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# αvβ3 Integrin Boosts the Innate Immune Response Elicited in Epithelial Cells through Plasma Membrane and Endosomal Toll-Like Receptors

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**We report that αvβ3 integrin strongly affects the innate immune response in epithelial cells. αvβ3 integrin greatly increased the response elicited via plasma membrane Toll-like receptors (TLRs) by herpes simplex virus or bacterial ligands. The endosomal TLR3, not the cytosolic sensor interferon gamma-inducible protein 16 (IFI16), was also boosted by αvβ3 integrin. The boosting was exerted specifically by αvβ3 integrin but not by αvβ6 or αvβ8 integrin. Current and previous work indicates that integrin-TLR cooperation occurs in epithelial and monocytic cells. The TLR response should be considered an integrin-TLR response.**

The Toll-like receptors (TLRs) constitute the first line of defense against invading pathogens (1). They include TLR1, TLR2, and TLR4 to TLR6, which are localized at the plasma membrane, and TLR3, TLR7, and TLR9, which are localized at endosomal membranes (1). TLR2 can be expressed as a homodimer or as a heterodimer with TLR1 or TLR6. The plasma membrane TLRs recognize pathogen-associated molecular patterns (PAMPs) present on the surfaces of bacteria (e.g., lipopolysaccharide [LPS], flagellin) or of viruses (e.g., virion envelope glycoproteins). The endosomal TLRs recognize viral or bacterial DNA or RNA.

Integrins are cell surface glycoproteins involved in cell-cell and cell-matrix interactions. They are composed of an α and a β subunit (2–4). They serve as receptors for several viruses, including some herpesviruses (5–10). Our laboratory investigated the role played by integrins in herpes simplex virus (HSV) entry and found that αvβ6 and αvβ8 integrins serve as interchangeable receptors for HSV entry into epithelial and neuronal cells (11). They bind the envelope glycoproteins gH and gL (gH/gL), a heterodimeric component of the fusion machinery, with high affinity (11). Their interaction with gH/gL promotes virion endocytosis and the displacement of gL from gH, most likely as part of the process of gH activation (12). An additional integrin involved in HSV entry is αvβ3, which serves two functions. It binds gH/gL at low affinity and routes HSV to lipid rafts and an acidic endosomal pathway of entry and thus serves as a routing factor (13). Importantly, it contributes to the innate immune response through a concerted action with TLR2 (14–16). In epithelial cell lines, including keratinocytic and neuronal cells, interferon alpha (IFN-α) and IFN-β, interleukin 2, and interleukin 10 are upregulated, and NF-κB is activated in response to HSV or to LPS (14). This response is strongly impaired in the absence of TLR2 or upon β3-integrin depletion. The HSV PAMP is gH/gL, which simultaneously binds αvβ3 integrin and TLR2 and thus cross-links the two receptors (14). The basis of the concerted αvβ3 integrin-TLR2 response rests in boosting by αvβ3 integrin of MYD88-dependent TLR2 signaling (16).

Here, we asked whether the concerted integrin-TLR response is a broader phenomenon that involves epithelial integrins other than αvβ3 (e.g., αvβ6 and αvβ8) or additional TLRs. To address this question, 293T cells and the HaCaT keratinocytes were depleted singly of β3, β6, or β8 integrin and transfected with plasma membrane or endosomal TLRs for overexpression. We report that (i) in contrast to the depletion of β3, the depletion of β6 or β8 integrin did not suppress the NF-κB response elicited by HSV virions or by specific TLR ligands; (ii) TLR4 and TLR5, as well as the endosomal TLR3, acted in concert with β3 integrin, but only in response to their specific non-HSV ligands; (iii) TLR2 was the sole plasma membrane TLR capable of responding to HSV; and (iv) the response of the cytoplasmic sensor IFN-γ-inducible protein 16 (IFI16) was not affected by β-integrin depletion. The results suggest that concerted integrin-TLR signaling is specific for β3 integrin, involves the plasma and endosomal TLRs, but not the cytosolic sensors, and boosts the innate response to a variety of PAMPs.

