGTCAC. All primers were synthesized by the Keck Biotechnology resource facility at Yale.

Results

LFA-1 induces mRNA stabilization

T cell LFA-1 engagement is partially responsible for effector T cell function, including pro-inflammatory cytokine production. Previous studies that examined LFA-1 engagement-mediated mRNA stabilization used LFA-1 activating antibodies rather than its natural ligand, ICAM-1. Our first goal was to show that LFA-1 engagement by ICAM-1 leads to stabilization of mRNA transcripts. Murine T cells were stimulated with the phorbol ester PMA (replacing CD3 engagement as the T cell gene activator) or PMA with ICAM. After 3 hours of stimulation, transcription was arrested. To assess the half life of the T cell transcripts present at the time of transcriptional arrest, cells were lysed

at 0, 15, 30, and 45 minutes after arrest. mRNA levels were evaluated at each time point by real time RT-PCR. The transcript level for the pro-inflammatory cytokine IFN-γ was compared to the stable, housekeeping mRNA encoding cyclophilin-α, at each time point. The results are illustrated in Figure 2. When cells are stimulated with PMA alone, less than 10% of the IFN-γ transcript is present in the cell 45 minutes after transcriptional arrest. However, when T cell β_2 integrin is engaged in addition to PMA recruitment, 60% of the IFN-γ transcript remains at 45 minutes after transcriptional arrest. These results suggest that LFA-1 engagement by ICAM-1 leads to stabilization of the mRNA transcript encoding the pro-inflammatory/immune cytokine IFN-γ.

LFA-1 engagement induces HuR nuclear-to-cytoplasmic translocation

IFN-γ mRNA transcripts contain destabilizing AREs in their 3'-UTRs. Since HuR is known to bind mRNA AREs and prevent degradation of those transcripts, and since LFA-1 engagement confers stability to IFN-γ transcripts, we hypothesize that LFA-1 stabilizes the IFN-γ transcripts through HuR. While it has been demonstrated in human T cells that LFA-1 engagement causes HuR nuclear-to-cytoplasmic translocation and subsequent mRNA binding, no one has ever shown a link between LFA-1 engagement and HuR translocation in the murine system. Therefore, we attempted to show that LFA-1 engagement causes HuR translocation in murine T cells. T cells were plated on cover slips and treated with LFA-1 activation buffer alone, or LFA-1 activation buffer in the presence of ICAM-1, for up to one hour. The LFA-1 activation buffer contains

$MnCl_2$ which induces a conformational change in LFA-1, allowing it to bind ICAM-1 more avidly. HuR was stained with a fluorescent antibody and compared to DAPI, a fluorescent marker which stains the nucleus. Each population of cells were stimulated for 15, 30, 45, and 60 minutes, then washed and fixed to a slide. In T cells stimulated with ICAM, HuR begins to translocate out of the nucleus as soon as 15 minutes after incubation with ICAM begins. After 60 minutes of adhesion onto ICAM, all visible HuR has translocated out of the nucleus and into the T cell cytoplasm (Figure 3). Incubation up to 60 minutes with the LFA-1 activation buffer alone does not induce translocation of HuR (data not shown). We conclude that, in murine T cells, LFA-1 engagement causes HuR nuclear-to-cytoplasmic translocation, where it binds to and stabilizes IFN-γ transcripts.

HuR is required for Rac-mediated mRNA stabilization

We have shown that LFA-1 engagement causes both HuR translocation and mRNA stabilization. Our next goal was to demonstrate directly that HuR is required for LFA-1-mediated mRNA stabilization. Previous work in our lab demonstrates the role of the Rho-GTPases Rac-1 and Rac-2 in LFA-1 engagement-mediated mRNA stabilization (Ramgolam et al, in preparation). To address whether HuR is required for mRNA stabilization, HuR was “knocked down” via RNA interference in the presence of constitutively active Rac-1 and Rac-2 constructs. RNA interference uses small (~21 nucleotide), double-stranded RNA sequences, complementary to the transcript of interest, which bind to and cause the degradation of that particular mRNA. RNA interference is much

more precise than antisense RNA (30). After RNA interference of HuR, mRNA stabilization was assessed in the presence of constitutively active Rac constructs which are capable of inducing mRNA stabilization (Ramgolam et al, in preparation). Unfortunately, due to the inherent inefficiency of T cell transfection, endogenous IFN-γ mRNA transcripts could not be quantitated to examine whether constitutive Rac/p38 activation causes mRNA stabilization. Instead, we co-transfected an mRNA reporter construct as a readout for stabilization.

The exogenous gene used for mRNA stabilization experiments is the rabbit β-globin gene attached to a serum-inducible promoter. The rabbit β-globin gene encodes an extremely stable transcript: transfection into human Jurkat T cells followed by serum-induced transcription generates transcripts that show no degradation 400 minutes after transcription. The rabbit β-globin gene becomes destabilized by fusing AREs into its 3'-UTR. By exchanging the natural 3'-UTR of rabbit β-globin with the 3'-UTR of uPAR, the transcript degrades and is undetectable 400 minutes after transcription in Jurkat T cells (9). Since stabilization of this destabilized rabbit β-globin transcript would presumably require HuR to bind the AREs and stabilize the transcript, this construct makes an ideal exogenous vector to analyze HuR-mediated mRNA stabilization.

We transfected Jurkat T cells with the rabbit β-globin construct containing the uPAR 3'-UTR, as well as a serum-inducible chloramphenicol acyltransferase (CAT) gene which encodes the stable CAT mRNA and is used to normalize the transfection efficiency as well a mRNA half life. Cells were then transfected with either a constitutively active Rac-1 or a constitutively active Rac-2 construct, both

of which have been shown to induce mRNA stabilization of rabbit β-globin transcripts containing the uPAR AREs (Ramgolam et al. in preparation). Each population of cells then received either a 21 nucleotide RNA duplex complementary to HuR (HuR siRNA), or a scrambled sequence of the HuR siRNA (scRNA). The results are shown in Figure 4. Both the constitutively active Rac-1 and Rac-2 constructs induced mRNA stabilization of the destabilized β-globin construct in the presence of the scrambled RNA duplex. When HuR was knocked down by at least 80% of its original level, neither constitutively active Rac-1 nor Rac-2 were able to induce mRNA stabilization of the modified β-globin construct. These results show that HuR is required for Rac-mediated mRNA stabilization. Since Rac-1 and Rac-2 are downstream of LFA-1, this suggests that HuR is required for LFA-1 engagement-mediated mRNA stabilization.

LFA-1 engagement activates both the p38 MAPK and JNK pathway

After demonstrating, within a murine system, that LFA-1 engagement results in HuR translocation and mRNA stabilization of transcripts encoding pro-inflammatory cytokines, we sought to elucidate the molecules mediating this signal transduction pathway. As stated in the introduction, the two most likely effector pathways are the p38 MAPK pathway and the JNK pathway. Cells were stimulated with ICAM-1 in the presence of LFA-1 activation buffer. After stimulation, the cells were lysed and the activity of p38 MAPK and JNK were quantitated using monoclonal antibodies (it is convention to quantitate p38

activity with antibodies against phosphorylated p38 MAPK, while quantitating JNK activity with antibodies against phosphorylated c-jun, the active form of the JNK substrate). After incubation of murine T cells with ICAM, p38 MAPK and JNK activity increased twelve-fold and 6.5-fold, respectively, when normalized to the total level of the respective proteins (Figure 5). These results suggest that LFA-1 engagement activates both the p38 MAPK pathway and the JNK pathway.

The JNK pathway does not play a role in LFA-1 mediated mRNA stabilization

Since LFA-1 engagement activates both the p38 MAPK and JNK pathways, it is possible that p38 MAPK, JNK, both, or neither mediate HuR translocation and subsequent mRNA stabilization. To analyze whether JNK is necessary for LFA-1 mediated mRNA stabilization, we used the JNK-specific pharmacologic inhibitor SP600125 (31). SP600125 competitively blocks the ATP binding site on JNK, inhibiting JNK's ability to phosphorylate c-jun. Using a concentration of 10 μM SP600125, we were able to inhibit JNK activity without inhibiting other MAP kinases, such as p38 MAPK (Figure 6a, data not shown). We then examined the effect of JNK inhibition on mRNA levels in T cells upon LFA-1 engagement. Cells were stimulated with PMA, PMA and ICAM, or PMA and ICAM in the presence of SP600125. After stimulation, the cells were lysed and their mRNA quantitated (Figure 6B). T cells stimulated with PMA and ICAM demonstrated a twelve-fold increase in IFN-γ mRNA levels when compared to PMA stimulation alone. The addition of 10 μM SP600125 to the T cells prior to

PMA and ICAM stimulation did not significantly alter mRNA levels; these T cells exhibited a 10-fold increase in IFN-γ mRNA levels compared to PMA stimulation. Since the major role of ICAM-1 stimulated LFA-1 engagement is to stabilize labile transcripts in this system, these results suggest that JNK is not required in LFA-1 mediated modulation of mRNA levels. However, this does not separate the LFA-1 effect on transcriptional vs. post-transcriptional modulation of mRNA levels. To distinguish this, we stimulated T cells with PMA, PMA/ICAM, and PMA/ICAM/SP600125. After stimulation, we arrested transcription and quantitated IFN-γ mRNA levels at various timepoints folowing transcriptional arrest. The addition of SP600125 did not abrogate the LFA-1 mediated mRNA stabilization 60 minutes after transcriptional arrest (Figure 6C). These results support that JNK is not required for LFA-1 mediated mRNA stabilization.

The JNK pathway does not play a role in LFA-1 mediated HuR translocation

Although JNK is not required for T cell integrin engagement-mediated mRNA stabilization, it is conceivable that JNK causes HuR translocation independent of HuR-mediated mRNA stabilization (and raising the possibility that LFA-1 mediated mRNA stabilization can also occur independently of HuR). To examine this possibility, we observed HuR translocation in T cells in the presence of SP600125. Stimulation of T cells with LFA-1 activation buffer and ICAM in the presence of 10 μM SP600125 has no effect on HuR translocation (Figure 7). Indeed, doubling the dose of SP600125 to 20 μM did not abrogate the HuR translocation caused by LFA-1 engagement. After 1 hour of stimulation,

all visible HuR is in the cytoplasm in the presence of the JNK inhibitor. Therefore, JNK appears to be required for neither LFA-1 mediated mRNA stabilization nor LFA-1 mediated HuR translocation.

p38 MAPK is required for LFA-1 mediated mRNA stabilization

After establishing that JNK is not involved in LFA-1 mediated HuR translocation and subsequent mRNA stabilization, we examined whether p38 MAPK is involved using the p38-specific pharmacologic inhibitor SB203580 (32). Like SP600125, SB203580 competitively blocks the ATP binding site of p38 MAPK, inhibiting the enzyme's ability to phosphorylate its substrates. A concentration of 1 μM of SB203580 reduced p38 MAPK activity to baseline levels without inhibiting other MAP kinases (Figure 8A, data not shown). We analyzed the effect of inhibiting p38 MAPK on LFA-1 modulation of mRNA levels. As in previous experiments, stimulation with PMA and ICAM causes a twelve-fold increase in IFN-γ levels when compared to PMA stimulation alone. Stimulating T cells in the presence of 1 μM SB203580, however, reduces IFN-γ levels to their baseline value (Figure 8B). This demonstrates that p38 MAPK is required for LFA-1 mediated modulation of IFN-γ mRNA levels.

