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# Direct binding to integrins and loss of disulfide linkage in interleukin-1 $\beta$ (IL-1 $\beta$ ) are involved in the agonistic action of IL-1 $\beta$

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There is a strong link between integrins and interleukin-1 $\beta$  (IL-1 $\beta$ ), but the specifics of the role of integrins in IL-1 $\beta$  signaling are unclear. We describe that IL-1 $\beta$  specifically bound to integrins  $\alpha\beta3$  and  $\alpha5\beta1$ . The E128K mutation in the IL1R-binding site enhanced integrin binding. We studied whether direct integrin binding is involved in IL-1 $\beta$  signaling. We compared sequences of IL-1 $\beta$  and IL-1 receptor antagonist (IL1RN), which is an IL-1 $\beta$  homologue but has no agonistic activity. Several surface-exposed Lys residues are present in IL-1 $\beta$ , but not in IL1RN. A disulfide linkage is present in IL1RN, but is not in IL-1 $\beta$  because of natural C117F mutation. Substitution of the Lys residues to Glu markedly reduced integrin binding of E128K IL-1 $\beta$ , suggesting that the Lys residues mediate integrin binding. The Lys mutations reduced, but did not completely abrogate, agonistic action of IL-1 $\beta$ . We studied whether the disulfide linkage plays a role in agonistic action of IL-1 $\beta$ . Reintroduction of the disulfide linkage by the F117C mutation did not affect agonistic activity of WT IL-1 $\beta$ , but effectively reduced the remaining agonistic activity of the Lys mutants. Also, deletion of the disulfide linkage in IL1RN by the C116F mutation did not make it agonistic. We propose that the direct binding to IL-1 $\beta$  to integrins is primarily important for agonistic IL-1 $\beta$  signaling, and that the disulfide linkage indirectly affects signaling by blocking conformational changes induced by weak integrin binding to the Lys mutants. The integrin-IL-1 $\beta$  interaction is a potential target for drug discovery.

The interleukin-1 (IL-1)<sup>2</sup> family is a group of 11 cytokines, which induces a complex network of pro-inflammatory cyto-

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<sup>2</sup> The abbreviations used are: IL-1, interleukin-1; IL1RN, IL-1 receptor antagonist; IL1R, IL-1 receptor; IGF1, insulin-like growth factor 1; SPR, surface plasmon resonance; Ni-NTA, nickel-nitrilotriacetic acid.

kines and, via expression of integrins on leukocytes and endothelial cells, regulates and initiates inflammatory responses (1). IL-1 $\beta$  is a key regulator of innate and adaptive immune systems. It plays a critical role in inflammatory diseases and is a major therapeutic target. It has a natural antagonist IL-1 receptor antagonist (IL1RN). IL-1 $\beta$  and IL1RN bind to IL-1 receptor (IL1R) and activate signaling via MyD88 adaptor. IL1RN regulates IL-1 $\beta$  pro-inflammatory activity by competing with IL-1 $\beta$  for binding sites of the receptor (1).

Integrins are a family of cell adhesion receptors that recognize extracellular matrix ligands and cell-surface ligands (2). They are transmembrane  $\alpha\beta$  heterodimers, and at least 18  $\alpha$  and 8  $\beta$  subunits are known (3). Integrins are involved in signal transduction upon ligand binding and their functions are in turn regulated by signals from within the cell (3). Cross-talk between integrins and cytokine receptors is an important signaling mechanism during normal development and pathological processes (4). We have reported that several cytokines including FGF1, insulin-like growth factor 1 (IGF1), neuregulin-1, and fractalkine (5–12) directly bind to integrins and generate a ternary complex (integrin-cytokine-cytokine receptor), and this process is critical for cytokine signaling.

Previous studies suggest the potential role of integrins in IL-1 $\beta$  pro-inflammatory action. IL-1 $\beta$  causes acute lung injury via integrin  $\alpha\beta5$  and  $\alpha\beta6$ -dependent mechanisms (13). Antibodies against  $\alpha4$ ,  $\alpha5$ ,  $\alpha v$ , and  $\beta1$  integrins inhibit IL-1 $\beta$ -stimulated invasion of articular cartilage by rheumatoid synovial fibroblasts (14). Multiple  $\beta1$  integrins mediate IL-1 $\beta$ -induced human airway smooth muscle cytokine release (15). Antibodies to  $\alpha6$  integrin inhibit IL-1 $\beta$ -induced neutrophil transmigration *in vivo* (16). In addition, IL-1 $\beta$  is abundant at tumor sites, where it may affect the process of carcinogenesis, tumor growth, and invasiveness (17). However, the specifics of the role of integrins in IL-1 $\beta$  in its agonistic action are unclear.

In the present study, we studied if integrins are directly involved in the agonistic action of IL-1 $\beta$ . IL-1 $\beta$  specifically bound to integrins. We identified several Lys residues that are involved in integrin binding. These Lys residues are not present in IL1RN. Mutating the Lys residues only partially reduced the agonistic action of IL-1 $\beta$ . A disulfide linkage is present in IL1RN, but not in IL-1 $\beta$ . Reintroduction of the missing disulfide linkage into IL-1 $\beta$  did not affect its agonistic action. Rein-

## Integrins in IL-1 $\beta$ signaling

roduction of the disulfide linkage into IL-1 $\beta$  together with Lys mutations markedly reduced the agonistic action of IL-1 $\beta$ . Thus, our results suggest the direct integrin binding plays a major role in the agonistic action of IL-1 $\beta$ .

