Published in final edited form as:

Hum Immunol. 2011 December ; 72(12): 1150–1159. doi:10.1016/j.humimm.2011.09.004.

Antibody Ligation of HLA Class I Molecules Stimulates Migration and Proliferation of Smooth Muscle Cells in a FAK Dependent Manner:

HLA-class I-mediated SMC migration and proliferation

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Abstract

Chronic rejection manifests as transplant vasculopathy which is characterized by intimal thickening of the vessels of the allograft. Intimal thickening is thought to result from the migration and proliferation of vascular smooth muscle cells (SMC) in the vessel media followed by deposition of extracellular matrix proteins. The development of post-transplant anti-HLA antibodies (Ab) is strongly correlated with the development of transplant vasculopathy and graft loss. Here we demonstrate that crosslinking of HLA class I molecules on the surface of human SMC with anti-HLA class I Ab-induced cell proliferation and migration. Class I ligation also increased phosphorylation of Focal Adhesion Kinase (FAK), Akt and ERK1/2 in SMC. Knockdown of FAK by siRNA attenuated class I-induced phosphorylation of Akt and ERK1/2, as well as cell proliferation and migration. These results indicate that ligation of HLA class I molecules induces SMC migration and proliferation in a FAK dependent manner, which may be important in promoting transplant vasculopathy.

Keywords

anti-HLA antibodies; human smooth muscle cells; focal adhesion kinase; cell migration; cell proliferation

Introduction

Transplantation is a life-saving procedure for patients with end-stage organ disease. Improvements in immunosuppression therapy and patient management have markedly reduced the incidence of acute rejection. However, chronic rejection remains the major limitation to long-term allograft survival. The hallmark of chronic rejection is transplant

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Conflict of interest: The authors declare that they have no conflicting interests.

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vasculopathy (TV), which is characterized by intimal thickening, interstitial fibrosis and occlusion of vessels of the graft [1, 2]. The occlusive neointimal layer that develops in the arteries of allografts is caused by the accumulation of proliferating vascular smooth muscle cells (SMC), endothelial cells (EC), macrophages, and T lymphocytes in the subendothelial layer [3] of vascular bed of allografts [4]. Although there is significant intimal proliferation, the tunica media of allograft is rarely thickened [3], suggesting that donor-derived vascular SMC migrate from tunica media into the lumen area and proliferate in the subendothelial space [4, 5].

The mechanisms underlying chronic rejection and TV are still poorly defined [5]. Numerous studies have shown that patients developing anti-donor HLA antibodies (Ab) following transplantation are at significantly higher risk of developing TV, supporting the important contribution of humoral immune responses to the mismatched donor HLA antigens in the disease process [6-9]. HLA antigens function as signal transduction molecules that regulate cell growth, cell cycle arrest and apoptosis [10]. Therefore, it is conceivable that anti-donor HLA Ab act directly on the smooth muscle of the allograft to transduce signals that elicit SMC migration and proliferation leading to intimal thickening.

Ab ligation of HLA class I molecules on cultured EC stimulates phosphorylation of Src and FAK which in turn causes activation of phosphoinositide 3-kinase (PI3K), Akt, mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and ERK signaling pathways that contribute to EC proliferation [11-14]. Crosslinking of HLA class I molecules on EC activates PI3K via a FAK-dependent phosphorylation of the p85 regulatory domain. Using siRNA, we showed that FAK plays a critical role in HLA class I induced cell survival and proliferation and focal adhesion assembly in EC [15]. The relevance of the HLA class I signaling pathway was further confirmed *in vivo*. Histological examination of biopsies from cardiac transplant patients showed that phosphorylated S6 ribosomal protein, a downstream target of mTORC1, was associated with diagnosis of antibody-mediated rejection (AMR) [16]. These findings were also validated in an animal model which showed phosphorylation of S6RP, S6K, ERK, mTOR and Akt in animals that received passive transfer of anti-donor MHC class I Ab [17].