**αvβ3 integrin acts in concert with plasma membrane TLRs to boost NF-κB activation in epithelial cells.** To investigate whether αvβ3 integrin acts in concert with plasma membrane TLRs other than TLR2, we made use of a 293T-derivative cell line in which β3 integrin was stably silenced by means of a lentivirus encoding a short hairpin RNA (shRNA; herein called 293TshB3 cells). Depletion of β3 integrin was ascertained through a 70% reduction in both β3 mRNA and β3 protein compared to those in cells transduced with a control shRNA (14, 15). We measured the NF-κB activation in 293T and 293TshB3 cells transfected with the appropriate TLRs and exposed to HSV or bacterial PAMPs. TLR transfection was necessary, since 293T cells fail to express TLRs. The $gD^{-/-}$ mutant of HSV was employed because this virus attaches to the target cells and is capable of eliciting an immediate response to incoming virions (17, 18). However, it fails to enter cells, and therefore it does not elicit the cellular response depen-

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dent on newly synthesized viral proteins. The latter response is a function of the extent of virus which entered the cell, and it may be affected by a reduction in virus entry consequent to the integrin silencing. In addition, some of the newly synthesized proteins encoded by HSV, e.g., infected cell protein 0 (ICP0) and UL41, counteract the immune response and thus reduce the overall NF-κB response (19–24). 293T and 293Tshβ3 cells were transfected with plasmids expressing TLR2, TLR2 plus TLR1, TLR2 plus TLR6, TLR4 (the latter in the presence of CD14 and MD2, also known as lymphocyte antigen 96), or TLR5, together with plasmids encoding firefly luciferase (Luc) under the NF-κB promoter (NF-κB–luc) and *Renilla* (Ren) luciferase (ratio, 160:1) (14). Cells were then exposed to HSV $gD^{-/-}$ (20 PFU equivalents/cell), LPS (TLR2- and TLR4-transfected cells; herein called L2), the triacylated lipoprotein PAM-3 (TLR2/TLR1-transfected cells; herein called L2-1), the diacylated lipoprotein FSL-1 (TLR2/TLR6-transfected cells; herein called L2-6), or flagellin (TLR5-transfected cells; herein called L5). Luciferase activity was measured in cell lysates by means of the Dual-Glo luciferase reporter assay system (Promega). Figure 1A and B show that depletion of β3 integrin strongly inhibited NF-κB activation induced by either HSV $gD^{-/-}$ or the specific TLR ligands. Inhibition was also seen in 293T cells transfected with TLR4 or TLR5, but only after stimulation with bacterial ligands (Fig. 1C). This indicates that (i) β3 integrin cooperates with the plasma membrane TLRs independently of the nature of the PAMP, be it viral or bacterial; and (ii) HSV does not elicit the signaling activity of TLR4 or TLR5.

Previously, our laboratory identified gH/gL as the envelope component that interacts with TLR2 to trigger the immune response (14, 17). Here, we confirmed that a soluble form of HSV gH/gL ($gH_t$/gL) (25) was sufficient to stimulate the TLR2-dependent NF-κB response only when β3 integrin was present (Fig. 1D). As expected, we did not detect any NF-κB activation through TLR4 or TLR5 upon exposure to $gH_t$/gL, strengthening the conclusion that HSV is not recognized by the latter TLRs.

Finally, to assess whether there was a contribution of the single TLR1 or TLR6 to the HSV-induced response, we measured the NF-κB response in 293T cells transfected with TLR1 or TLR6 in the absence of TLR2. Figure 2A shows that neither TLR1 nor TLR6 elicited NF-κB activation upon exposure to HSV, implying that the presence of TLR2 was critical. To determine whether TLR2 acted as a homodimer or as a heterodimer, 293T cells were transfected with the same quantity of TLR2 (4 ng) alone or in combination with TLR1 (4 ng) or TLR6 (4 ng). These conditions led to a similar extent of NF-κB activation, irrespective of whether TLR2/TLR2 homodimers or TLR1/TLR2 or TLR6/TLR2 heterodimers could be formed (Fig. 2B). The results suggest that TLR2 is necessary and sufficient for recognition and response to HSV at the plasma membrane level.