### Results

#### IL-1 $\beta$ binds to soluble and cell-surface integrins

We studied whether IL-1 $\beta$  binds to purified soluble integrin  $\alpha\text{v}\beta\text{3}$  in ELISA-type binding assays in the presence of  $\text{Mn}^{2+}$  to fully activate  $\alpha\text{v}\beta\text{3}$ . Soluble  $\alpha\text{v}\beta\text{3}$  bound to immobilized IL-1 $\beta$  in a dose-dependent manner (Fig. 1*a*), suggesting that IL-1 $\beta$  directly binds to integrin  $\alpha\text{v}\beta\text{3}$ . We performed surface plasmon resonance (SPR) studies, in which soluble  $\alpha\text{v}\beta\text{3}$  was immobilized to sensor chip and IL-1 $\beta$  was in the solution phase (Fig. 1*b*). The data show that IL-1 $\beta$  binds to  $\alpha\text{v}\beta\text{3}$  at  $K_D = 4.28 \times 10^{-7} \text{ M}$  ( $k_a = 5.13 \times 10^{-3} \text{ mol}^{-1}\text{s}^{-1}$ ,  $k_d = 5.13 \times 10^{-3} \text{ s}^{-1}$ ) in the presence of 1 mM  $\text{Mn}^{2+}$  (Fig. 1*b*).

It has been reported that Chinese hamster ovary (CHO) cells express IL1R at very low levels (18). To study if IL-1 $\beta$  binds to CHO cells through IL1R, we incubated CHO cells with FITC-labeled IL-1 $\beta$  in the presence of 1 mM EDTA to reduce contribution of integrins and analyzed the binding in flow cytometry. We found that IL-1 $\beta$  bound to MCF7 breast cancer cells as positive controls, but did not show detectable binding to parent CHO cells (Fig. 1*c*). We detected weak binding of IL-1 $\beta$  to CHO cells that have been transfected with human IL1R (data not shown). These findings suggest that the contribution of endogenous IL1R in CHO cells to IL-1 $\beta$  binding is very little if any.

We studied whether IL-1 $\beta$  binds to integrins on the cell surface in adhesion assays using CHO and  $\beta\text{3}$ -CHO cells. Under physiological cation conditions (in DMEM, in which integrins are not active because of  $>1 \text{ mM} [\text{Ca}^{2+}]$ ), IL-1 $\beta$  did not support adhesion of CHO (Fig. 1*d*) or  $\beta\text{3}$ -CHO cells well (Fig. 1*e*). We found that WT IL-1 $\beta$  supported adhesion of parent CHO cells or  $\beta\text{3}$ -CHO cells in 1 mM  $\text{Mg}^{2+}$ , in which integrins are activated (Fig. 1, *d* and *e*). Cyclic RGDfV, a specific inhibitor to  $\alpha\text{v}\beta\text{3}$  (19), suppressed adhesion of  $\beta\text{3}$ -CHO cells to IL-1 $\beta$  (Fig. 1*e*), suggesting that IL-1 $\beta$  specifically binds to  $\alpha\text{v}\beta\text{3}$  when it is activated. These results suggest that IL-1 $\beta$  specifically binds to activated integrins, but does not bind to integrins in physiological cation conditions. We used DMEM in integrin binding to mimic physiological cation conditions throughout the present study.

#### The E105K and E128K mutations in IL1R-binding site in IL-1 $\beta$ enhance integrin binding

It has been reported that the E105K and E128K mutations in the IL1R-binding site of IL-1 $\beta$  suppress the IL-1 $\beta$  function (20). We discovered that the E105K and E128K mutations enhanced adhesion of  $\beta\text{3}$ -CHO cells (Fig. 2*a*). In SPR study using soluble  $\alpha\text{v}\beta\text{3}$  that was immobilized to the sensor chip, E128K in the solution phase showed enhanced affinity to  $\alpha\text{v}\beta\text{3}$   $K_D = 2.41 \times 10^{-8} \text{ M}$  ( $k_a = 4.93 \times 10^{-4} \text{ mol}^{-1}\text{s}^{-1}$ ,  $k_d = 1.19 \times 10^{-3} \text{ s}^{-1}$ ) (Fig. 2*b*). These findings indicate that  $K_D$  to E128K is an order of magnitude lower than that of WT IL-1 $\beta$ . CHO cells adhered to E128K in DMEM (Fig. 2*c*). CHO cells do not express  $\alpha\text{v}\beta\text{3}$ , suggesting that other integrins in CHO cells are also involved in IL-1 $\beta$  binding. We studied which integrins in CHO cells are

involved in E128K binding. The  $\alpha\text{5}\beta\text{1}$ -deficient B2 variant of CHO cells (21) showed little or no binding to E128K (Fig. 2*d*), suggesting that integrin  $\alpha\text{5}\beta\text{1}$  is involved in the adhesion of CHO cells to E128K and that both  $\alpha\text{5}\beta\text{1}$  and  $\alpha\text{v}\beta\text{3}$  are involved in adhesion of  $\beta\text{3}$ -CHO cells to E128K IL-1 $\beta$ . It is unclear why E105K and E128K IL-1 $\beta$  mutants show enhanced integrin binding, because these mutations are located within the IL1R-binding site of IL-1 $\beta$ , we suspect that IL-1R binding induces integrin binding because of conformational changes and that the E105K and E128K mutants mimic IL1R-bound form.