In this study, we characterized the effects of HLA class I Ab on SMC activation, proliferation and migration. The data demonstrate that binding of Ab to HLA class I molecules increases the phosphorylation of FAK, Akt and ERK and stimulates SMC proliferation and migration. HLA Ab-mediated cell migration and proliferation were inhibited by depleting FAK protein expression. This study shows that FAK plays an important role in class I-induced SMC migration and proliferation which may contribute to the development of TV.

Materials and Methods

Antibodies

Antibodies against phospho-Akt (Ser473), phospho-ERK1/2 (Thr202/Tyr204), Akt, and ERK1/2 were obtained from Cell Signal Technology (Beverly, MA). Ab against phospho-FAK (Tyr576) was purchased from Invitrogen (Carlsbad, CA). Ab to FAK protein was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Ab to β -actin was obtained from Abcam (Cambridge, MA). Vinculin Ab was purchased from Sigma-Aldrich (St. Louis, MO). The mAb EMR8-5 which recognizes the denatured heavy chain of HLA class I used for Western blotting analysis was purchased from MBL International (Woburn, MA). W6/32, a murine mAb against a monomorphic epitope on HLA class I antigens, was purchased from the American Type Culture Collection (Manassas, VA). The mouse and human IgG isotype controls were from Sigma-Aldrich (St. Louis, MO) and Jackson

ImmunoResearch Laboratories (West Grove, PA), respectively. The murine anti-HLA-A2 mAb was generously provided by Dr. Jar-how Lee, One Lambda Inc. Human mAb directed against HLA-A24/A32 was provided by Dr. Arend Mulder, Leiden University, the Netherlands.

Cell Culture

Primary human aortic SMC1 was generously provided by Dr. Judith A. Berliner, University of California, Los Angeles and SMC2 was obtained from Lonza (Basel, Switzerland). The HLA-A and B locus typings of the SMC used in these experiments were SMC1 HLA-A*24, A*32; B*1501, B*39 and SMC2 HLA-A*02, A*02; B*1501, B*35. Cell culture plates were coated with 0.1% gelatin in PBS solution for 30 min before seeding the cells. Cells were maintained in M199 medium supplemented with sodium pyruvate (1 mM) (Irvine Scientific, Santa Ana, CA), penicillin (100 units/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml) (Invitrogen, Calsbad, CA) and 20% (v/v) HyClone fetal bovine serum (FBS) (Thermo Scientific, Logan, UT). Cells were used for experiments during passages 7-10. Cells were cultured to a confluence of 80% and incubated for 16 h in serum-free medium prior to treatment.

Small interfering RNA (siRNA) transfection

Transfection of FAK siRNA and HLA siRNA was performed as we previously described [15, 18]. The siRNA targeting FAK (5'-GGU UCA AGC UGG AUU AUU U-3') [15], the siRNA targeting HLA class I heavy chain (5'-GCA GAG AUA CAC CUG CCA U-3') [18] and the non-targeting siRNA duplexes against firefly GL2 luciferase (5'-CGU ACG CGG AAU ACU UCG A -3') [19] were synthesized by Dharmacon, Inc. (Lafayette, CO). SMC at a density of 60-80% confluency were transfected with 100 nM siRNA using *TransIT*-TKO transfection reagent (Mirus Bio LLC, Madison, WI) according to the manufacturer's instructions. Experiments were conducted 48h after transfection.

HLA class I expression measured by flow cytometry

HLA class I antigen expression was analyzed by flow cytometry using mAb W6/32 (IgG2a), the allele-specific murine mAb against HLA-A2 (IgG2a), or the human mAb against HLA-A24 and A32 (IgG1) as previously described [15].

Western blotting analysis

Western blotting was performed as previously described [15]. Protein bands were quantitated by densitometry and analyzed using ImageJ (<http://rsb.info.nih.gov/ij/>). The phosphorylation of individual proteins was normalized to the β -actin loading control. Densitometry results are expressed as fold change of phosphorylation compared to isotype control.