**β3 integrin acts in concert with the endosomal TLR3 to induce IFN-β production but has no effect on the cytosolic sensor IFI16.** To address the question of whether β3 integrin acts in concert with the endosomal TLRs, we measured IFN-β production in response to TLR3 induction. Since we failed to detect a significant and consistent IFN-β response to HSV in 293T cells transfected with TLR3, we employed HaCaT keratinocytes, which are good producers of IFN-β *in vitro* (15). HaCaT and HaCaTshβ3 (15) were transfected with TLR3, and 24 h later, they were stimulated with poly(I·C) (herein called L3) for an additional 24 h. IFN-β released in the medium was quantified by means of a commercial enzyme-linked immunosorbent assay (ELISA) kit (PBL Assay Science). Figure 3A shows that the absence of β3 integrin dramatically reduced the TLR3-dependent production of IFN-β in HaCaT cells. We could not measure the IFN-β response

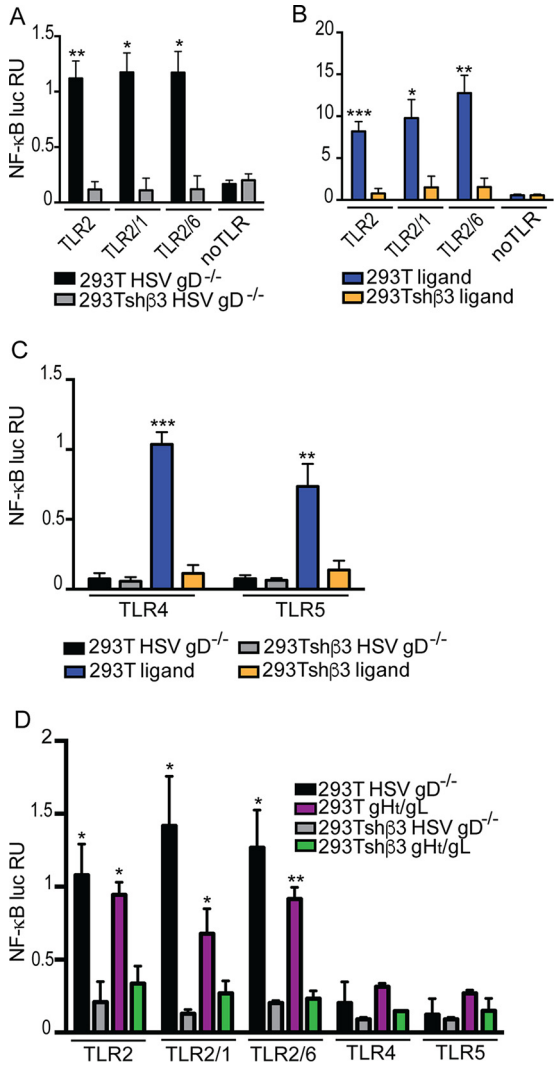


**FIG 1** NF-κB response in wild-type (wt) and β3-integrin-silenced 293T cells upon stimulation of plasma membrane TLRs. wt-293T (293T) and β3-integrin-silenced 293T (293Tshβ3) cells were transfected with a mixture of plasmids encoding firefly luciferase under the NF-κB promoter (Luc), *Renilla* luciferase (Ren; ratio, 160:1), and the indicated TLR(s) (50 ng/well for each TLR in 24-well plates) by means of Lipofectamine 2000 (Life Technologies) as described previously (14). The transfected cells were maintained in culture for 2 days in preexhausted 293T medium (14) and then exposed to HSV $gD^{-/-}$ (20 PFU equivalents/cell) (A, C, and D) for 6 h or to the ligand specific to each TLR (B and C). The ligands (Invivogen) (L2 for TLR2, L2-1 for TLR2/1, L2-6 for TLR2/6, L2 for TLR4, and L5 for TLR5, as indicated in the text) were added at 100 ng/ml for 6 h. For panel D, the transfected cells were exposed to soluble glycoprotein $gH_t$/gL (100 μM) for 6 h. NF-κB activity was measured by means of the Dual-Glo luciferase reporter assay system (Promega), as previously detailed (14), and expressed as relative units (NF-κB luc RU). For each sample, the Luc/Ren ratio was determined. The Luc/Ren value obtained with the transfected, untreated 293T cells was considered the NF-κB basal activity value and was subtracted. Each value represents the average from three independent experiments ± the standard errror of the mean (SEM). Data were analyzed with the unpaired *t* test, comparing results for 293T and 293Tshβ3 cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