It is still possible that low-level IL1R contributes to binding to E128K to CHO or  $\beta\text{3}$ -CHO cells. IL1RN competes with IL-1 $\beta$  for binding to IL1R and block IL-1 $\beta$  signaling. We studied if IL1RN can affect the adhesion of CHO and  $\beta\text{3}$ -CHO cells to E128K. We found that IL1RN did not affect adhesion of CHO and  $\beta\text{3}$ -CHO cells to E128K (Fig. 2*e*). As controls, we stably expressed IL1R in CHO cells or  $\beta\text{3}$ -CHO cells (designated IL1R-CHO and IL1R- $\beta\text{3}$ -CHO cells, respectively). IL1RN dose-dependently reduced the adhesion of IL1R-CHO cells or IL1R- $\beta\text{3}$ -CHO cells (Fig. 2*e*). These findings suggest that integrins contribute to the binding of E128K to CHO and  $\beta\text{3}$ -CHO cells.

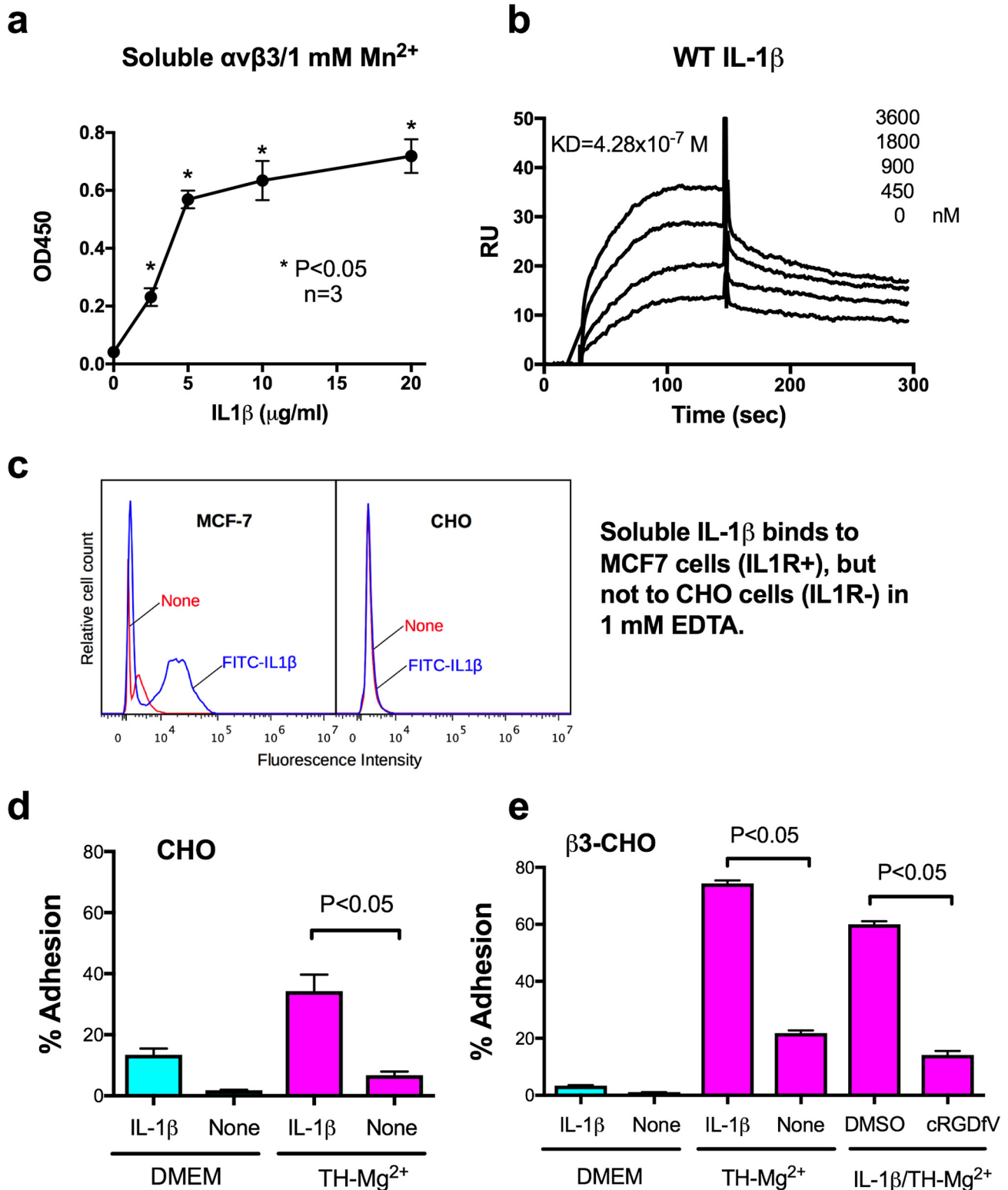
#### Critical Lys residues of IL-1 $\beta$ for integrin binding are exposed on the surface of IL-1 $\beta$ -IL1R signaling complex

We studied which amino acid residues are critical for integrin binding using E128K that binds to integrins well in cell adhesion assays in DMEM. Our previous studies found that Lys or Arg residues in integrin ligands play a critical role in integrin binding (5, 11, 12, 22). We found that the Lys residues at positions 55, 63, 65, 74, and 88 are present in IL-1 $\beta$  that is agonistic, but are not present in IL1RN that is not agonistic (Fig. 3*a*). Interestingly, these Lys residues are exposed to the surface in the IL1R/IL-1 $\beta$  complex (Fig. 3*b*). We thus hypothesized that these Lys residues are involved in integrin binding in IL-1 $\beta$ , and the loss of the Lys residues and thereby the loss of integrin binding are related to the loss of agonistic action in IL1RN. We thus tested if the Lys residues are involved in integrin binding of E128K by mutating them to Glu individually or in combination. We found that mutating these Lys residues effectively suppressed the binding of E128K to  $\beta\text{3}$ -CHO or CHO cells (Fig. 3, *c* and *d*). These results suggest that these Lys residues play a critical role in IL-1 $\beta$  binding to integrins  $\alpha\text{v}\beta\text{3}$  and  $\alpha\text{5}\beta\text{1}$ .