Cell proliferation assay

Cell proliferation was determined using CFSE as we previously described [13]. SMC transfected with control, HLA class I heavy chain siRNA or FAK siRNA were starved, labeled with 2 µM of CFSE (Invitrogen, Carlsbad, CA) and stimulated with Ab to HLA class I (W6/32) or HLA A24/A32, or control murine or human IgG for 48 hours. Cell proliferation was analyzed on a FACSCalibur and calculated using the Modfit LT software (Verity Software House, Topsham, ME) and Proliferation Wizard Model. The freshly CFSE labeled SMC were used as the parental cells and their homogeneity was verified at the start of each experiment to be >85%. The proliferation index (PI) is the sum of the cells in all generations divided by the computed number of parental cells present at the start of

experiment [20]. The PI ratio represents the PI of test culture divided by PI of IgG isotype control or non-treated (NT) culture.

In vitro wound healing assay for migration

The *in vitro* wound healing assay was performed as described previously [18] with slight modifications. Briefly, SMC were transfected with control, FAK or HLA class I heavy chain siRNA, or for some experiments pre-treated with mitomycin C at 10 µg/ml for 2 h to inhibit cell proliferation [18, 21]. The cell monolayers were scratched with a pipet tip and stimulated with W6/32, HLA-A2, or mIgG at 37°C for 24 h, fixed and stained with standard Fluka Giemsa Stain (Sigma-Aldrich). Wound closure was measured using the Cellprolifer (Broad Institute of MIT, Cambridge, MA) program to calculate the number of migrating cells in the wound area compared to the number of cells present in a non-wound area.

Statistical Analyses

The one-way analysis of variance (ANOVA) with Bonferroni correction post hoc analysis was used for comparisons, with $P < 0.05$ considered significant. Data in the graph are presented as mean $\pm$ the standard error of the mean (SEM).

Results

Ligation of HLA class I molecules by anti-HLA class I Ab induces SMC proliferation and migration

To determine the effect of Ab ligation of HLA class I molecules on SMC proliferation, SMC were stimulated with the anti-HLA class I mAb W6/32 and cell proliferation was measured using the intravital dye CFSE. Treatment of SMC with various concentrations of anti-class I mAb W6/32 for 48 h stimulated a dose dependent increase in proliferation compared to cells treated with isotype control mIgG (Fig. 1A). The highest proliferation index (PI=29) was observed in SMC treated with 1.0 µg/ml of anti-class I mAb compared to cells treated with isotype control IgG (PI=21) ($P < 0.05$). Treatment with FBS, a potent stimulator of EC proliferation, yielded a similar degree of cell proliferation to that induced by HLA class I ligation (PI=31). These data indicate that ligation of class I molecules by anti-HLA Ab induces SMC proliferation.

To further elucidate the effect of class I Ab on SMC, we examined the capacity of HLA-class I Ab to induce SMC migration. SMC were treated with different doses of HLA-class I Ab or FBS and tested for migration using the *in vitro* wound healing assay. As shown in Fig. 1B, SMC stimulated with FBS migrated across the wound and completely closed the gap within 24 h. Similarly, we found that treatment of SMC with class I Ab, but not IgG isotype control, stimulated increased migration across the wound margin at 24 h of incubation (Fig. 1B). The wound repair process involves both cell migration and proliferation. To exclude the confounding effect of cell proliferation in the wound healing assay, SMC were pretreated with mitomycin C to prevent cell proliferation [18, 21].

Stimulation of SMC with anti-class I Ab promoted cell migration in cells treated with mitomycin C compared to SMC treated with control IgG ($P < 0.05$) (Fig 1C). These results indicate that HLA class I-mediated wound closure can be mediated by cell migration in the absence of proliferation.