As previously stated, the experiment does not separate transcriptional and post-transcriptional events. To examine whether p38 MAPK is required for LFA-1 mediated mRNA stabilization, T cells were transcriptionally arrested after stimulation with PMA and ICAM in the presence of SB203580. Quantitation of IFN-γ mRNA levels after transcriptional arrest shows that the addition of the p38

MAPK inhibitor causes a loss of mRNA stabilization upon LFA-1 engagement by ICAM. Interestingly, IFN-γ mRNA levels in T cells stimulated with PMA/ICAM/SB203580 drop below those in T cells stimulated with PMA alone (this result is evident in repeated experiments, although statistical significance was not reached). Together, these data demonstrate that p38 MAPK is required for LFA-1 engagement-mediated mRNA stabilization.

MKK3 gene-deleted T cells demonstrate loss of p38 MAPK activity

We have pharmacologic evidence that the p38 MAPK pathway is required for LFA-1 engagement mediated mRNA stabilization. Pharmacologic models are imprecise, however, given the inherent lack of specificity of pharmacologic inhibitors. We therefore investigated the p38 MAPK pathway in a more precise, gene-deleted mouse system. We received MKK3 gene deleted mice as a kind gift from Dr. Richard Flavell. (as stated, MKK3 is the MAPK kinase which activates p38 MAPK in peripheral T cells (27). Phenotypically, these mice appear normal, albeit smaller in overall size and with smaller spleens than their C57BL/6 wild type counterparts. We bred MKK3 knockout ($MKK3^{-/-}$) mice (backcrossed into the C57BL/6 strain) with wild type ($MKK3^{+/+}$) C57BL/6 mice to generate heterozygous MKK3 ($MKK3^{+/-}$) mice.

To confirm that the $MKK3^{-/-}$ mice actually have a deficiency in the p38 MAPK signaling pathway, we isolated T cells from $MKK3^{+/+}$, $MKK3^{+/-}$, and $MKK3^{-/-}$ mice. We activated the T cells with LFA-1 activation buffer and ICAM and analyzed p38 MAPK activity by Western blot. Each population of T cells also had

an unstimulated control group against which p38 MAPK activity was normalized (Figure 9A). The MKK3$^{+/-}$ had a 50% reduction in p38 MAPK activity upon LFA-1 engagement when compared to MKK3$^{+/+}$ T cells, and the MKK3$^{-/-}$ cells had a 75% reduction in p38 MAPK activity when compared to the wild type cells. In contrast, upon LFA-1 engagement, there is no correlation between JNK activity and the number of wild-type MKK3 alleles in T cells (Figure 9B). These results indicate that MKK3 gene-deleted mice do have a selective defect p38 MAPK pathway, with other MAPK signaling pathways intact.

MKK3$^{-/-}$ T cells have reduced IFN-γ mRNA levels upon LFA-1 engagement

Since pharmacologically inhibiting p38 MAPK causes a decrease in IFN-γ mRNA levels upon LFA-1 engagement, we hypothesized that knocking out MKK3 would also cause a reduction in IFN-γ levels upon T cell integrin engagement, although it is possible that multiple upstream kinases can co-activate p38 upon LFA-1 engagement. There are at least 2 known MAPK kinases that activate p38 MAPK (MKK3 and MKK6), and it is possible that MKK6, or some other currently unknown MAPK kinase, activates p38 MAPK and causes the HuR translocation and subsequent mRNA stabilization independent of MKK3. To examine whether MKK3 is, in fact, the MAPK kinase responsible for LFA-1 mediated mRNA stabilization via p38 MAPK, we isolated MKK3$^{+/+}$, MKK3$^{+/-}$, and MKK3$^{-/-}$ T cells and analyzed IFN-γ mRNA levels upon integrin engagement. After stimulation with PMA and ICAM, MKK3$^{+/-}$ T cells contained 50% less IFN-γ mRNA than wild type T cells, while MKK3$^{-/-}$ T cells express 80% less IFN-γ than wild type T cells

(Figure 10). These results suggest that MKK3 is required for IFN-γ mRNA modulation upon T cell integrin engagement.

MKK3 KO T cells exhibit a loss of LFA-1 mediated mRNA stabilization

As stated previously, the prior experiment does not separate transcription vs. post-transcriptional modulation of mRNA levels. To examine whether loss of the MKK3 gene causes instability of mRNA, T cells were stimulated as in previous experiments, mRNA transcription arrested, and IFN-γ mRNA levels quantitated at various timepoints after transcriptional arrest (Figure 11). As expected, in $MKK3^{+/+}$ mice, stimulation with PMA and ICAM increases the half life of IFN-γ mRNA levels, with 80% of transcripts present 45 minutes after transcriptional arrest. If the MKK3 gene is completely knocked out, T cells largely lose the mRNA stabilization conferred by LFA-1 engagement: mRNA levels are 30% of their original value 45 minutes after transcriptional arrest. Surprisingly, the T cells with only one copy of the MKK3 gene exhibit complete stabilization of IFN-γ mRNA transcripts when compared to wild type T cells, despite reduced p38 MAPK activity compared to wild type T cells. These results suggest that MKK3 is required for LFA-1 mediated mRNA stabilization through p38 MAPK. Furthermore, MKK3 appears to act in a threshold-dependent manner rather than a dose-dependent manner for its mRNA stabilization phenotype.

MKK3 KO T cells exhibit a loss of LFA-1 mediated HuR nuclear-to-cytoplasmic translocation

Since MKK3$^{-/-}$ T cells demonstrate a loss of T cell integrin-mediated mRNA stabilization, we hypothesized that loss of MKK3 in T cells may also cause a loss of LFA-1 mediated HuR nuclear-to-cytoplasmic translocation. We isolated MKK3$^{+/+}$, MKK3$^{+/-}$, and MKK3$^{-/-}$ T cells and stimulated them with ICAM in the presence of LFA-1 activation buffer. LFA-1 activation buffer alone caused no HuR translocation in any of the three populations of T cells (data not shown). While stimulating wild type T cells with ICAM causes complete HuR translocation, stimulation of MKK3$^{-/-}$ T cells with ICAM causes no visible movement of HuR from the nucleus into the cytoplasm (Figure 12). This confirms our suspicion that MKK3 is required for LFA-1 mediated HuR translocation and subsequent mRNA stabilization. Furthermore, in MKK3$^{+/-}$ T cells, ICAM stimulation causes complete HuR translocation from the nucleus to the cytoplasm. This mirrors the finding in the previous experiment that the MKK3$^{+/-}$ T cells phenotypically behave identically to MKK3$^{+/+}$ T cells with respect to HuR translocation and subsequent mRNA stabilization despite having reduced p38 MAPK activity levels.

Discussion

Our results support the hypothesis that the p38 MAPK pathway, acting through Rac-1/Rac-2 and the MAPK kinase MKK3, plays an integral role in peripheral T cell integrin engagement-mediated HuR translocation and

subsequent mRNA stabilization. The pathway we propose from our data is illustrated in Figure 13. Previous work in our laboratory demonstrates that LFA-1 activation leads to Rac-1 and Rac-2 activation (Ramgolam et al., in preparation). The Rac proteins activate JNK, via MKK4 and MKK7, as well as p38 MAPK through MKK3 and MKK6. JNK has been implicated in T cell differentiation (23) and, per our results, does not play a role in LFA-1 mediated mRNA stabilization. MKK3, acting through p38 MAPK, is required for T cell integrin-mediated mRNA stabilization. Activation of p38 MAPK alone through MKK6, however, is not sufficient to induce mRNA stabilization (data not shown). These data suggest that p38 is necessary but not sufficient for LFA-1 mediated mRNA stabilization. Therefore, signaling protein(s) separate from p38 MAPK must be involved in LFA-1 engagement-mediated mRNA stabilization. These proteins have to be activated at or above the level of MKK3 within the signaling cascade. One candidate class of proteins that may be involved in LFA-1 mediated mRNA stabilization is cytoskeletal proteins. Integrin engagement on T cells causes a shift in proteins within the immunologic synapse that is cytoskeleton-dependent (33). In addition, observation of T cells, upon LFA-1 engagement by ICAM, reveals striking morphologic changes by the T cell, whereupon it spreads along the ICAM and develops numerous lamellopodia. Furthermore, experiments studying LFA-1 engagement in the presence of cytochalasin D, a pharmacologic inhibitor of actin polymerization, show that T cells require cytoskeletal rearrangement for MAP kinase activation (3), mitogenesis (34), and mRNA stabilization (Ramgolam, unpublished data) upon integrin engagement. While

our lab has shown a loss of T cell integrin-mediated mRNA stabilization upon inhibition of actin polymerization, a loss of HuR translocation upon actin polymerization has not been evaluated. However, in natural killer cells, LFA-1 engagement was found to cause Vav-1 phosphorylation, which in turn was upstream of subsequent cytoskeletal reorganization (35). Thus, it is possible that in T cells, Vav, Rac, and/or MKK3 induce cytoskeletal rearrangement in addition to p38 MAPK activation, and that both p38 MAPK activity and cytoskeletal rearrangement are required for LFA-1 mediated HuR translocation and subsequent mRNA stabilization.

An obvious question stems from our data. What is the signal or signals that mediate p38 MAPK activation-induced HuR translocation? One candidate protein is p38 MAPK-activated protein kinase 2 (MK2). MK2 is a molecule that resides in the cytoplasm and, upon activation by p38 MAPK, binds to its chaperone, heat shock protein 27 (hsp-27), and translocates to the nucleus (36). Transfection of cells with a constitutively active MK2 construct induced stabilization of cytokine transcripts containing AREs (IL-6 and IL-8), while a dominant negative MK2 construct interfered with p38 MAPK-mediated mRNA stabilization (37). Recent work calls into question whether p38/MK2 operates in series or in parallel with HuR. With an elegant series of experiments, they have identified two distinct domains within the ARE of IL-8, both of which are necessary for mRNA destabilization in a quiescent cell. HuR and MK2 bind separately to each domain. HuR binds to an auxiliary domain within the IL-8 ARE which causes modest mRNA stabilization. HuR binding to the auxiliary

domain, however, increases the affinity of MK2 binding to the core domain of the IL-8 ARE. MK2 binding to the core domain leads to further mRNA stabilization by inhibiting deadenylation of the 3' tail of the IL-8 transcript (38). These data suggest that MK2 and HuR operate in parallel and not in series as we propose. This does not necessarily contradict our model. Firstly, the study only examines IL-8's transcript. While the same mechanism may apply to IFN-γ, it has not yet been confirmed. Secondly, we only examine HuR translocation after manipulating MKK3. While we conclude from this data that MKK3 is required for HuR translocation, and therefore infer that it acts via its substrate, p38 MAPK, we also established that either MKK3 or a signaling protein proximal to MKK3 *must* activate a protein in addition to p38 MAPK to achieve HuR translocation. It is conceivable that the other protein (or proteins) activated by MKK3 are responsible for HuR translocation, and that the coordinated action of HuR and p38/MK2 are required for mRNA stabilization.

The next step beyond discovering the pathway transducing LFA-1 engagement-mediated mRNA stabilization is defining the significance of this mRNA stabilization to proper immune function *in vivo.* T cells in general, and T cell LFA-1 activation specifically, have been implicated in numerous immune/inflammatory diseases. The role of LFA-1 in rheumatoid arthiritis has been known for some time (39). The inflammatory infiltrate within the synovium of joints in patients suffering from rheumatoid arthritis is rich with T cells, and it has been shown that LFA-1 engagement by endothelial ligands plays an integral role in development of the infiltrate. Whether LFA-1 engagement mediates

proinflammatory signaling responsible for the development of rheumatoid arthritis through mRNA stabilization has not been investigated.