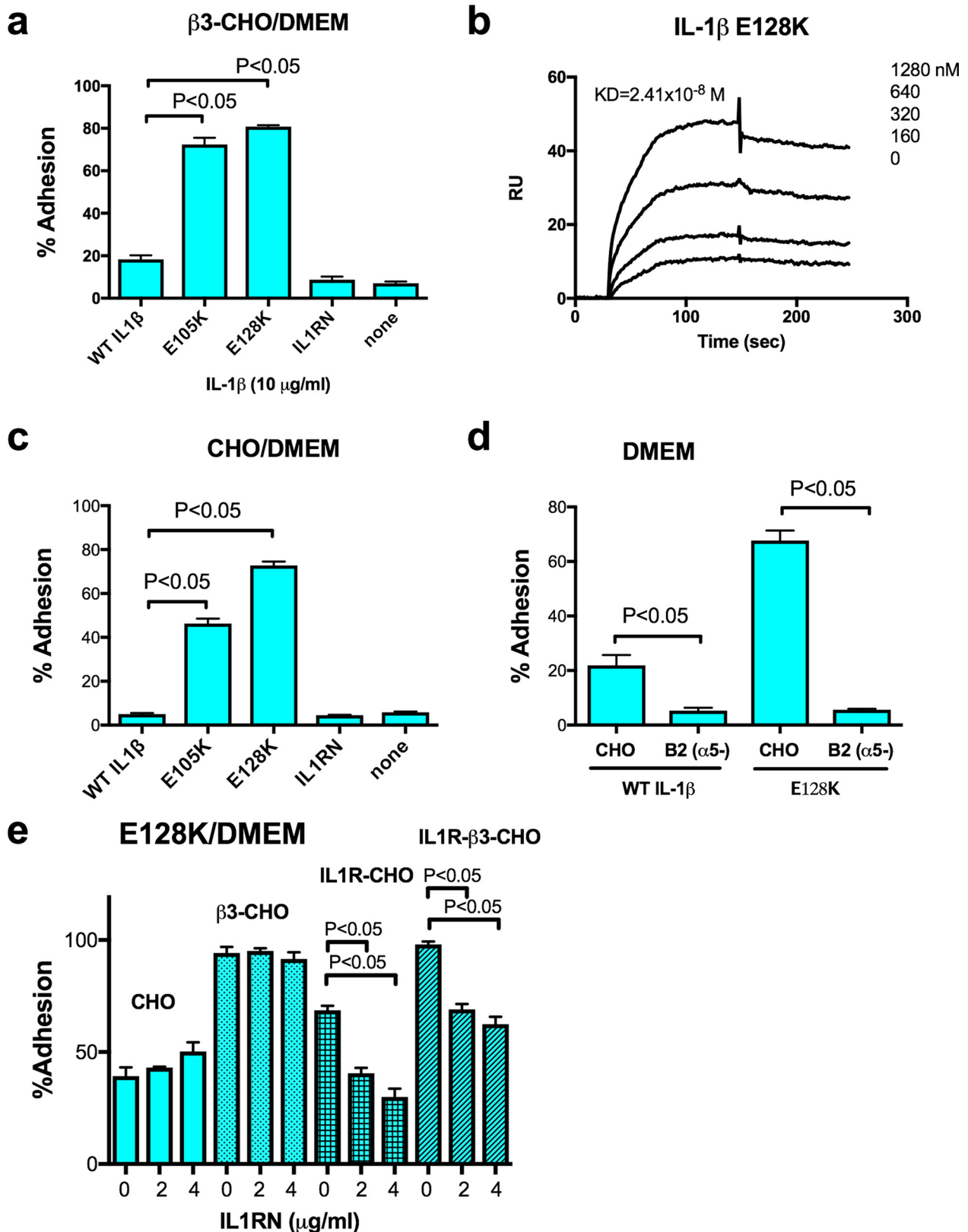
#### Role of integrin binding and the disulfide linkage in agonistic action of IL-1 $\beta$

IL1R is expressed in many cancer cell types (<http://www.proteinatlas.org/ENSG00000115594-IL1R1/pathology>)<sup>3</sup> (27) and may affect the process of carcinogenesis, tumor growth, and invasiveness (17). MCF7 breast cancer cells express IL1R and induce robust activation of NF- $\kappa\text{B}$  following IL-1 $\beta$  stimulation (23). We determined NF- $\kappa\text{B}$  activation using MCF7 cells that stably express NF- $\kappa\text{B}$  reporter gene as described (24). WT IL-1 $\beta$  induced robust NF- $\kappa\text{B}$  activation (Fig. 4*a*). IL1RN as a negative control did not induce NF- $\kappa\text{B}$  activation (Fig. 4, *b* and *c*). We used several IL-1 $\beta$  mutants in which Lys residues at posi-