Decreasing HLA class I expression impairs class I-mediated smooth muscle cell proliferation and migration

To further confirm the role of HLA class I molecules in SMC proliferation, we used siRNA to selectively knockdown HLA class I antigen expression in SMC. SMC1 and SMC2 were

transfected with HLA class I heavy chain siRNA or non-targeting control siRNA. Transfection with HLA class I heavy chain siRNA markedly reduced expression of HLA class I on the surface of SMC as demonstrated by the decreased binding of the pan reactive W6/32 mAb and the allele specific HLA-A2 mAb on SMC2 and the HLA-A24/32 mAb on SMC1 (Fig. 2A). Transfection with HLA class I heavy chain siRNA decreased expression of HLA class I (Fig. 2B), but had no effect on expression of β -actin (Fig. 2B) or proteins involved in the class I signaling pathway including FAK, ERK and AKT (Fig. 3C). Treatment of SMC transfected with control siRNA with mAb W6/32 or mAb HLA-A24/32 stimulated significant cell proliferation (Fig. 2C). In contrast, transfection with HLA class I heavy chain siRNA appreciably inhibited class I-mediated SMC proliferation (Fig. 2C).

To further characterize the impact of HLA class I protein depletion on class I induced cell migration, SMC transfected with control siRNA or HLA class I heavy chain siRNA were stimulated with anti-class I Ab and tested for migration using the wound healing assay. As shown in Fig. 2D, transfection with the HLA class I heavy chain siRNA inhibited mAb HLA-A2 and mAb W6/32-induced migration of SMC compared to SMC transfected with control siRNA.

Ligation of HLA class I molecules by anti-HLA class I Abs induces protein phosphorylation in SMC

Previous studies from our laboratory have shown that ligation of class I molecules on EC induces phosphorylation of intracellular proteins that regulate cell survival and proliferation [11, 13, 15]. In view of these results, experiments were performed to determine whether ligation of HLA class I molecules on SMC induces phosphorylation of proteins that have been implicated in class I mediated EC proliferation. For this, SMC were incubated with mAb W6/32 at various concentrations ranging from 0.01 to 10.0 μ g/ml and the cell lysates were studied for protein phosphorylation by Western blot. Increased phosphorylation of FAK Tyr576, Akt Ser473 and ERK1/2 Thr202/Tyr204 was observed at concentrations ranging from 0.1 μ g/ml to 10.0 μ g/ml with maximal effects observed at 1.0 μ g/ml (Fig. 3A). Increased protein phosphorylation was observed as early as 5 min following the addition of anti-class I mAb and stayed at high levels at 60 min (Fig. 3B). Inhibition of HLA class I expression by siRNA impaired anti-HLA-A2 mAb induced phosphorylation of FAK, Akt, and ERK without altering their total protein expression level (Fig. 3C).

FAK knockdown inhibits HLA class I-mediated phosphorylation of Akt and ERK in SMC

As demonstrated above, rapid increases in phosphorylation of FAK, Akt and ERK are prominent events in response to class I ligation on SMC. Previous studies have shown that the capacity of FAK to transmit signals to downstream targets after class I ligation is dependent on its ability to interact with several intracellular signaling molecules [11, 13, 15]. To examine the cause-effect connections in the signal transduction pathways induced by class I ligation, we determined the effect of siRNA knockdown of FAK on Akt and ERK activation. SMC transfected with FAK siRNA showed a substantial decrease in FAK and p-FAK expression without altering the levels of Akt, ERK1/2, β -actin or vinculin (Fig. 4A). Treatment of SMC transfected with non-targeting control siRNA followed by stimulation with anti-class I Ab for various times showed a time dependent increase in protein phosphorylation of Akt Ser473 and ERK1/2 Thr202/Tyr204 in SMC (Fig. 4A). Transfection of SMC with FAK siRNA significantly reduced class I-stimulated phosphorylation of Akt at Ser473 and ERK1/2 at Thr202/Tyr204 (Fig. 4A), demonstrating that class I-mediated activation of Akt and ERK are dependent on FAK.