Within the context of vascular biology, T cells play a vital role in vascular pathology such as atherosclerosis, chronic vascular rejection, and angioplasty restenosis. Among the characteristics shared by the various forms of vascular pathology is endothelial dysfunction and injury. This endothelial injury leads to alterations in the endothelial cell's gene expression profile, including the up-regulation of cell adhesion molecules used for leukocyte adhesion. In a study examining atherosclerotic plaques, the intimal neovasculature within the plaque displayed significantly elevated levels of E-selectin, ICAM-1, and VCAM-1 expression when compared to normal arterial luminal endothelium. The increased expression of these adhesion molecules was strongly associated with intimal accumulation of leukocytes, particularly T cells (40). Chronic graft rejection has also been established as primarily a vascular phenomenon mediated by T cell invasion into the vasculature, causing dense intimal fibrosis that is not unlike the mechanism of post-injury restenosis. Furthermore, inhibition of the CD28 and CD40 signaling pathways within T cells has shown great promise in attenuating chronic graft rejection in rodents (41). Percutaneous transluminal coronary angioplasty (PTCA) has been shown to increase the concentration of $CD4^+$, IFN-γ expressing T cells. This increase in T cell density can be attenuated by treating patients with statins following PTCA, which have been shown to interfere with T cell signaling (42). Taken together, these data suggest that atherosclerosis, chronic graft rejection, and angioplasty restenosis

are all forms of vascular pathology that share the mechanism of T cell adhesion to injured vascular endothelium, followed by subintimal invasion with subsequent neointimal proliferation and fibrosis.

The T cell integrin LFA-1 has been implicated in T cell adhesion to endothelium and the subsequent development of atherosclerosis (43). In fact, oxidized low density lipoprotein (LDL), long known to be an important contributing factor for atherosclerosis, has been shown to be a potent trigger for LFA-1 activation and subsequent leukocyte adhesion and transmigration into atherosclerotic plaques (44). Angioplasty restenosis shares many mechanisms of pathogenesis with atherosclerosis. PTCA with additional stent implantation is associated with a local recruitment of LFA-1 positive lymphocytes to the site of the procedure (45). Furthermore, studies in Germany show that assessing the expression of LFA-1, and the monocyte counterpart Mac-1, in patients prior to angioplasty may predict the subsequent development of angioplasty restenosis (46).

While T cells, acting through LFA-1 engagement by endothelial ICAM-1, have been implicated in vascular pathology, the role of signaling events downstream of LFA-1 engagement in vascular pathology have not been examined. We aim to determine whether LFA-1 mediated mRNA stabilization of pro-inflammatory cytokine transcripts is an important step in the development of vascular pathology. Preliminary data suggest that MKK3 deficient mice reject HY-mismatched skin grafts over a longer time course than their wild type counterparts (Figure 14). An HY-mismatch model refers to a syngeneic skin graft

taken from a male donor and grafted onto a female recipient. A protein encoded on the Y chromosome of the male donor graft (the HY protein) generates a minor histocompatability mismatch within the female recipient, causing a chronic graft rejection (47), but not one that requires immunosupression for graft viability. In our preliminary experiments, the MKK3 knockout mice take an average of 7 days longer to reject HY-mismatched skin grafts than wild type BL/6 mice (data not shown as statistical significance was not reached). We also have the HY-mismatch model in place for aorta allograft experiments. In both wild type and MKK3 knockout mice populations, sections of thoracic aorta are taken from a male donor and grafted onto a female recipient abdominal aorta. Again, the donor HY antigen induces a recipient T cell response and chronic graft rejection. Because the graft is vascular, rejection will more closely approximate vascular pathology than skin graft rejection.

It is important to note that MKK3 gene deleted mice are deficient in IL-12 production. IL-12 is a heterodimeric cytokine that plays an integral role in differentiating naïve $CD4^+$ T cells into Th1 cells by inducing IFN-γ production (28). The deficiency in IL-12 production in MKK3 knockout mice does not affect our presented *in vitro* results since our experiments focus on naïve T cells or T cell lines and therefore do not require IL-12. The IL-12 defect is important, however, in discussing the extension of these experiments to an *in vivo* system, particularly when using a graft rejection model. As stated, graft rejection is primarily a T cell-mediated phenomenon. Specifically, a cell-mediated, Th1 phenotype for $CD4^+$ T cells is perhaps the most important component for

vascular rejection (1). Without IL-12 production capabilities, MKK3 deficient naïve T cells are not capable of differentiating into a Th1 phenotype. Therefore, it would be impossible to determine whether delayed graft rejection was truly a result of impaired mRNA stabilization of pro-inflammatory cytokine transcripts upon naïve T cell integrin engagement, or whether the delayed rejection is a result of the mouse's inability to generate Th1 cells.

As a solution, our lab is in the process of generating a conditional, "floxed" HuR knockout mouse. When crossing the HuR-floxed mouse with a T cell-specific (lck-promoter) cre recombinase transgenic mouse, a T cell HuR knockout mouse will be generated. If the mouse has a normal repertoire of T cells, we will be able to identify the role of HuR in some of the T cell, *in vivo* models. Knocking out T cell HuR would not only more definitively show that HuR is responsible for integrin engagement-mediated mRNA stabilization, it would allow us to further define the nature of HuR's association with relevant mRNAs. These recombinant tools of genetic manipulation would give us the power to more precisely interrogate the role of HuR in T cell integrin engagement-mediated mRNA stabilization, as well as the effects of this mRNA stabilization upon vascular rejection and other forms of vascular pathology.

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Figure 1: The effect of purification on T cell concentration of murine splenocytes. Murine splenocytes were isolated and purified as described in the Methods section. A sample of splenocytes was taken after purification. The samples were analyzed by flow cytometry using an antibody against the T cell-specific protein CD3 conjugated to the fluorescent marker FITC, as well as an antibody against the NK cell-specific protein NK1.1 conjugated to the fluorescent marker PE.

Figure 1

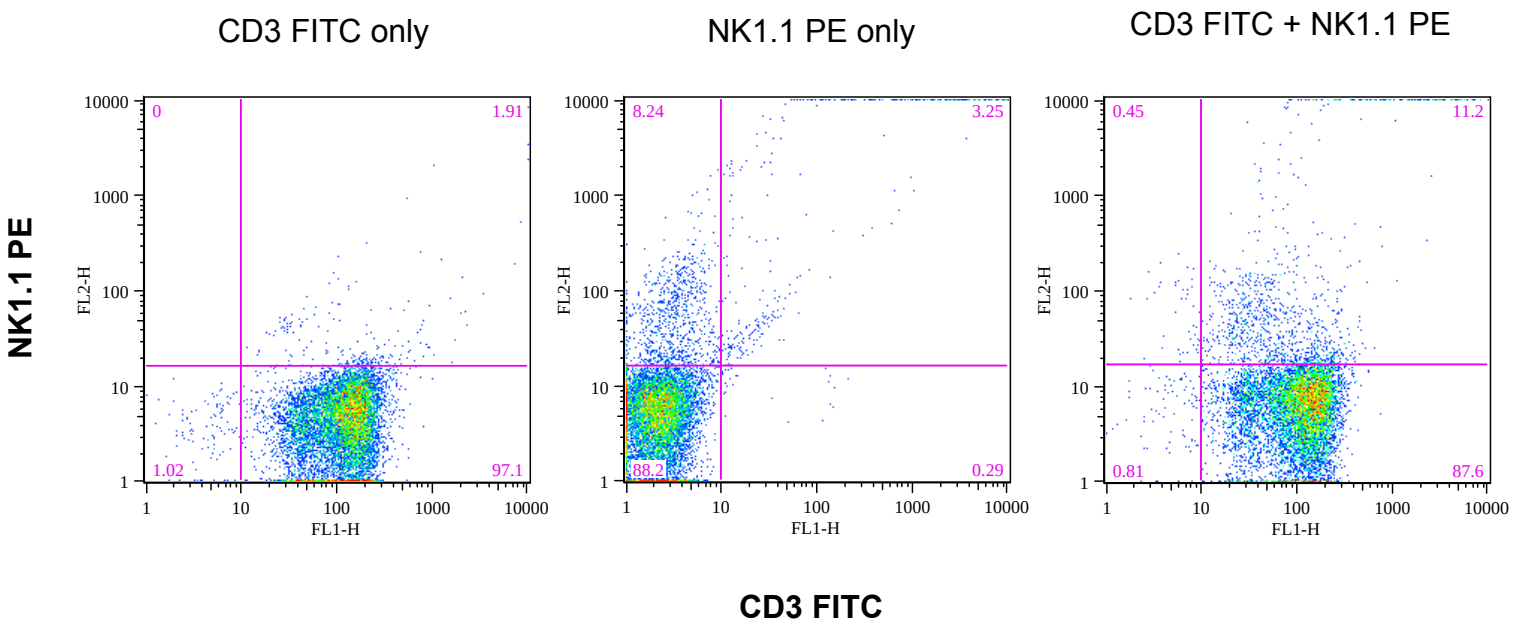

CD3 FITC only
NK1.1 PE only
CD3 FITC + NK1.1 PE
NK1.1 PE
CD3 FITC
FL2-H
FL1-H
10000
1000
100
10
1
0
1.91
1.02
97.1
8.24
3.25
88.2
0.29
0.45
11.2
0.81
87.6

Figure 2: The effect of LFA-1 engagement on IFN-γ mRNA stabilization in murine T cells. Mouse splenic T-cells were isolated as described, then immunostimulated with either PMA or PMA plus ICAM for 3 hr, after which the transcriptional inhibitor DRB was added (time 0). The RNA was harvested at time 0 as well as at the indicated times for quantitative, real time RT-PCR using probes complementary to IFN-γ. In this and all future mRNA stabilization experiments, IFN-γ mRNA levels were normalized against the stable transcript cyclophilin at each time point.