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**Figure 1. Specific binding of IL-1 $\beta$  to integrin  $\alpha\beta 3$  in an activation-dependent manner is shown.** We studied if IL-1 $\beta$  binds to integrin  $\alpha\beta 3$ . *a*, IL-1 $\beta$  binds to soluble integrin  $\alpha\beta 3$  in the presence of 1 mM Mn $^{2+}$ . Wells of 96-well microtiter plate were coated with IL-1 $\beta$  and remaining protein-binding sites were blocked with BSA. Soluble  $\alpha\beta 3$  in HEPES-Tyrode's buffer + 1 mM Mn $^{2+}$  was added to wells and incubated. Bound  $\alpha\beta 3$  was determined using anti- $\beta 3$  mAb. The data are shown as means  $\pm$  S.E. of triplicate experiments. *b*, IL-1 $\beta$  binds to  $\alpha\beta 3$  in surface plasmon resonance study. SPR study of the interaction between  $\alpha\beta 3$  and WT IL-1 $\beta$ . Recombinant soluble  $\alpha\beta 3$  was immobilized to a sensor chip and IL-1 $\beta$  is in a solution phase. Mg $^{2+}$  (1 mM) was included in the binding buffer. *c*, FITC-IL-1 $\beta$  does not bind to CHO cells (IL1R-negative), but binds to MCF7 cells (IL1R-positive). CHO cells or MCF7 cells were incubated with FITC-labeled IL-1 $\beta$  (1  $\mu$ g/ml) in PBS/1 mM EDTA for 1 h at 4  $^{\circ}$ C, and bound FITC was measured in flow cytometry. *d* and *e*, binding of IL-1 $\beta$  to cell-surface  $\alpha\beta 3$ . Parent CHO cells (*d*) and  $\beta 3$ -CHO cells (*e*). Integrins are activated in the presence of Mg $^{2+}$  (HEPES-Tyrode's buffer + 1 mM Mg $^{2+}$ , TH-Mg $^{2+}$ ), but not in DMEM, which contains > 1 mM Ca $^{2+}$ . Wells of 96-well microtiter plate were coated with IL-1 $\beta$  (10  $\mu$ g/ml), and incubated with parent IL1R-negative CHO cells or CHO cells that express recombinant  $\alpha\beta 3$  ( $\beta 3$ -CHO cells). Bound cells were quantified using endogenous phosphatase activity. The data are shown as means  $\pm$  S.E. of triplicate experiments.

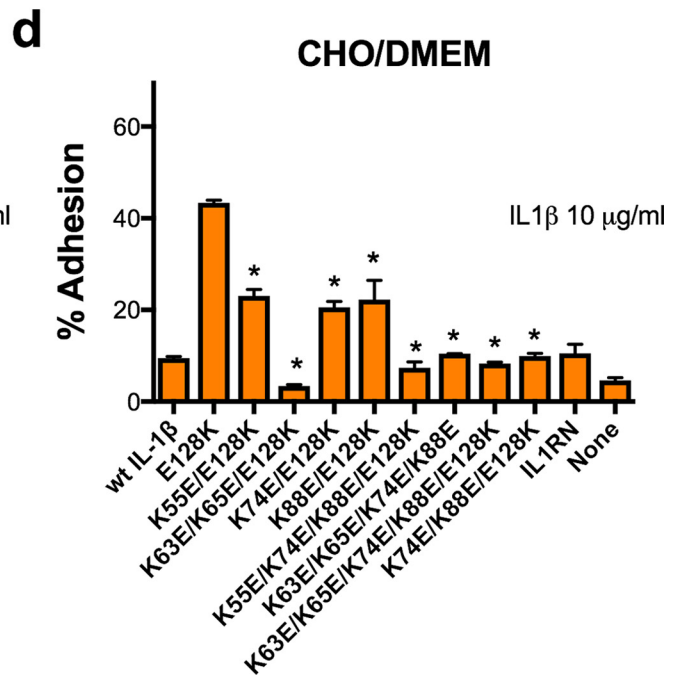
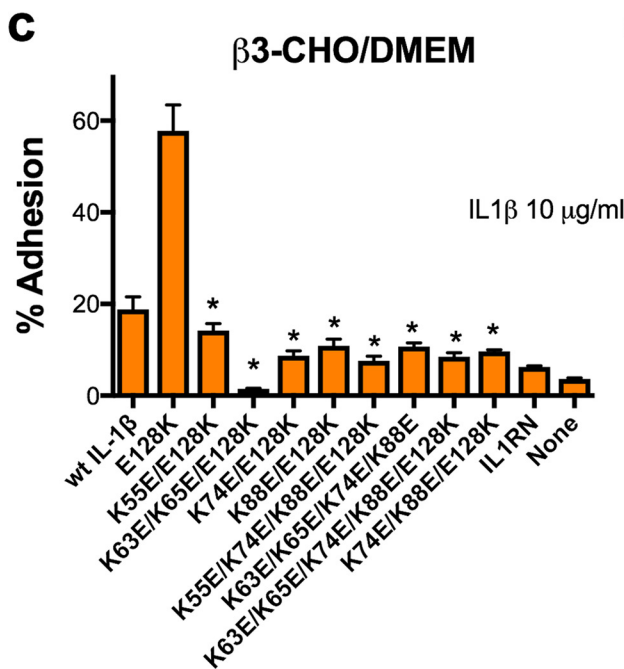
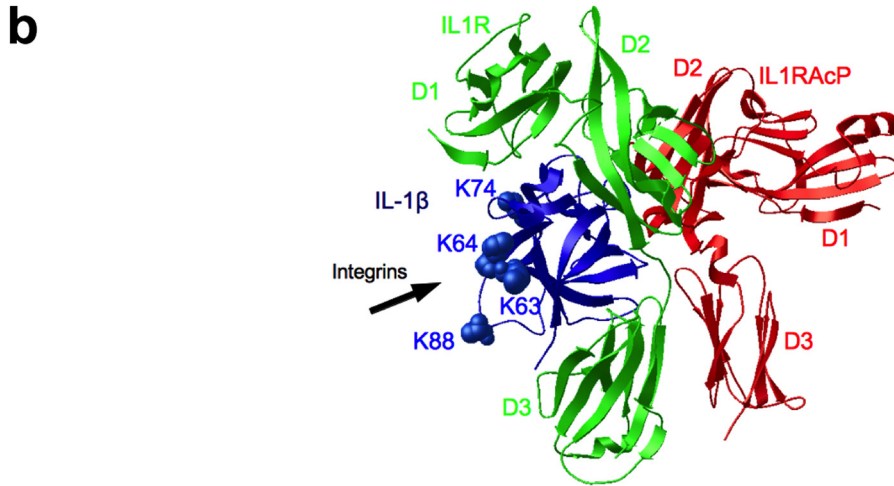