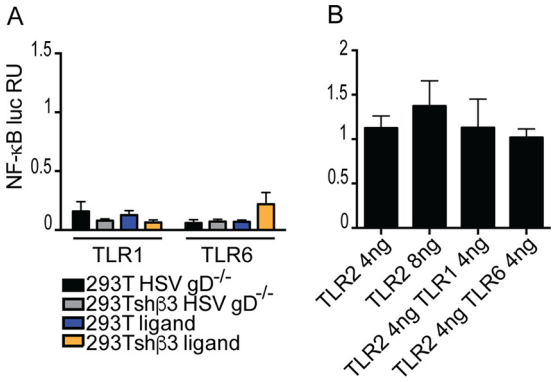


**FIG 2** Effect of β3-integrin silencing on the signaling activity of plasma membrane TLRs other than TLR2. (A) 293T and 293Tshβ3 cells were transfected with TLR1 or TLR6 (50 ng/well for each TLR in 24-well plates) plus Luc and Ren plasmids (as detailed in the legend to Fig. 1). The transfected cells were maintained for 2 days in preexhausted 293T medium and then exposed to HSV $gD^{-/-}$ (20 PFU equivalents/cell) or the cognate TLR ligand (L2-6 for TLR6 or L2-1 for TLR1) at a concentration of 100 ng/ml for 6 h. (B) 293T cells were transfected with TLR2 or mixtures of TLR2 and TLR1 or TLR2 and TLR6 at the indicated DNA quantities/well (in 24-well plates) plus Luc and Ren plasmids. Cultures were maintained in preexhausted medium for 2 days and then exposed to HSV $gD^{-/-}$ for 6 h, as specified in the legend to panel A. NF-κB activity was measured and expressed as detailed in the legend to Fig. 1. Each value represents the average from three independent experiments ± SEM.

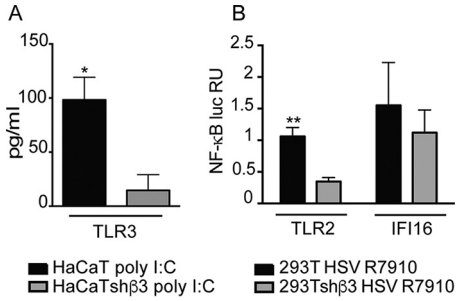


**FIG 3** Effect of β3-integrin depletion on IFN-β production and NF-κB activation upon stimulation of the endosomal TLR3 or of the cytosolic sensor IFI16. (A) HaCaT and HaCaTshβ3 cells (15) were transfected with TLR3 (150 ng/well in 12-well plates); 24 h later cells were exposed to L3 poly(I·C) (2 μg/well in 12-well plates; Sigma) for an additional 24 h. The culture media were harvested and centrifuged to remove cells and debris. The secreted IFN-β (pg/ml) was quantified with a commercial ELISA kit (PBL Assay Science). Each column represents the average from three independent experiments ± SEM. Data were analyzed with the unpaired *t* test, comparing results for HaCaT and HaCaTshβ3 cells. *, $P < 0.05$. (B) 293T and 293Tshβ3 cells were transfected with plasmids encoding TLR2 or IFI16 (50 ng/well of each in 24-well plates), plus Luc and Ren plasmids, as detailed in the legend to Fig. 1. After 2 days, cells were infected with HSV R7910 (20 PFU/cell) and harvested 6 h later. NF-κB activity was measured and expressed as detailed in the legend to Fig. 1. Each value represents the average from three independent experiments ± SEM. Data were analyzed with the unpaired *t* test, comparing results for 293T and 293Tshβ3 cells. **, $P < 0.01$.

upon HSV induction in these cells, since they are positive for TLR2 (15), and the contribution of TLR3 could not be separated from that of TLR2 (15). Importantly, TLR3 is activated in response to infection with HSV-1 and Kaposi's sarcoma-associated herpesvirus (26–28), and mutations are associated with a high incidence of HSV encephalitis (26, 27). Thus, integrin cooperation with TLR3 may be an important pathogenic factor in herpesvirus infections.