Figure 2

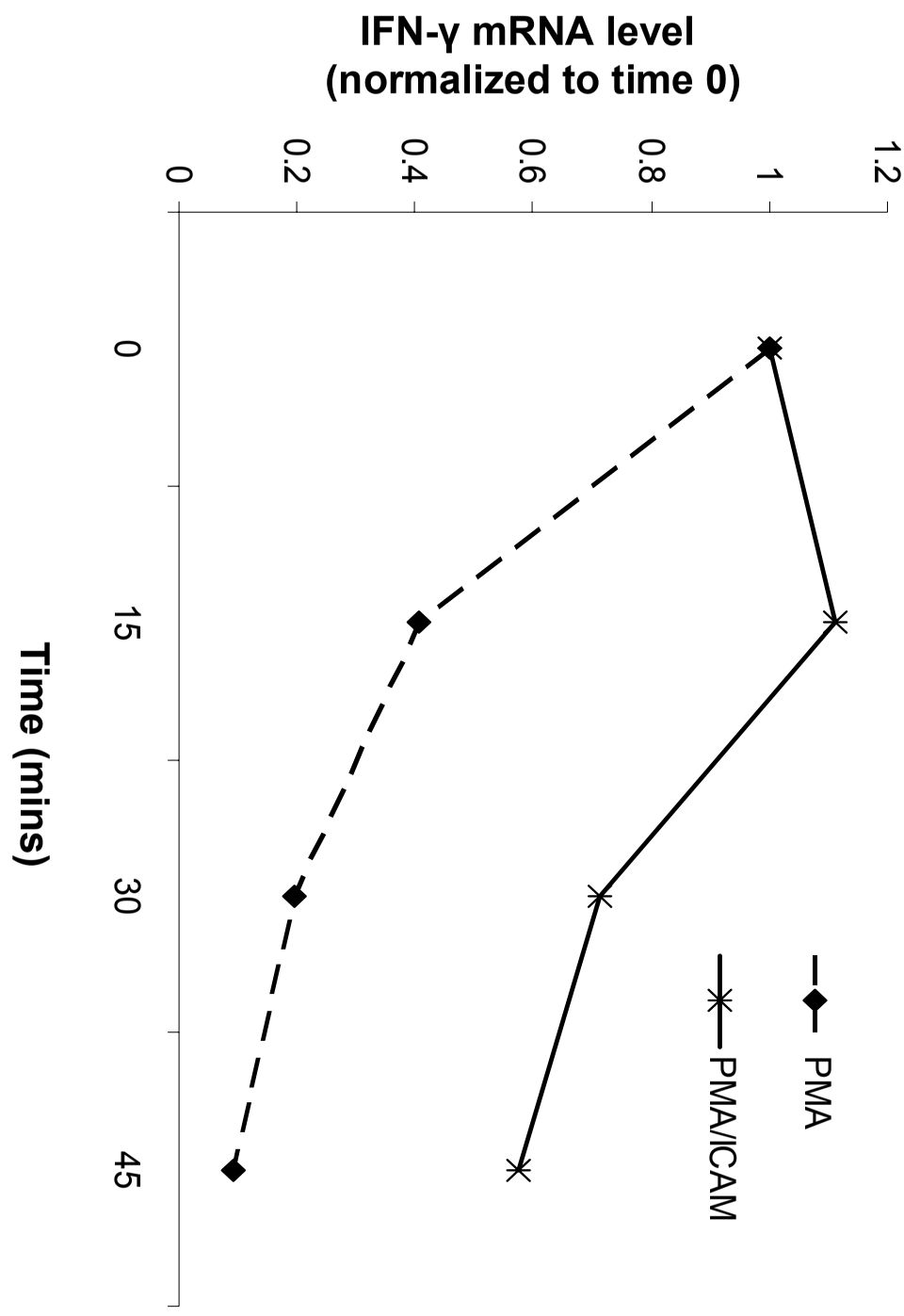

IFN-γ mRNA level
(normalized to time 0)
0
0.2
0.4
0.6
0.8
1
1.2
0
15
30
45
Time (mins)
PMA
PMA/ICAM

Figure 3: The effect of LFA-1 engagement on HuR nuclear-to-cytoplasmic translocation in murine T cells. T cells were isolated from C57BL/6 mouse spleens and resuspended in LFA-1 activation buffer (2 mM MnCl2), following which they were plated on coverslips coated with 2.5 μg/ml murine Intercellular Adhesion Molecule-1 (ICAM-1) or poly-L-lysine for 15, 30, 45 and 60 minutes. Immunofluorescent analysis was performed with anti-HuR mAb (red) and the nuclear marker DAPI (blue). Magnification 100X.

Figure 3

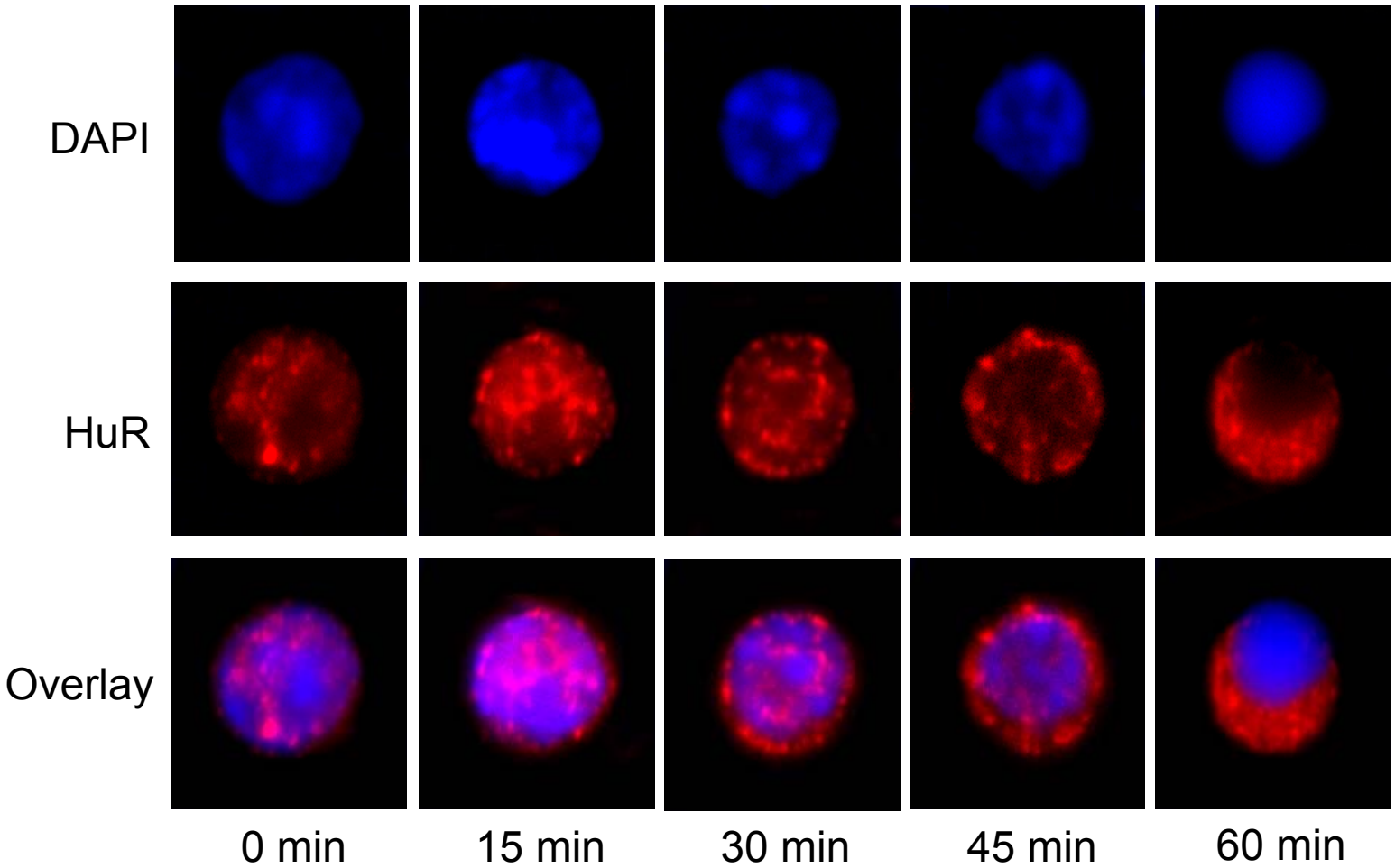


Figure 4: The effect of HuR knockdown on Rac-1-mediated mRNA stabilization of ARE-containing transcripts in immortalized human T cells. Jurkat T cells were transfected with a rabbit β-globin construct containing the 3'-UTR of uPAR, as well as chloramphenicol acetyl transferase to normalize transfection levels. The T cells were also transfected with a constitutively active Rac-1 construct (Rac1 V12, Panel A), or a constitutively active Rac-2 construct (Rac2 L61, Panel B). The cells were then split into two populations, with half receiving a double-stranded RNA interference sequence specific for HuR (HuRsiRNA) while the other half received a scrambled double stranded RNA sequence (ScRNA). RNA interference succeeded in knocking HuR down by at least 80% compared to baseline levels (top panels). AUF-1 was used to normalize protein levels. The lower panels show the effect of knocking down HuR on Rac-1-mediated mRNA stabilization. The T cells were stimulated by the addition of serum and, following the withdrawal of serum, were lysed at various time points and mRNA levels analyzed at each time point using quantitative, real time RT-PCR using probes for rabbit β-globin.