Because phosphorylated FAK is found in focal adhesions associated with stress fibers, experiments were designed to evaluate whether disruption of the actin cytoskeleton

prevented activation of Akt and ERK following class I ligation on SMC. SMC were pretreated with cytochalasin D or latrunculin A and then stimulated with anti-class I Ab. As shown in Fig. 4 B, both latrunculin A and cytochalasin D attenuated W6/32-induced phosphorylation of FAK at Tyr576, Akt at Ser473 and ERK1/2 at Thr202/Tyr204. These results indicate that the integrity of actin cytoskeleton is required for class I-induced SMC activation

FAK plays an important role in HLA class I-mediated cell proliferation and migration

FAK regulates the cytoskeleton and controls essential cellular processes such as survival, migration, and differentiation [22, 23]. Inhibition of FAK tyrosine phosphorylation attenuates the migration of SMC induced by different stimuli [24-26]. We next determined if knockdown of FAK inhibits class I-mediated cell proliferation and migration. SMC were transfected with FAK or control siRNA, labeled with CFSE and stimulated with anti-class I Ab or isotype control mIgG. Treatment with anti-class I Ab stimulated a 33% increase in the proliferation of SMC transfected with control siRNA above cells treated with isotype control IgG (Fig. 5A). Conversely, knockdown of FAK prevented HLA class I stimulated increases in SMC proliferation (Fig. 5A). The PI of cells transfected with control siRNA and stimulated with anti-class I Ab was 27, while cells transfected with FAK siRNA and stimulated with isotype control or class I Ab both had a PI of 15. These data demonstrate that FAK was required for HLA class I-mediated proliferation of SMC.

We also examined the effect of FAK siRNA on class I-mediated cell migration. As shown in Fig. 5B, SMC transfected with control siRNA and stimulated with anti-class I Ab increased migration across the wound. In contrast, class I-induced migration of SMC transfected with FAK was significantly less than that induced in cells transfected with control siRNA, demonstrating that class I-mediated cell migration is dependent upon FAK.

Discussion

The data presented here demonstrate that anti-HLA class I Ab may contribute to the process of TV by binding directly to class I molecules expressed on SMC and inducing SMC proliferation and migration. These findings are consistent with recent experimental models of transplant arteriosclerosis which showed that passive transfer of Ab to HLA class I molecules caused significant smooth muscle cell proliferation and neointimal thickening in the grafted human artery transplanted into T and B cell deficient SCID/Beige Mice [27]. Similarly, studies by Uehara et al have also shown that repeated administration of anti-donor MHC class I Ab can cause transplant arteriosclerosis in the transplanted heart in a complement-independent manner [28].

While animal models using passive transfer of anti-MHC antibodies have shown repeatedly that anti-donor MHC Ab elicit TV [27-31], the mechanism underlying this process is not well understood. In this study, we observed that mAb directed against monomorphic or polymorphic residues on HLA class I molecules stimulate the phosphorylation of proteins involved in cell survival and proliferation including FAK Tyr576, Akt Ser473 and ERK1/2 Thr202/Tyr204 and causes the proliferation of SMC. In addition, we showed for the first time that treatment with anti-class I Ab stimulated SMC migration. SiRNA-mediated protein depletion of FAK inhibited anti-HLA class I-induced proliferation and migration indicating that the capacity to stimulate SMC migration and proliferation is dependent upon FAK. Taken together, direct binding of HLA Ab to SMC induces proliferation and migration of SMC suggesting this as a possible mechanism to explain the link between donor specific HLA antibodies and the development of TV.