Next, we asked whether the boosting activity by β3 integrin was limited to the membrane-bound TLRs or also included the cytosolic sensors. As a prototype, we chose IFI16, an intracellular protein that senses viral DNA in the nucleus as well as in the cytosol of infected cells, including cells infected with herpesviruses, which promote IFI16 translocation to the cytosol (29–34). IFI16 is known to induce NF-κB activation, also in response to HSV-1 (32, 35). We transfected 293T and 293Tshβ3 cells with plasmids encoding IFI16 or TLR2, together with the luciferase plasmids, as detailed above. Cells were then infected with HSV R7910 (20 PFU/cell). This recombinant carries a partial deletion in the gene encoding the ICP0 protein which counteracts the NF-κB and IFN responses (36). $gD^{-/-}$ HSV was not used in these experiments, since IFI16 is activated only upon virus entry. Figure 3B shows that β3-integrin depletion did not significantly modify the NF-κB response elicited via IFI16, in contrast to the response elicited via TLR2. Altogether, these results suggest that cooperation between β3 integrin and pathogen recognition receptors involves the membrane-bound TLRs but not the cytosolic sensor IFI16.

**αvβ6 and αvβ8 integrins do not boost the TLR-dependent response in epithelial cells.** We asked whether integrins other than β3 cooperate with TLRs to trigger NF-κB signaling in epithelial cells. We focused on αvβ6 and αvβ8 integrins, which serve as coreceptors for HSV entry and interact with HSV gH/gL at high affinity (11). These properties are in contrast to those of αvβ3 integrin, which does not serve as a coreceptor for HSV entry and interacts with gH/gL at lower affinity (14). 293T and 293Tshβ3 cells were transfected with small interfering RNA (siRNA) specific to β6 or β8 integrin (Dharmacon) (11), together with TLRs and luciferase plasmids, as above. Cells were exposed to $gD^{-/-}$ HSV or to specific TLR ligands. As shown in Fig. 4A and B, depletion of β6 or β8 integrin did not lead to a significant inhibition in the NF-κB response upon exposure to HSV $gD^{-/-}$ (Fig. 4A) or to TLR ligands (Fig. 4B, L2 for TLR2, L2-1 for TLR2/1, L2-6 for TLR2/6), implying that αvβ6 and αvβ8 integrins do not contribute to the innate response to incoming HSV virions. Previous data from our laboratory demonstrated that β6 and β8 siRNA did not have any off-target effects (11). Transfection of an unrelated control siRNA (gray bars in Fig. 4A and B) did not have any significant effect on the NF-κB response to HSV or to TLR ligands. Silencing of β6 (Fig. 4C) and β8 integrins (Fig. 4D) ranged between 60 and 70%, as determined by quantitative real-time PCR (Applied Biosystems).

To confirm the above data on the lack of effect of β6 and β8 integrins, we carried out two additional series of experiments. To better analyze the role of αvβ6 integrin, 293T and 293Tshβ3 cells transfected with TLR2 and luciferase plasmids were incubated with a function-blocking monoclonal antibody (MAb) to αvβ6 integrin prior to exposure to $gD^{-/-}$ HSV virions. Figure 4E shows that the NF-κB response was elicited irrespectively of the presence of the neutralizing antibody. The antibody alone did not elicit any response. With respect to αvβ8 integrin, we made use of a recombinant carrying a form of gH mutated in the RGD motif, named $gH_{ADA}$ (11, 25). This virus maintains the ability to infect through αvβ8 integrin (RGD independent) but does not infect through αvβ6 integrin (RGD dependent). Since our control was HSV $gD^{-/-}$ throughout the experiments, exposure to the $gH_{ADA}$ mutant was carried out in the presence of the neutralizing MAb to gD, HD1 (37). Figure 4F shows that $TLR2^{+}$ 293T cells exposed to HSV-$gH_{ADA}$ exhibited a reduced NF-κB response compared to