Figure 4

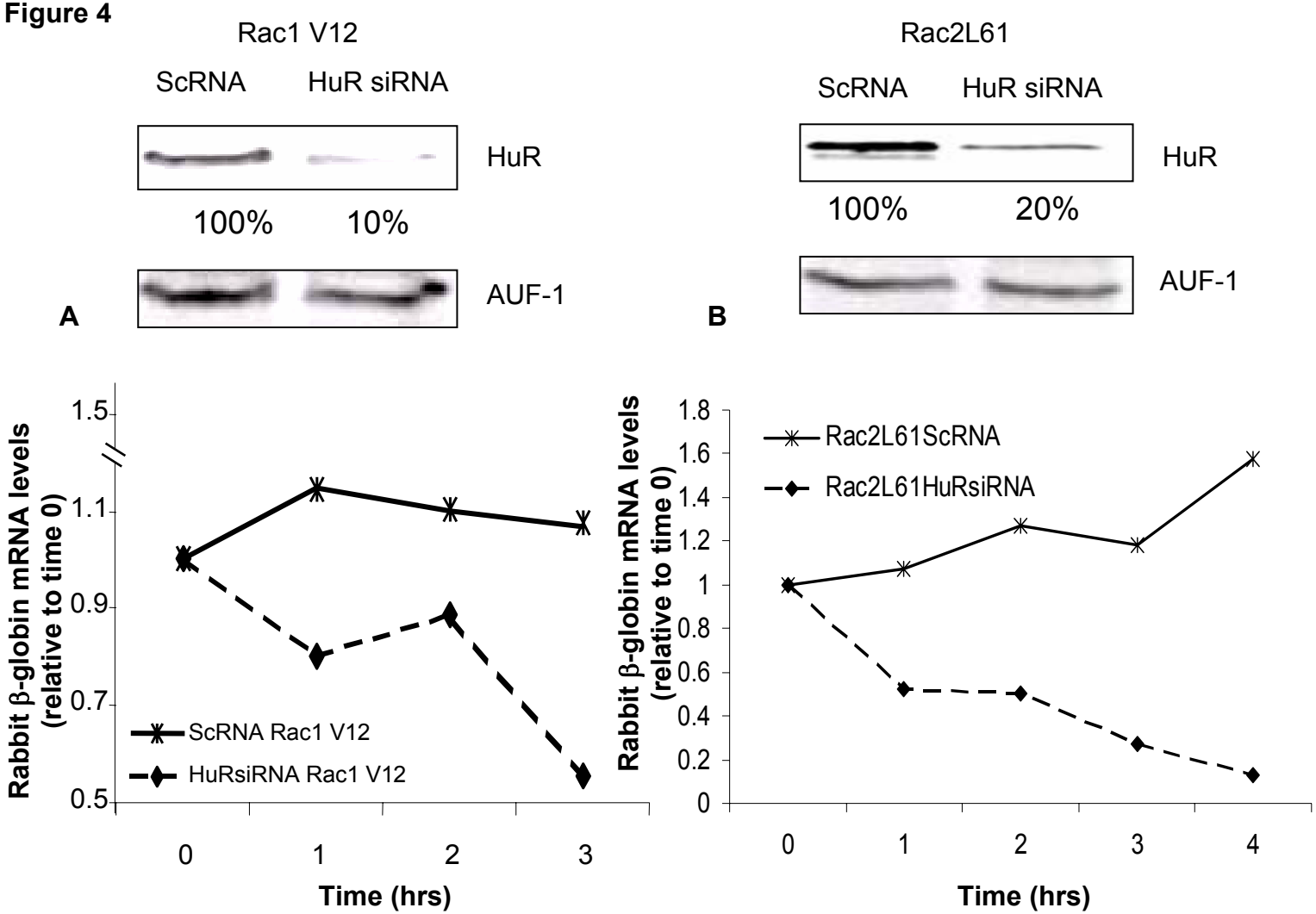
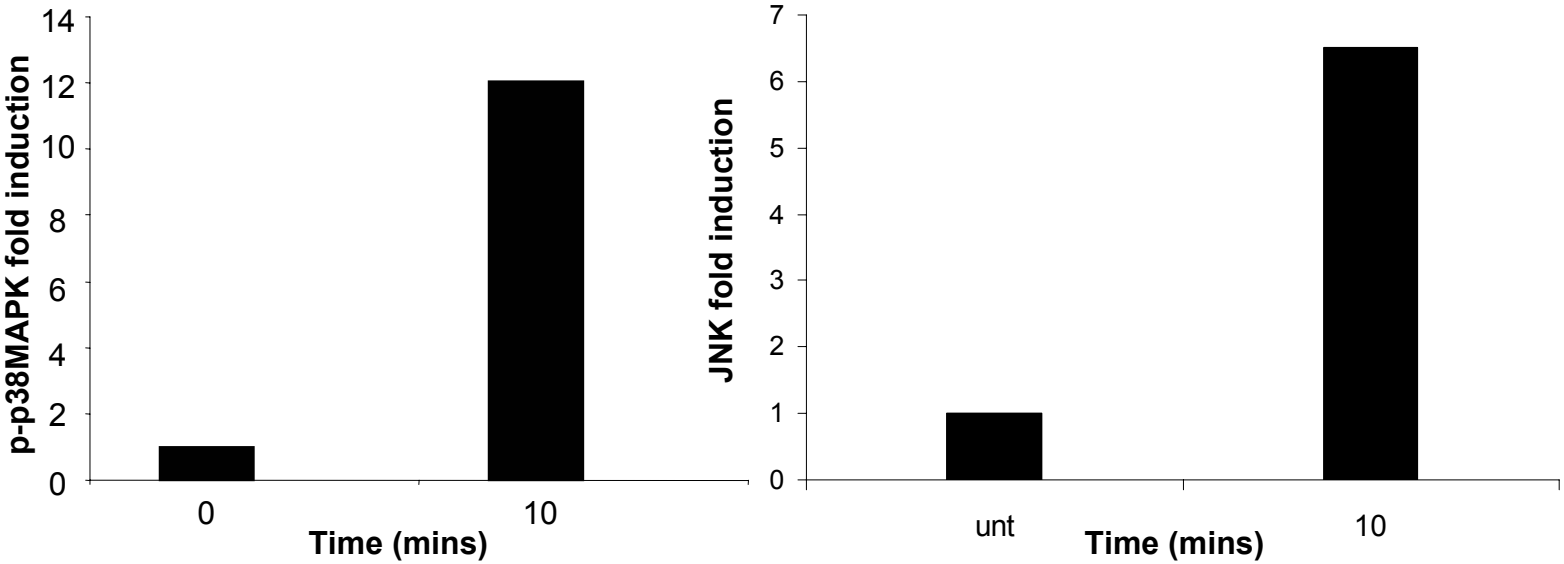
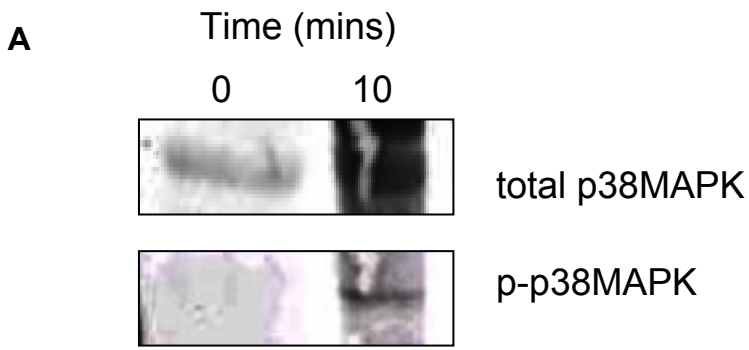

Rac1 V12
ScRNA
HuR siRNA
HuR
100%
10%
AUF-1
A
Rac2L61
ScRNA
HuR siRNA
HuR
100%
20%
AUF-1
B
Rabbit β-globin mRNA levels (relative to time 0)
1.5
1.1
0.9
0.7
0.5
ScRNA Rac1 V12
HuRsiRNA Rac1 V12
0
1
2
3
Time (hrs)
Rabbit β-globin mRNA levels (relative to time 0)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Rac2L61ScRNA
Rac2L61HuRsiRNA
0
1
2
3
4
Time (hrs)

Figure 5: The effect of LFA-1 engagement upon p38 MAPK and JNK activation in murine T cells. Murine T cells were suspended in LFA-1 activation buffer and plated on msICAM-1 coated dishes. Adherent and nonadherent cells were collected and lysed at the noted time points. The cell lysates were assayed for total and phosphorylated protein by Western blot analysis. Panel A shows p38 MAPK activity following LFA-1 engagement by msICAM-1. Phosphorylated p38 MAPK was normalized to total p38 MAPK within the Western blot. Panel B shows JNK activity. C-jun is a substrate for JNK, and phosphorylated c-jun is the customary readout for JNK activity, which was normalized against total JNK within the Western blot.

Figure 5


p-p38MAPK fold induction
14
12
10
8
6
4
2
0
0
10
Time (mins)
JNK fold induction
7
6
5
4
3
2
1
0
unt
10
Time (mins)


A
Time (mins)
0
10
total p38MAPK
p-p38MAPK

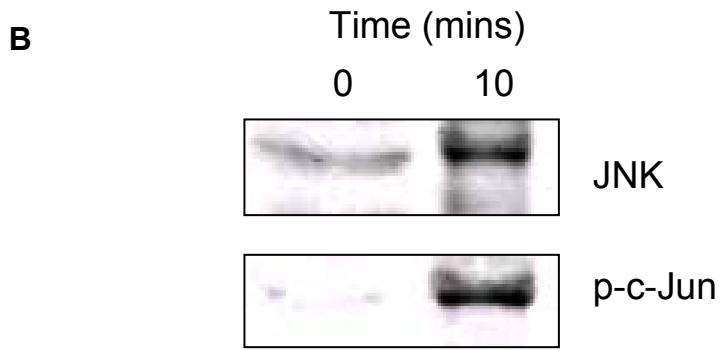

B
Time (mins)
0
10
JNK
p-c-Jun

Figure 6: The effect of JNK activity upon LFA-1 engagement-mediated mRNA stabilization in murine T cells. Panel A illustrates a sample dose response curve for SP600125, a JNK-specific pharmacologic inhibitor. Murine T cells were suspended in LFA-1 activation buffer and plated on msICAM-1 coated dishes with varying concentrations of SP600125. Adherent and nonadherent cells were collected and lysed at the noted time points. JNK activity was quantitated by normalizing phospho-c-jun against total JNK by Western blot analysis. Panels B & C: Murine T cells were stimulated for 3 hours with PMA, PMA and ICAM-1 or PMA, ICAM-1 and SP600125. Panel B: immediately following stimulation, the cells were lysed and their mRNA quantitated via quantitative, real time RT-PCR using primers for IFN-γ. Panel C: Transcription was inhibited following stimulation. RNA was harvested at the time of transcriptional arrest as well as the indicated time points and the mRNA quantitated via quantitative, real time RT-PCR using primers for IFN-γ.

Figure 6

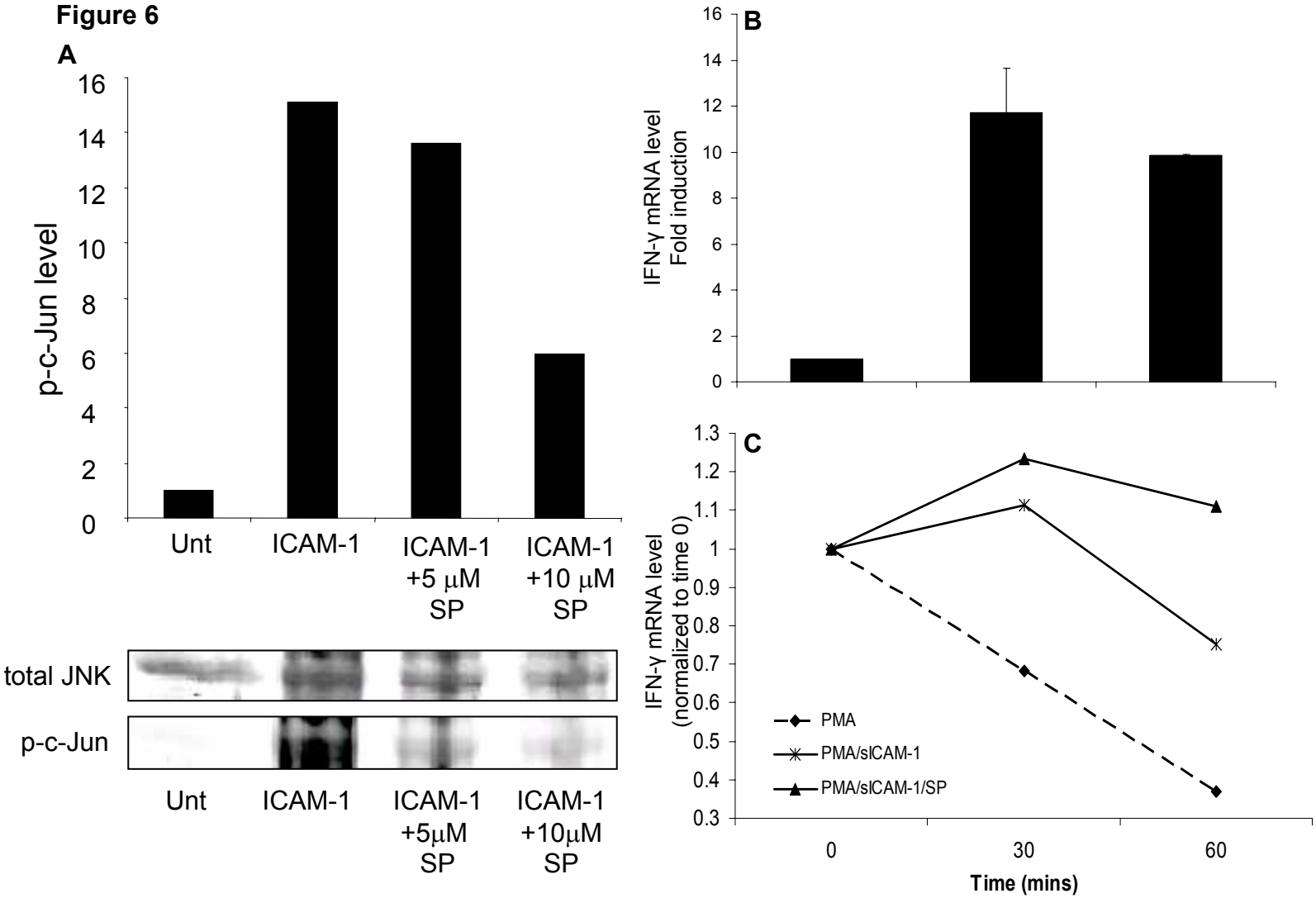

A
p-c-Jun level
16
14
12
10
8
6
4
2
0
Unt
ICAM-1
ICAM-1 +5 μM SP
ICAM-1 +10 μM SP
total JNK
p-c-Jun
Unt
ICAM-1
ICAM-1 +5μM SP
ICAM-1 +10μM SP
B
IFN-γ mRNA level Fold induction
16
14
12
10
8
6
4
2
0
C
IFN-γ mRNA level (normalized to time 0)
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
PMA
PMA/sICAM-1
PMA/sICAM-1/SP
0
30
60
Time (mins)

Figure 7: Effect of JNK inhibition on LFA-1 engagement-mediated HuR translocation in murine T cells. T cells were isolated from C57BL/6 mouse spleens, resuspended in LFA-1 activation buffer containing vehicle or the two noted doses of SP600125, and plated on coverslips coated with murine ICAM-1 or poly-L-lysine for 60 minutes. Immunofluorescent analysis was performed with anti-HuR mAb (red) and the nuclear marker DAPI (blue). Magnification 100X.