FAK is an important tyrosine kinase that regulates the organization of the actin cytoskeleton and modulates cell migration and proliferation. FAK has been shown to be a central regulator of class I induced EC proliferation [15]. We found that ligation of class I molecules on the surface of SMC promoted a time and dose dependent increase in the phosphorylation of FAK. FAK phosphorylation at Tyr576 and Tyr577 in the kinase domain [22] promotes maximal FAK catalytic activation that prompts the formation of signaling complexes with other proteins including PI3K, Akt [15] and ERK [12]. Consistent with this model, we found that class I ligation led to the phosphorylation of Akt at Ser473 and ERK1/2 at Thr202/Tyr204. Using siRNA-mediated protein depletion of FAK, we further explored its contribution to class I induced protein phosphorylation in SMC. siRNA-mediated protein depletion of FAK or HLA class I molecules, but not non-targeting control siRNA, markedly impaired HLA class I induced phosphorylation of Akt Ser473 and ERK1/2 Thr202/Tyr204. These results indicate a FAK-dependent pathway for class I induced phosphorylation of Akt and ERK. To provide an independent line of evidence linking FAK to activation of Akt and ERK following class I ligation, we treated SMC with the pharmacological agents Cytochalasin D and Latrunculin A, at concentrations known to disrupt the actin cytoskeleton and FAK activity. We found that phosphorylation of ERK1/2 at Thr202/Tyr204 and Akt at Ser473 were inhibited by pretreatment of SMC with these pharmacologic inhibitors.

The importance of FAK in cell proliferation was supported by our finding that FAK siRNA depletion was accompanied by a marked inhibition of class I induced SMC proliferation. siRNA knockdown of class I molecules in primary SMC also prevented HLA class I Ab-induced cell proliferation. These effects are consistent with previous findings showing that FAK plays an essential role in HLA Ab-mediated EC proliferation [15]. HLA class I-mediated FAK signaling elicits EC cell proliferation through its ability to activate the PI3K/Akt pathway and mTOR [11, 13, 15]. mTOR is present in two complexes in the cell, mTORC1 which regulates protein synthesis and proliferation [32-34] and mTOR complex 2 (mTORC2) which participates in cell survival and cytoskeletal reorganization [35, 36]. HLA class I ligation activates PI3K, via a FAK-dependent phosphorylation of the p85 regulatory domain [15]. The PI3K activation recruits 3-phosphoinositide dependent protein kinase (PDK1) and Akt to cell membrane [37], where phosphorylation of Akt by 3-phosphoinositide dependent protein kinase (PDK1) results in activation of mTORC1. mTORC1 phosphorylates S6 kinase, S6 ribosomal protein and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) which together increase protein translation and cell proliferation [34, 37-39]. Consistent with this model, our previous work showed that siRNA knockdown of mTOR, regulatory associated protein of mTOR (Raptor) and rapamycin-insensitive companion of mTOR (Rictor), suppressed class I induced EC proliferation [13]. Thus, FAK likely contributes to cell proliferation by setting the mTORC1 or mTORC2 pathway in motion.

Aberrant SMC migration is important to the development of TV [40, 41]. This is the first study to show that HLA class I signaling can directly promote SMC migration through the activation of FAK. FAK, once localized to focal adhesions, is thought to be one of the principal effectors in linking signals initiated by integrins [42, 43] and growth factor receptors to cytoskeleton, thus controlling migration [22, 44]. Phosphorylation of FAK Tyr861 has been implicated in FAK-dependent SMC migration [43, 45] and FAK-related non-kinase (FRNK), a dominant-negative inhibitor of FAK, has been shown to prevent SMC migration by inhibiting FAK signaling [46-48]. We observed that transfection of SMC with siRNA targeting FAK impaired HLA class I induced SMC migration. Therefore, agents that block FAK activation may be useful in the prevention and treatment of TV.

Recent studies from our group showed that HLA class I molecules physically interact with the adhesion molecule integrin $\beta 4$ on EC to elicit cell proliferation and migration [18]. HLA class I required integrin $\beta 4$ expression in order to cause phosphorylation of Src, Akt and ERK after crosslinking. Numerous studies have shown that ligation of integrin molecules on SMC also leads to a similar pattern of phosphorylation of FAK, ERK and Akt and subsequent proliferation and migration. In view of these considerations, it will be of interest to determine whether class I molecules and integrins cooperate to transduce signals in SMC.