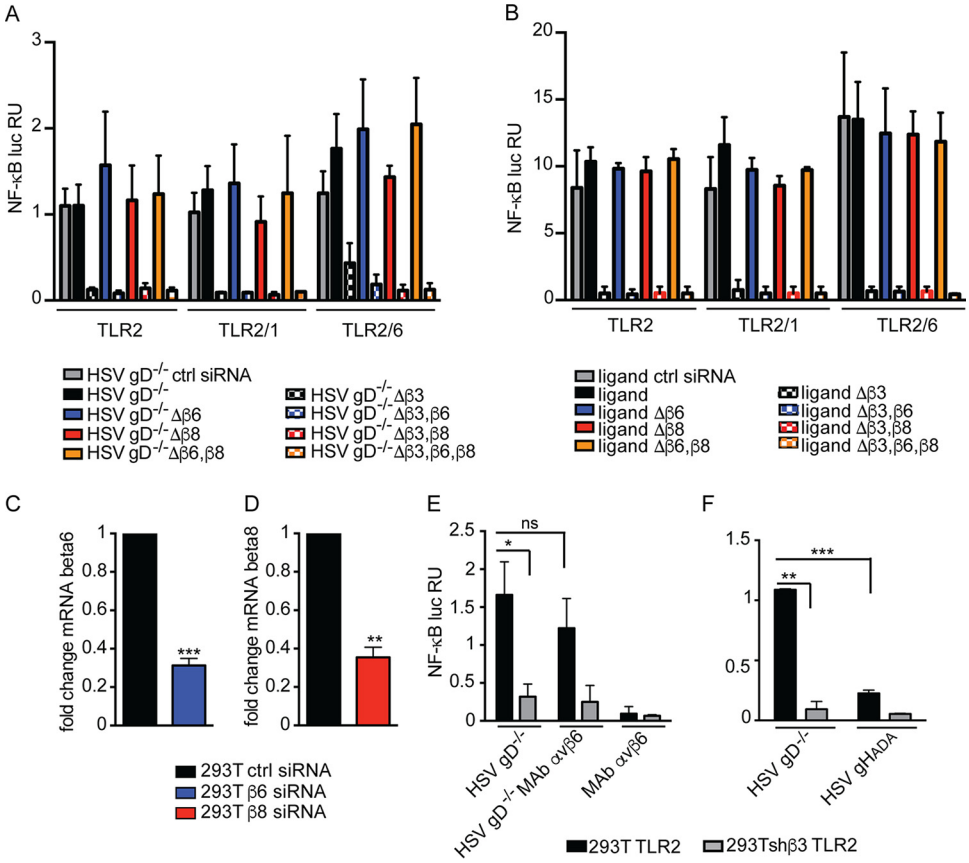


**FIG 4** Analysis of αvβ6 or αvβ8 integrin contribution to NF-κB activity elicited by plasma membrane TLRs. (A and B) 293T and 293Tshβ3 (Δβ3) cells were depleted of β6 integrin (Δβ6) or of β8 integrin (Δβ8) by means of specific siRNAs (10 uM; Dharmacon), as described in reference 11, or transfected with an unrelated control siRNA (ctrl siRNA). At the same time, cells were transfected with the indicated TLR(s) (50 ng/well for each TLR in 24-well plates) plus Luc and Ren plasmids, as detailed in the legend to Fig. 1. After 2 days, cells were exposed to HSV $gD^{-/-}$ (20 PFU equivalents/cell) (A) or the cognate TLR ligands (Invivogen) at a concentration of 100 ng/ml (B) for 6 h (L2 for TLR2, L2-1 for TLR2/1, or L2-6 for TLR2/6). Each value represents the average ± SEM from at least three independent experiments. (C and D) 293T cells were transfected as for panels A and B and silenced for β6 integrin (β6 siRNA) or β8 integrin (β8 siRNA) or with an unrelated control siRNA (ctrl siRNA). After 2 days, RNA was extracted and cDNA was synthesized with a high-capacity cDNA reverse-transcription kit (Applied Biosystems). Quantitative real-time PCR was performed by means of TaqMan primers and probes specific for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β6 integrin or β8 integrin. The fold change in mRNA expression was calculated by means of the comparative threshold cycle ($\Delta\Delta C_T$) method. Each value represents the average ± SEM from at least three independent experiments. Data were analyzed with the unpaired *t* test. **, $P < 0.01$; ***, $P < 0.001$. (E) 293T and 293Tshβ3 cells were transfected with TLR2 plus Luc and Ren plasmids, as detailed in the legend to Fig. 1. After 2 days, cells were exposed to a function-blocking monoclonal antibody (MAb) to αvβ6 (10 μg/ml) (Millipore MAB2077Z) for 1 h at 37°C. Cells were then exposed to HSV $gD^{-/-}$ as above. NF-κB activity was measured after an additional 6 h. (F) 293T and 293Tshβ3 cells were transfected with TLR2 plus Luc and Ren plasmids as above. After 2 days, cells were exposed either to HSV $gD^{-/-}$ or to HSV $gH_{ADA}$. The latter virus was incubated with the neutralizing MAb to gD HD1 (1.5 μg/ml) (37) for 1 h at 37°C prior to cell exposure to the virus. As MAb HD1 blocks HSV entry, this treatment mirrors infection with HSV $gD^{-/-}$. After 6 h, NF-κB activity was measured and expressed as detailed in the legend to Fig. 1. Each column represents the average of the results of three independent experiments ± SEM. Data were analyzed with unpaired *t* test, comparing results for the groups, as indicated in the graphs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cells exposed to HSV $gD^{-/-}$, indicating that αvβ8 integrin does not play a major role in eliciting NF-κB activation. Altogether, these data rule out that αvβ6 and αvβ8 integrins boost TLR2-dependent NF-κB activation.