Figure 7

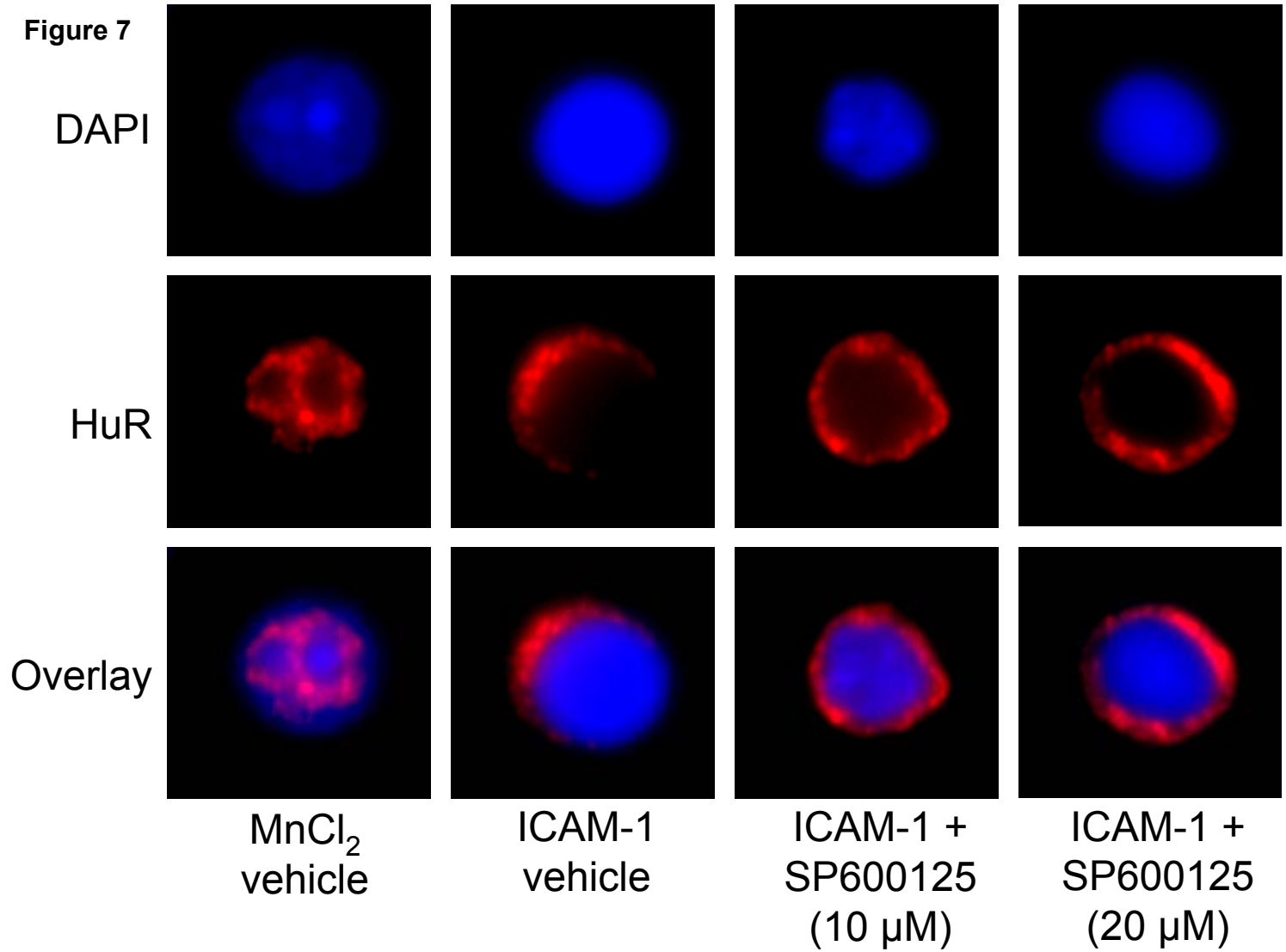

DAPI
HuR
Overlay
MnCl2 vehicle
ICAM-1 vehicle
ICAM-1 + SP600125 (10 μM)
ICAM-1 + SP600125 (20 μM)

Figure 8: The effect of p38 MAPK activity on mRNA stabilization in murine T cells. Panel A illustrates a sample dose response curve for SB203580, a p38 MAPK-specific pharmacologic inhibitor. Murine T cells were suspended in LFA-1 activation buffer and plated on msICAM-1 coated dishes with varying concentrations of SB203580. Adherent and nonadherent cells were collected and lysed at the noted time points. P38 MAPK activity was quantitated by normalizing phospho-p38 MAPK against total p38 MAPK by Western blot analysis. Panels B & C: Murine T cells were stimulated with PMA, PMA and ICAM-1 or PMA, ICAM-1 and SB203580. Panel B: immediately following stimulation, the cells were lysed and their mRNA quantitated via quantitative, real time RT-PCR using primers for IFN-γ. Panel C: Transcription was inhibited following stimulation. RNA was harvested at the time of transcriptional arrest as well as the indicated time points and the mRNA quantitated via quantitative, real time RT-PCR using primers for IFN-γ.

Figure 8

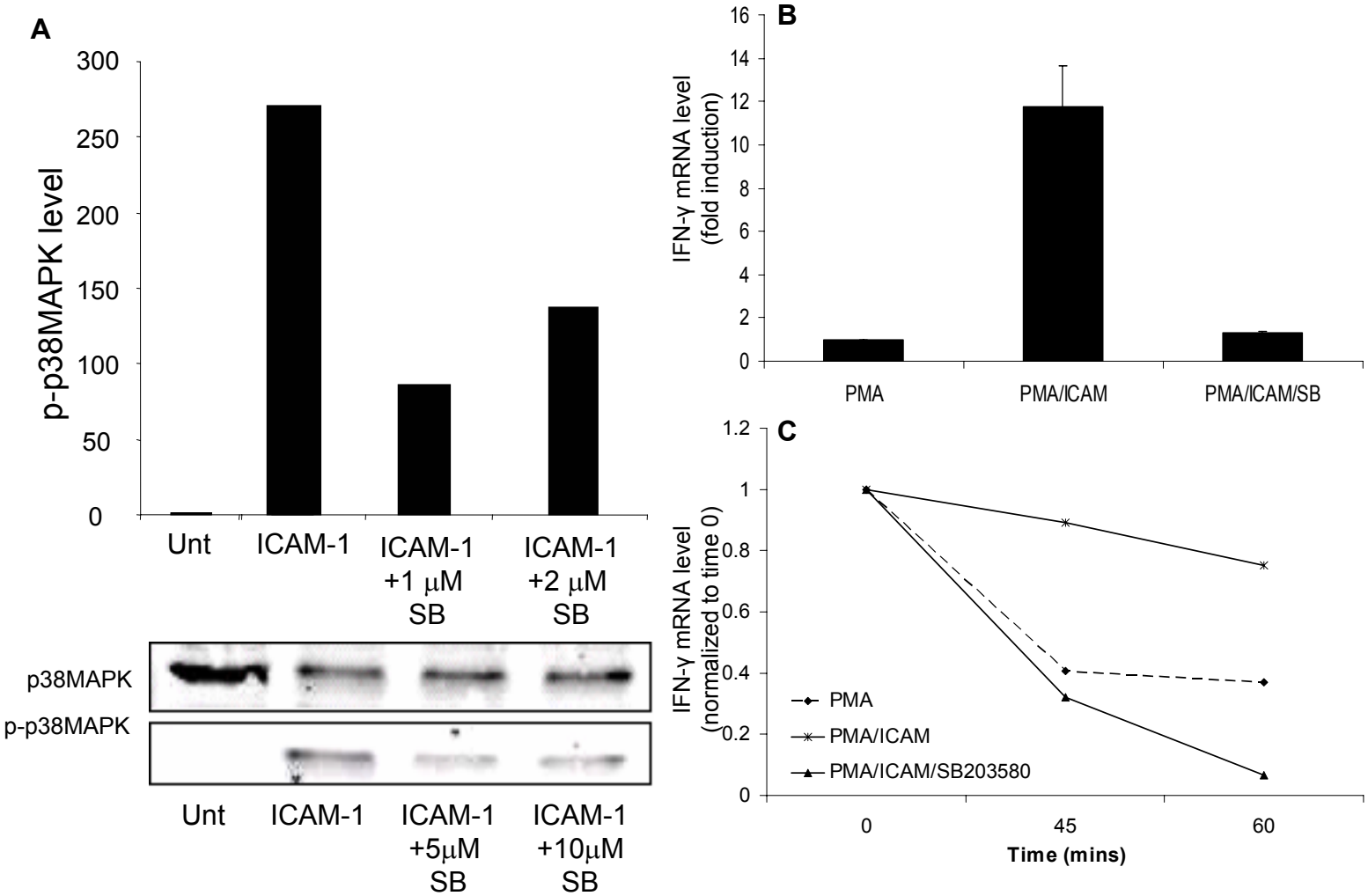

A
p-p38MAPK level
300
250
200
150
100
50
0
Unt
ICAM-1
ICAM-1 +1 μM SB
ICAM-1 +2 μM SB
p38MAPK
p-p38MAPK
Unt
ICAM-1
ICAM-1 +5μM SB
ICAM-1 +10μM SB
B
IFN-γ mRNA level (fold induction)
16
14
12
10
8
6
4
2
0
PMA
PMA/ICAM
PMA/ICAM/SB
C
IFN-γ mRNA level (normalized to time 0)
1.2
1
0.8
0.6
0.4
0.2
0
PMA
PMA/ICAM
PMA/ICAM/SB203580
0
45
60
Time (mins)

Figure 9: The effect of MKK3 gene deletion upon p38 MAPK and JNK expression in murine T cells. Murine splenic T cells were suspended in LFA-1 activation buffer and plated on msICAM-1 coated dishes. Adherent and nonadherent cells were collected and lysed after 1 hour. p38 MAPK activity (A) and JNK activity (B) were assessed via Western blot analysis. Activity was measured as a function of the phosphorylated enzyme (A) or its substrate (B) normalized against the total concentration of the MAPK. Data are presented as the mean value ± SEM (n=3).