These studies were designed to characterize the molecular and functional effects of anti-HLA class I antibodies in SMC using a cell culture model that allowed us to isolate the specific effects of anti-HLA antibodies on SMC in the absence of complement and immune cells. Therefore, the interpretation of these findings will require further validation in human cardiac transplants with evidence of transplant vasculopathy. This study did not rule out other alternative mechanisms that might contribute to aberrant SMC proliferation and migration, such as indirect mitogenic effect of cytokines and growth factors released from EC and SMC induced by class I ligation [49].

In summary, we show that exposure of SMC to anti-HLA class I Ab triggers proliferation and migration in a FAK-dependent manner. These mechanistic studies provide new insights to explain how HLA antibodies may contribute to the development of TV.

Acknowledgments

We thank Dr. Judith A. Berliner in Department of Pathology and Laboratory Medicine at UCLA for generously providing SMC for this research.

Funding: This work was supported by American Heart Association Western States Affiliation Postdoctoral Fellowship to F. L. and the National Institute of Allergy and Infectious Diseases grant RO1 AI 042819 (E.F.R.); NIH grant U01AI077821 (E.F.R.); the National Heart, Lung and Blood Institute grant RO1 HL 090995 (E.F.R.).

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Abbreviations

Ab	antibody
FAK	focal adhesion kinase
TV	transplant vasculopathy
SMC	smooth muscle cells
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
EC	endothelial cells
siRNA	Small interfering RNA
PI	proliferation index

Figure 1.

Ligation of class I molecules by mAb W6/32 increases SMC cell proliferation and migration. **A.** SMC (SMC1) were labeled with CFSE and stimulated with various concentrations of W6/32, isotype control mIgG, or 20% FBS for 48 h. Cell proliferation was measured by flow cytometry and analyzed using the Modfit LT software. The freshly CFSE-labeled SMC were used as the parental cells and their homogeneity was verified at the start of each experiment. The graphed data represent the ratio of the mean PI of the HLA Ab treated culture divided by the mean PI of non-treated culture (NT) $\pm$ SEM in 6 independent experiments. **B.** SMC (SMC2) were stimulated with mIgG (1.0 $\mu\text{g/ml}$), W6/32 (0.1 $\mu\text{g/ml}$ and 1.0 $\mu\text{g/ml}$) and 10% FBS for 24 h and their migration across an artificial wound was measured. The graph shows the mean percentage $\pm$ SEM of wound closure in 12 experiments. **C.** SMC (SMC2) were pretreated with mitomycin C at 10 $\mu\text{g/ml}$ for 2h to inhibit cell proliferation, stimulated with mIgG, mAbW6/32 or 10% FBS and assayed for their ability to migrate. The graph shows the mean percentage $\pm$ SEM of wound closure in 9 experiments. Differences for panels A-C were analyzed by one-way ANOVA followed by post hoc analysis (* $p < 0.05$).

Figure 2.