**Conclusions.** Previous investigations highlighted that αvβ3 integrin positively regulates TLR2 signaling in epithelial cell lines (14–17). Concerted αvβ3 integrin-TLR2 signaling plays a critical role in eliciting a robust type I IFN response, NF-κB activation, and secretion of a specific set of cytokines, and it is exerted through an increase in MyD88 recruitment to TLR2. Here, we asked how broad is the integrin regulation of TLR signaling in epithelial cells. We report that the response of all plasma membrane TLRs was greatly boosted by αvβ3 integrin. TLR2 and its heterodimers 2/1 and 2/6 responded to HSV or to bacterial PAMPs, whereas TLR4 and TLR5 responded only to bacterial PAMPs. The signaling of poly(I·C)-activated TLR3, an example of endosomal TLRs, was also boosted by αvβ3 integrin. In contrast, the cytosolic sensor IFI16 was not affected by β3 integrin depletion, indicating that αvβ3 integrin specifically boosts the membrane-bound sensors. On the integrin side, we analyzed αvβ6 and αvβ8 integrins, known to interact with HSV. Neither one played a significant role in regulating TLR2 signaling. Altogether, in epithelial cells, the cooperation of integrin with TLRs appears to be a broader phenomenon than detected so far, in that it involves both the plasma membrane and the endosomal TLRs. In turn, the integrin involvement is rather specific and involves the αvβ3 and not the αvβ6 and αvβ8 integrins. It is worth noting that the integrin-TLR cooperative response provides a significant contribu-

tion to the overall innate response in epithelial cells, since the depletion of β3 integrin or the absence of TLR2 resulted in a strong reduction in type I IFN production and NF-κB activation (14).

Previously, the integrin regulation of TLRs has been documented in nonepithelial cells (38). Thus, αMβ2 integrin (CD11b/CD18) regulates TLR signaling in monocytes/macrophages and in dendritic cells. The regulation can be negative or positive, depending on the cell type. CD11b positively regulates TLR4 signaling in mouse dendritic cells but not in macrophages (39). High-avidity ligation of β2 integrin in macrophages inhibited type I IFN receptor and TLR signaling (40). CD11b deficiency in mice enhanced TLR-mediated responses in macrophages (41). β3 integrin and α1β3 integrin cooperate with TLR2 and TLR2/1, respectively, to sense bacterial lipopeptides in human monocytes and macrophages and HEK293 cells (42, 43).

In summary, integrin-TLR cooperation occurs in both epithelial and monocytic cells. We propose that what was previously thought to be a TLR-elicited response is instead a response elicited in concert with integrins and that integrins might be targets for the control of the innate immune response to different pathogens.

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C.C., T.G., and G.C.-F. conceived the experiments; C.C. carried out the experiments; C.C., T.G., and G.C.-F. analyzed the results; and C.C. and G.C.-F. wrote the manuscript.

The authors declare no competing interests.

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