Figure 9

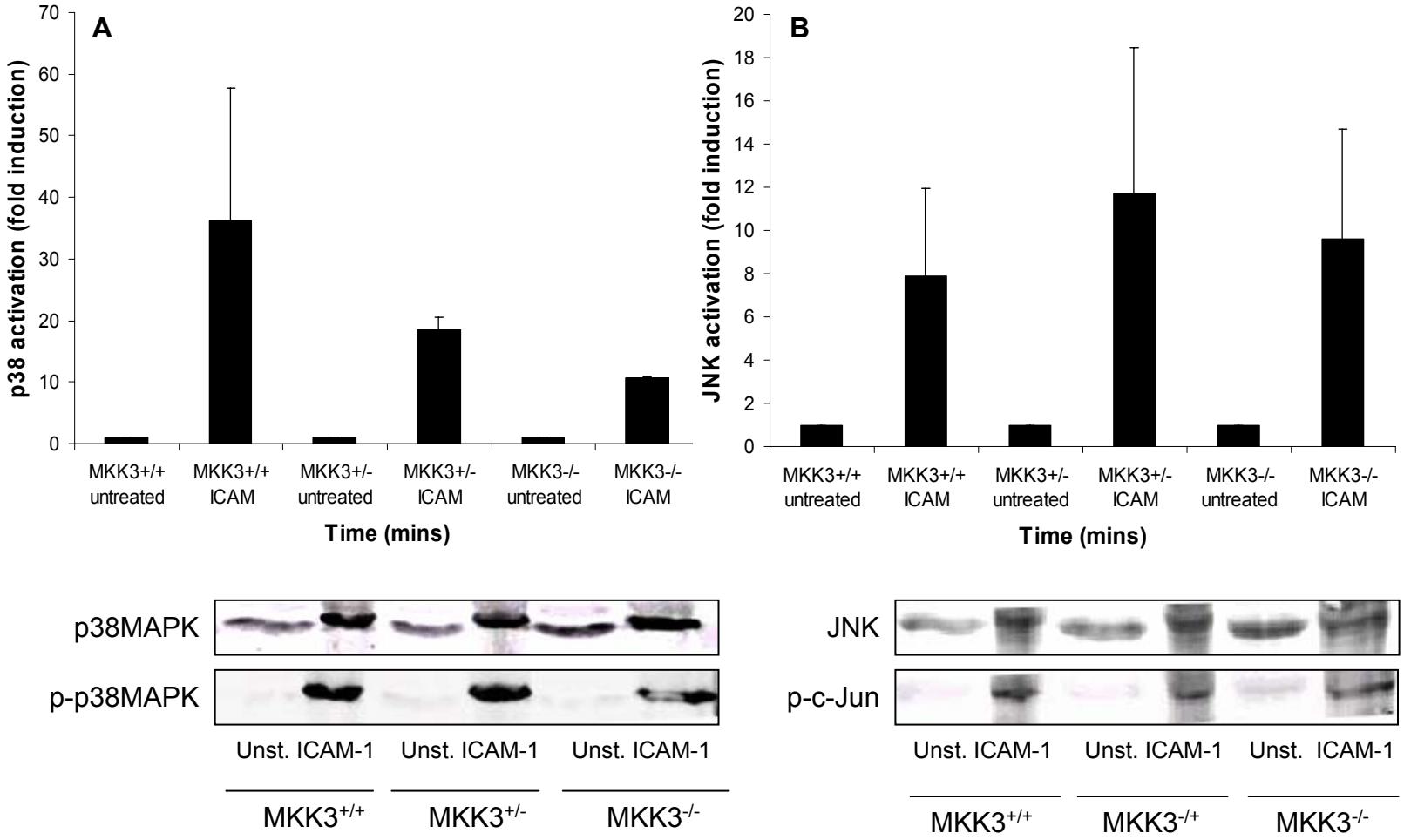

A
p38 activation (fold induction)
70
60
50
40
30
20
10
0
MKK3+/+ untreated
MKK3+/+ ICAM
MKK3+/- untreated
MKK3+/- ICAM
MKK3-/- untreated
MKK3-/- ICAM
Time (mins)
B
JNK activation (fold induction)
20
18
16
14
12
10
8
6
4
2
0
MKK3+/+ untreated
MKK3+/+ ICAM
MKK3+/- untreated
MKK3+/- ICAM
MKK3-/- untreated
MKK3-/- ICAM
Time (mins)
p38MAPK
p-p38MAPK
Unst. ICAM-1
Unst. ICAM-1
Unst. ICAM-1
MKK3+/+
MKK3+/-
MKK3-/-
JNK
p-c-Jun
Unst. ICAM-1
Unst. ICAM-1
Unst. ICAM-1
MKK3+/+
MKK3-/+
MKK3-/-

Figure 10: The effect of MKK3 gene deletion upon interferon gamma mRNA levels in murine T cells. Murine splenic T cells were isolated from MKK3+/+, MKK3+/-, and MKK3-/- mice and stimulated with PMA and ICAM for 3 hours. After stimulation, the T cells were lysed and IFN-g mRNA levels were quantitated via quantitative, real time RT-PCR using primers complementary for IFN-γ. IFN-γ mRNA levels were normalized against IFN-γ mRNA levels in MKK3+/+ T cells stimulated with PMA alone. Data are presented as the mean value ± SEM (n=3).

Figure 10

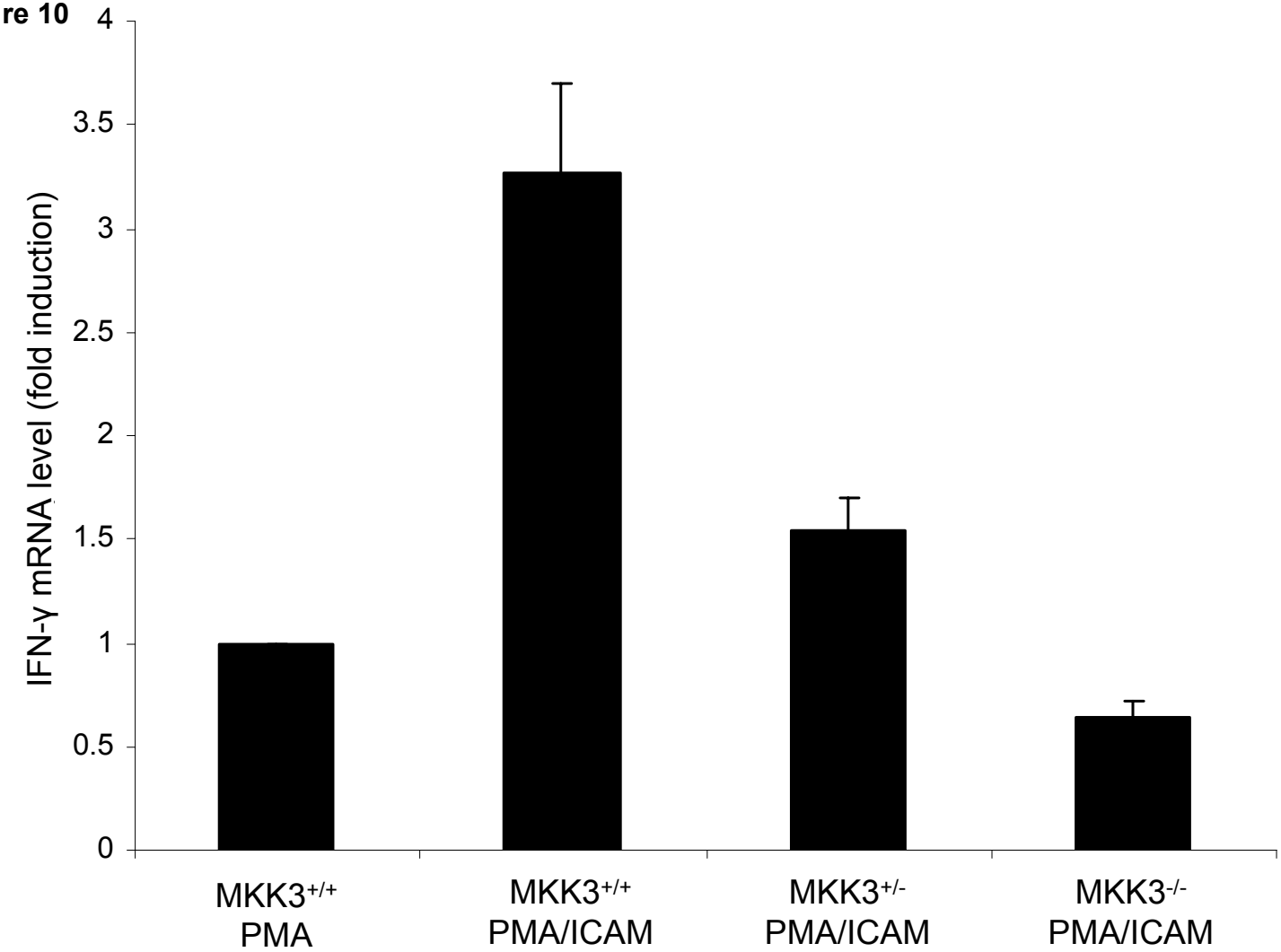

IFN-γ mRNA level (fold induction)
4
3.5
3
2.5
2
1.5
1
0.5
0
MKK3+/+ PMA
MKK3+/+ PMA/ICAM
MKK3+/- PMA/ICAM
MKK3-/- PMA/ICAM

Figure 11: The effect of MKK3 gene deletion upon LFA-1 engagement-mediated mRNA stabilization of interferon gamma transcripts in murine T cells. Murine splenic T cells were isolated from MKK3+/+, MKK3+/-, and MKK3-/- mouse splenocytes and stimulated with PMA and ICAM for 3 hours. After stimulation, transcription was inhibited by the addition of DRB. At the time of transcriptional arrest as well as the indicated time points after addition of DRB, T cells were lysed, mRNA harvested, and IFN-γ levels analyzed via quantitative, real time RT-PCR. Data are presented as the mean value ± SEM (n=3).