Decreased HLA class I expression prevents class I-induced cell proliferation and migration in SMC. **A.** The surface expression of HLA class I was analyzed by flow cytometry in SMC1 and SMC2 without siRNA or transfected with control or HLA class I heavy chain siRNA by using the pan-specific mAb W6/32 (SMC2) or the allele specific mAbs against HLA-A2 (SMC2) and HLA-A24/A32 (SMC1). Unstained cells are shown as a solid peak. **B.** SMC2 were transfected with 50, 100 and 200 nM of control siRNA or siRNA duplex against HLA class I heavy chain and analyzed by Western blotting with Ab against the HLA class I heavy chain (Clone EMR8-5) and β -actin that served as loading control. **C.** cells (SMC1) were transfected with control or the HLA class I siRNA, labeled with CFSE and stimulated with 1.0 µg/ml W6/32, human mAb HLA-A24/32 or IgG isotype controls for 48 h. Cell proliferation was measured by flow cytometry and analyzed using the Modfit LT software. The graphed data represent the ratio of the mean PI of the HLA Ab treated culture divided by the mean PI of the isotype control treated culture $\pm$ SEM in 6 independent experiments. **D.** Cells (SMC2) were transfected with control or HLA class I siRNA, stimulated with 1.0 µg/ml IgG isotype control, mAb W6/32 or mAb HLA-A2 for 24 h and their migration across an artificial wound was measured. The graph shows mean percentage of wound closure ($\pm$ SEM) of 9 experiments. Differences for panels C and D were analyzed by one-way ANOVA followed by post hoc analysis (* $p < 0.05$). The HLA genotypes of the SMC used in the experiments were SMC1 HLA-A*24, A*32; B*1501, B*39 and SMC2 HLA-A*02, A*02; B*1501, B*35.

Figure 3.

Decreased HLA class I expression attenuates HLA class I mediated protein phosphorylation in SMC. SMC2 (A) were treated with various concentrations of mAb W6/32 or isotype control mIgG for 15 min or (B) treated with mAb W6/32 at 1.0 $\mu\text{g/ml}$ for various times or (C) transfected with 100 nM of HLA class I heavy chain or control siRNA and stimulated with 1.0 $\mu\text{g/ml}$ mAb HLA-A2 for various times. Stimulated cells were lysed and immunoblotted with Ab against p-FAK, p-Akt and p-ERK1/2. The membrane was immunoblotted with β -actin to confirm equal loading. The densitometry results are expressed as the mean fold-change ($\pm$ SEM) in phosphorylation of the HLA class I treated SMC compared to treatment with isotype control IgG of 5 independent experiments. Differences for panels A-C were analyzed by one-way ANOVA followed by post hoc analysis (* $p < 0.05$).

Figure 4.

FAK is required for HLA class I-mediated protein phosphorylation in SMC. **A.** SMC2 were transfected with 100 nM of FAK siRNA. Transfected cells were stimulated with 1.0 μ g/ml mAb W6/32 for various times. SMC were lysed and immunoblotted with antibodies to p-FAK, p-Akt, p-ERK1/2, FAK, Akt, ERK1/2, vinculin and β -actin. The bands were quantitated by densitometry and normalized to β -actin. The densitometry results are expressed as the fold-change in phosphorylation of the HLA class I treated SMC compared to treatment with isotype control IgG (mean $\pm$ SEM) of 5 independent experiments. Differences in protein phosphorylation were analyzed by one-way ANOVA followed by post hoc analysis (* p <0.05). **B.** SMC2 were pretreated with 0.5 μ M latrunculin A or 1.0 μ M cytochalasin D for 30 min and then stimulated with 1.0 μ g/ml mAb W6/32 for different time points. Protein phosphorylation was immunoblotted and analyzed (3 independent experiments).

Figure 5.

Inhibition of FAK protein expression decreases cell proliferation and migration stimulated by class I ligation. **A.** SMC1 were transfected with control or FAK siRNA, labeled with CFSE and stimulated with mAb W6/32, or isotype control mIgG for 48 h. Cell proliferation was measured by flow cytometry and analyzed using the Modfit LT software. The graphed data represent the ratio of the mean PI of the HLA Ab treated culture divided by the mean PI of the isotype control treated culture $\pm$ SEM in 6 independent experiments **B.** SMC2 were transfected with control or FAK siRNA, stimulated with 1.0 μ g/ml mIgG or W6/32 for 24 h and migration across an artificial wound was measured. The graph shows mean percentage of wound closure ($\pm$ SEM) of 9 experiments. Differences for panels A and B were analyzed by one-way ANOVA followed by post hoc analysis (* $p < 0.05$)