**Figure 2. The E105K and E128K mutations in the IL1R-binding sites of IL-1 $\beta$  markedly enhance integrin binding.** *a* and *c*,  $\beta$ 3-CHO cells (*a*) and CHO cells (*c*) (both IL1R-negative) adhered much better to E105K and E128K than to WT IL-1 $\beta$  or IL1RN in DMEM. Adhesion assays were performed as described in Fig. 1. The data are shown as means  $\pm$  S.E. of triplicate experiments. *b*, surface plasmon resonance study of the interaction between  $\alpha$ v $\beta$ 3 and E128K IL-1 $\beta$ . Recombinant soluble  $\alpha$ v $\beta$ 3 was immobilized to a sensor chip and IL-1 $\beta$  is in a solution phase. Mg<sup>2+</sup> (1 mM) was included in the binding buffer. The results suggest that E128K has much higher affinity to  $\alpha$ v $\beta$ 3 than to WT IL-1 $\beta$ . *d*, CHO cells (integrin  $\alpha$ 5 $\beta$ 1-positive) adhere to E128K, but the B2 variant of CHO cells ( $\alpha$ 5 $\beta$ 1-negative) did not in DMEM. Adhesion assays were performed as described in Fig. 1. *e*, adhesion of CHO and  $\beta$ 3-CHO cells with or without transfection of IL1R to E128K in the presence of IL1RN. Adhesion assays were performed as described in Fig. 1.



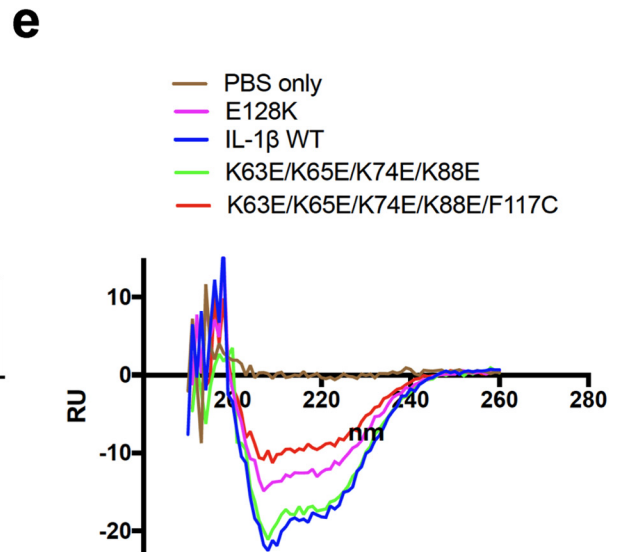
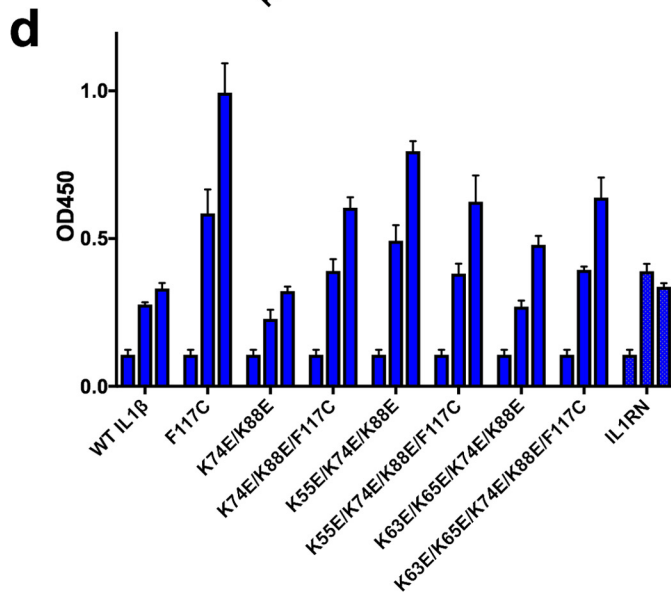
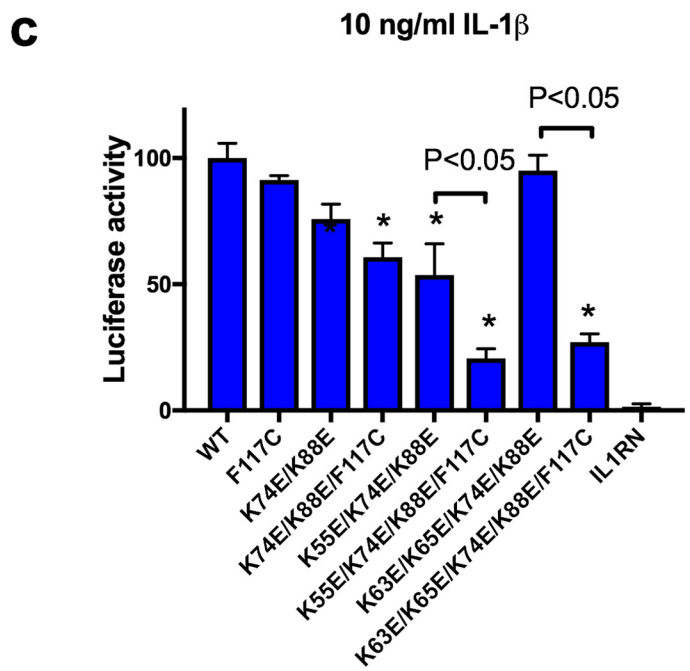
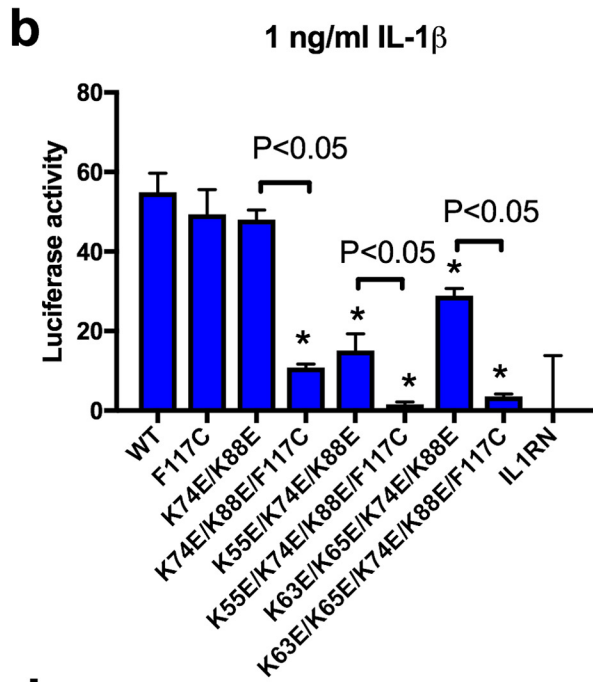
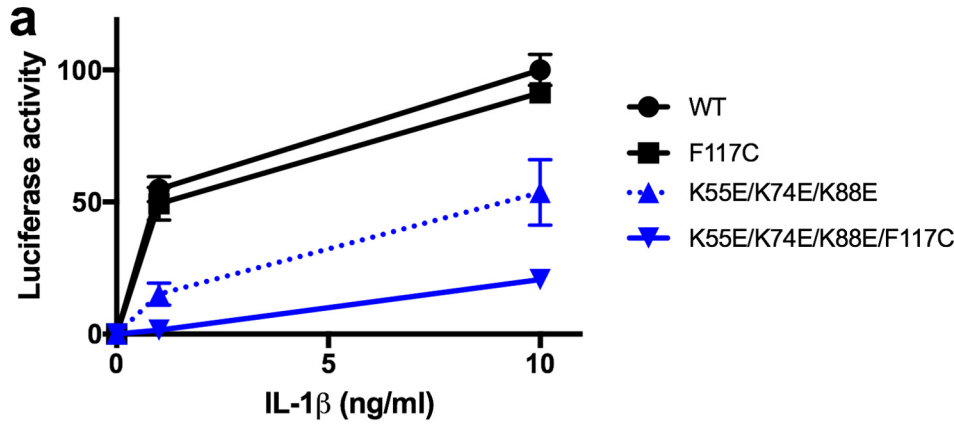
**a**