Figure 11

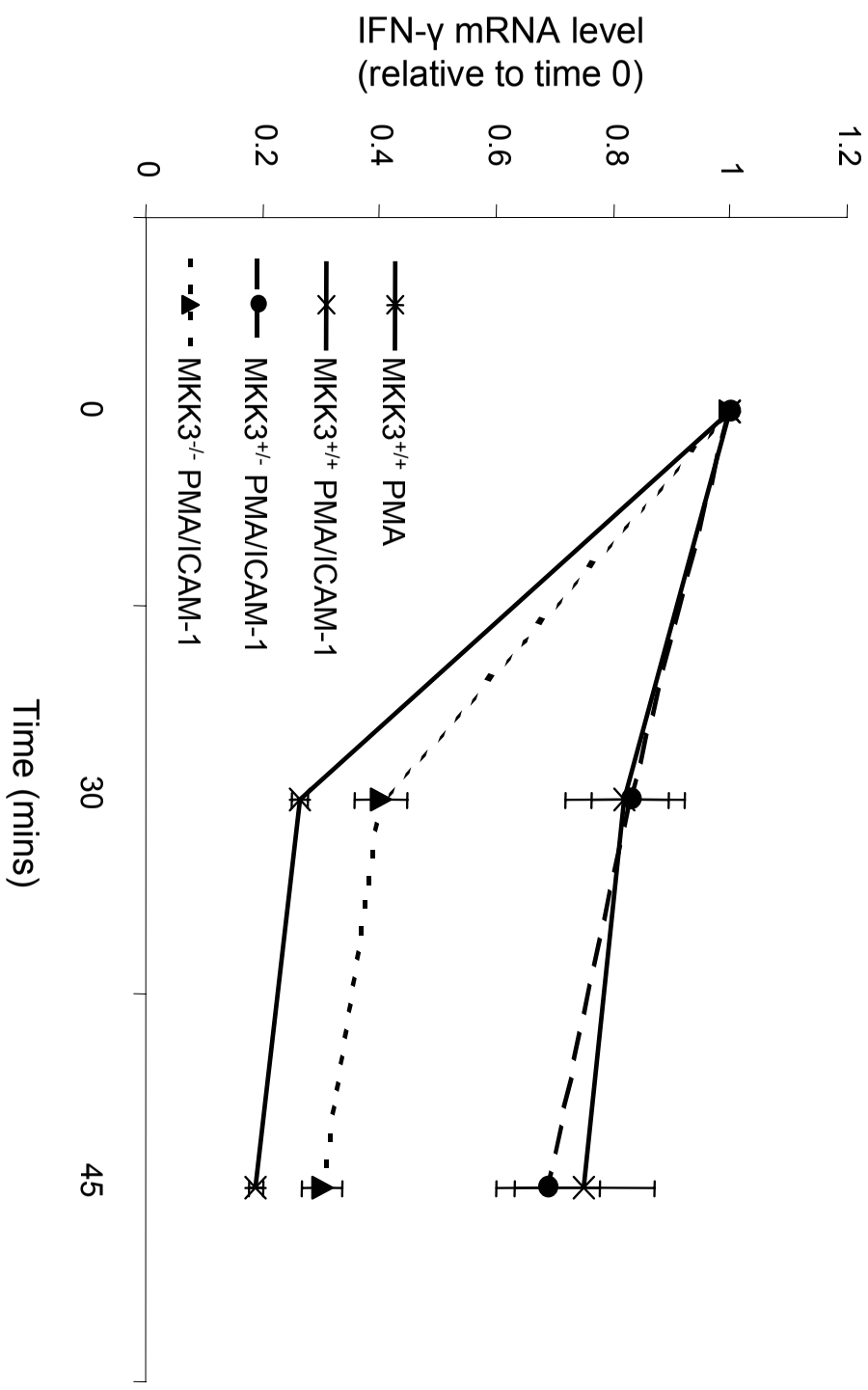

IFN-γ mRNA level
(relative to time 0)
0
0.2
0.4
0.6
0.8
1
1.2
0
30
45
Time (mins)
MKK3+/+ PMA
MKK3+/+ PMA/ICAM-1
MKK3+/- PMA/ICAM-1
MKK3-/- PMA/ICAM-1

Figure 12: The effect of MKK3 gene deletion upon HuR nuclear-to-cytoplasmic translocation in murine T cells. T cells were isolated from MKK3+/+, MKK3+/-, and MKK3-/- mouse spleens. The cells were then treated with LFA-1 activation buffer and plated on ICAM-1 coated coverslips for 60 minutes. Immunofluorescent analysis was performed with anti-HuR mAb (red) and the nuclear marker DAPI (blue). Magnification 100X.

Figure 12

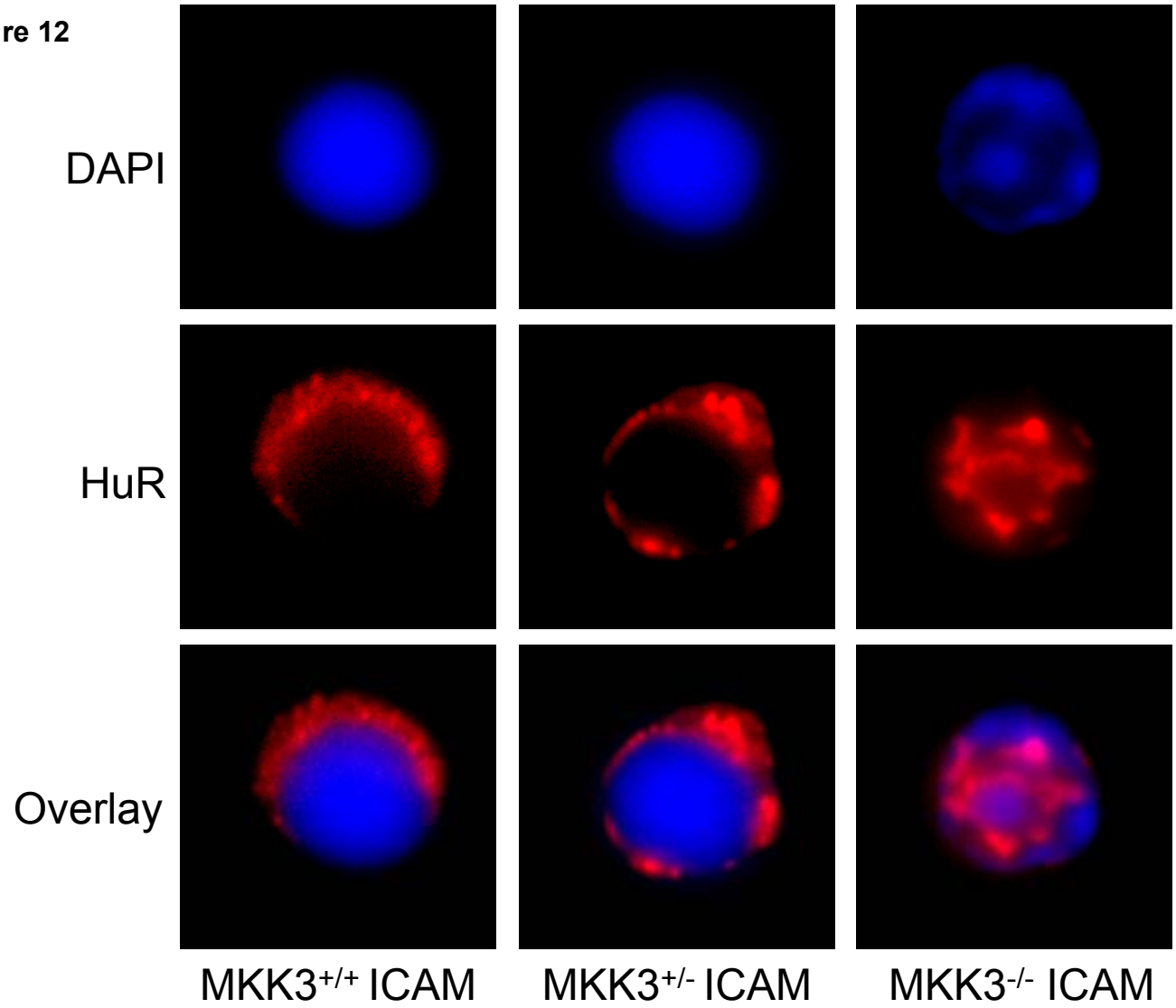


Figure 13: Proposed LFA-1 signal transduction pathway.

Figure 13

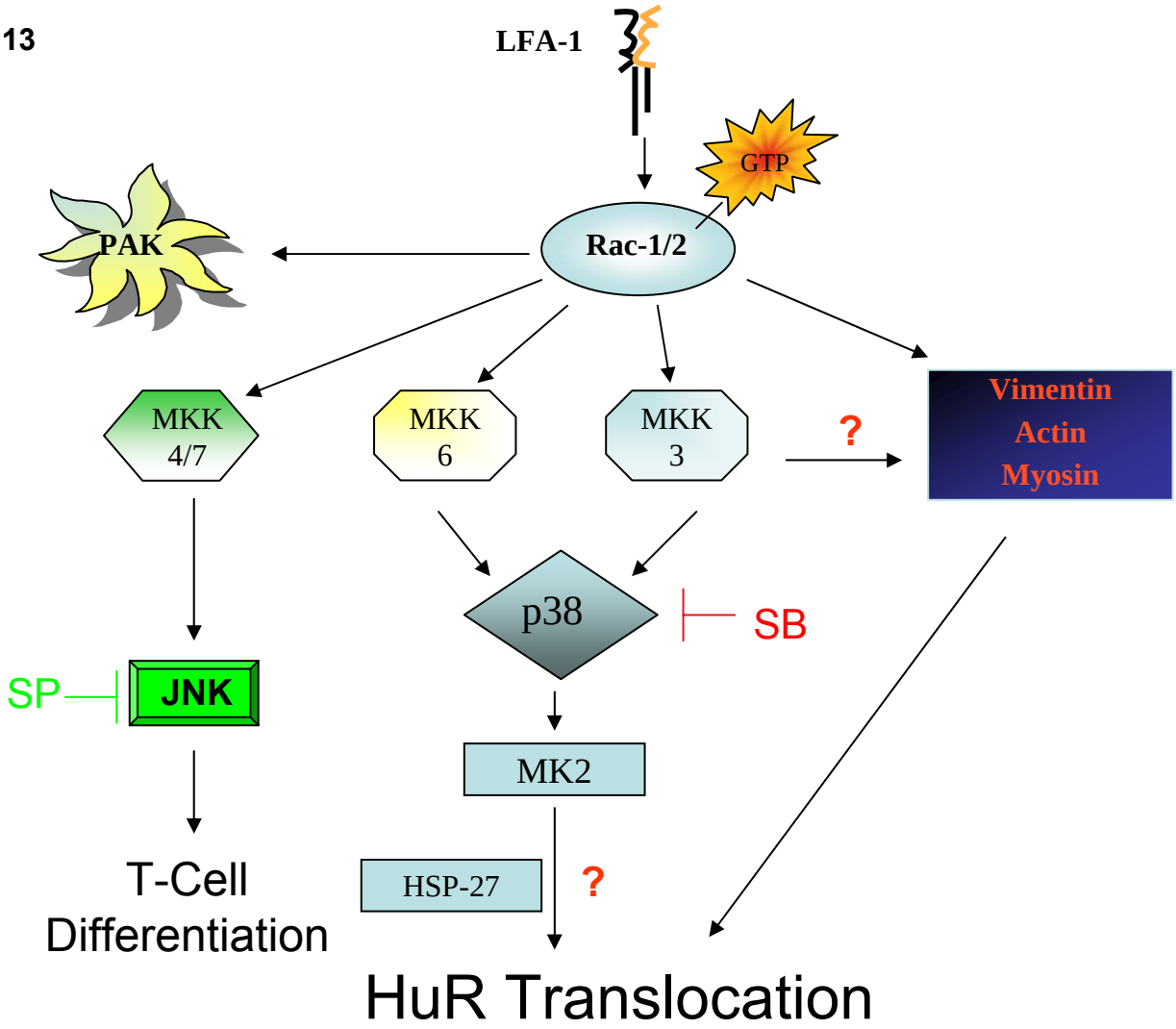

LFA-1
GTP
PAK
Rac-1/2
MKK 4/7
MKK 6
MKK 3
?
Vimentin
Actin
Myosin
p38
SB
SP
JNK
MK2
T-Cell Differentiation
HSP-27
?
HuR Translocation

Figure 14: The effect of MKK3 gene deletion upon rejection of skin grafts in mice. Preliminary pictures of skin grafts taken from wild type and MKK3-/- C57BL/6 male donors and grafted onto wild type and MKK3-/- C57BL/6 female recipients, respectively. The pictures are taken at 14 and 28 days after graft transplant. Rejection of the graft causes contraction of the transplanted skin. Therefore, a larger area of transplanted tissue indicates a greater amount of viable graft remaining on the recipient. We define rejection at 100% contraction of the graft (when the recipient's skin has completely grown over the graft site, such as MKK3+/+ at 28 days).

Figure 14