IL-1 $\beta$	1	APVRSLNCTL	RDSQQKSLVM	SGPYELKALH	LQQDMEQQV	VFSMSFVQGE
IL1RN	7	SSKMQAFRI	WDVNQKTFYL	RNN-QLVAGY	LQGNVNLLE	KIDVVPPIE--
		55	63/65	74	88	
		E	E E	E	E	
IL-1 $\beta$	51	ESNDKIPVAL	GLKEKNLYLS	CVLKDDKPTL	QLESVDPKNY	-PKKKMEKRF
IL1RN	53	----PHALFL	GIHGGMCLSL	CVKSGDETRL	QLEAVNITDL	SENRRQDKRF
		105	117	128		
		K	C	K		
IL-1 $\beta$	100	VFNKIINNK	LEFESAQFPN	WYISTSQAEN	MPVFLGGTRG	-QDITDFTM
IL1RN	99	AFIRSDSGPT	TSFESAACPG	WFLCTAMEAD	QPVSLTNMPD	EGVMVTKFYF
IL-1 $\beta$	149	QFVSS				
IL1RN	149	QED				



**Figure 3. Identification of amino acid residues critical for integrin binding in IL-1 $\beta$  by mutagenesis is shown.** We used the E128K mutant of IL-1 $\beta$  for mapping integrin-binding site in IL-1 $\beta$ . *a*, alignment of IL-1 $\beta$  (PDB ID 9ILB) and IL1RN (PDB ID 1IRA). The positions shown are of Lys residues (blue), E105 and E128 involved in IL1R binding (red), and disulfide linkage (yellow). The alignment shows that the several Lys residues (Lys-55, Lys-63, Lys-64, Lys-74, and Lys-88) are present in IL-1 $\beta$  but are changed to other neutral amino acids in IL1RN. A disulfide linkage is present in IL1RN, but not present in IL-1 $\beta$  because of mutation (the C117F mutation). *b*, the Lys residues (Lys-55, Lys-63, Lys-64, Lys-74, and Lys-88) are exposed to the surface in the IL1R/IL-1 $\beta$ /IL1RAcP complex (PDB ID 4DEQ). The arrow indicates the predicted integrin binding site in IL-1 $\beta$ . Lys residues exposed to the surface of IL-1 $\beta$  that are not in the IL1R-binding sites were mutated to Glu. *c* and *d*, the ability of  $\beta$ 3-CHO ( $\alpha$ v $\beta$ 3+,  $\alpha$ s $\beta$ 1+) or CHO cells ( $\alpha$ s $\beta$ 1+) to the IL-1 $\beta$  mutant in adhesion assays in DMEM. The data are shown as means  $\pm$  S.E. of triplicate experiments. \*, the binding to integrins is significantly low compared with E128K ( $p < 0.05$ ,  $n = 3$ ). The results suggest that several Lys residues in IL-1 $\beta$  are critical for integrin binding. Note that IL1RN did not bind to integrins under the conditions used.

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tions 55, 63, 65, 74, and/or 88 are mutated to Glu. We found that several integrin-binding–defective mutations reduced, but did not completely abrogate, IL-1 $\beta$ –induced NF- $\kappa$ B activation. Our results suggest mutating the Lys residues critical for integrin binding is insufficient to abrogate the agonistic activity of IL-1 $\beta$  (Fig. 4, *a–c*). A disulfide linkage is present in IL1RN, but not in IL-1 $\beta$  because of natural mutation (the C117F mutation) (Fig. 3*a*). We hypothesized that the loss of the disulfide linkage is as a potential factor in the agonistic action of IL-1 $\beta$ . We studied whether reintroduction of the disulfide linkage by the F117C mutation affects the agonistic activity of IL-1 $\beta$ . Interestingly, the F117C mutation by itself did not significantly reduce NF- $\kappa$ B activation by IL-1 $\beta$ , suggesting that the disulfide linkage by itself is not directly related to the agonistic activity of IL-1 $\beta$ . The F117C mutation itself did not affect integrin binding functions of the WT and mutant IL-1 $\beta$  in adhesion assays (data not shown). Notably, the combined F117C and Lys mutations (e.g. K63E/K65E/K74E/K88E/F117C) markedly reduced the remaining agonistic action of the Lys mutants (e.g. K63E/K65E/K74E/K88E). These findings suggest that the loss of integrin binding and the reintroduction of disulfide linkage work synergistically in reducing agonistic action of IL-1 $\beta$ .

It is possible that introducing multiple mutations reduces their ability to bind to IL1R or changes global conformation of IL-1 $\beta$ . We determined if IL-1 $\beta$  mutants still bind to IL1R using ELISA-type binding assays (Fig. 4*d*). We immobilized WT and mutant IL-1 $\beta$  and incubated with soluble recombinant IL1R, and bound IL1R was quantified. WT and mutant IL-1 $\beta$  bound to IL1R, which suggests that they all have the ability to bind to IL1R. We found that the IL-1 $\beta$  mutants that have four Lys mutations with and without reintroduction of disulfide linkage have CD spectra that are similar to WT IL-1 $\beta$ , suggesting that they are properly folded (Fig. 4*e*).

## Discussion

The present study establishes that IL-1 $\beta$  specifically binds to integrins  $\alpha$ v $\beta$ 3 and  $\alpha$ 5 $\beta$ 1. This is consistent with previous reports that integrins are related to IL-1 $\beta$  signaling (see Introduction). We identified several Lys residues of IL-1 $\beta$  that are critically involved in integrin binding. These Lys residues are all exposed to the surface in IL-1 $\beta$ /IL1R complex. IL1RN did not bind to integrins in physiological cation conditions in which E128K binds to integrins. Interestingly, these Lys residues are changed to other amino acid residues in IL1RN, which suggests that the Lys residues are involved in agonistic action of IL-1 $\beta$  through direct binding to integrins. We suspect that the loss of Lys residues is related to the loss of agonistic action of IL1RN. Because IL-1 $\beta$  concentrations in body fluid are very low (e.g. <1 ng/ml), it is unlikely that soluble IL-1 $\beta$  directly binds to integrins on the surface. One possible scenario is that IL-1 $\beta$  is highly

concentrated on the cell surface by binding to IL1R and then integrin is recruited to the IL-1 $\beta$ /IL1R complex.

We also found that the two known point mutations in the IL1R-binding site (E105K and E128K) enhanced the ability of IL-1 $\beta$  to bind to integrins by reducing the  $K_D$  by an order of magnitude. It is unclear how the mutations enhanced integrin binding, but we suspect that integrin binding of IL-1 $\beta$  enhances binding of IL-1 $\beta$  to integrins in physiological cation conditions. Interestingly, the Lys mutations that affect integrin binding showed lower agonistic activity, but they did not completely abrogate the agonistic action of IL-1 $\beta$ . The F117C mutation markedly reduced the remaining agonistic activity of the Lys mutants. The F117C mutation did not affect agonistic activity or integrin binding of WT IL-1 $\beta$ . One possibility is that the reintroduced disulfide linkage suppressed conformational changes induced by weak integrin binding in the Lys mutants. The disulfide linkage did not block conformational changes by strong integrin binding. We thus propose that integrin binding to IL-1 $\beta$  plays a major role in its agonistic action. It has been reported that the removal of the disulfide linkage of IL1RN by the C116F mutation made IL1RN agonistic (25). In our preliminary studies, the C116F mutation in IL1RN did not induce detectable activation of NF- $\kappa$ B in reporter assays (data not shown), suggesting that the loss of disulfide linkage is not directly related to the agonistic activity of IL1RN. This finding is consistent with the fact that IL1RN does not have critical Lys residues for integrin binding (Fig. 3*a*) and did not bind to integrins well in physiological cation conditions (Fig. 3, *c* and *d*). The disulfide linkage is likely to block conformational changes induced by weak, if any, integrin binding to IL1RN.

In conclusion, the present study identified integrin binding to IL-1 $\beta$  as a new critical component for an agonistic action of IL-1 $\beta$ . We propose that integrin-IL-1 $\beta$  interaction is a novel potential target for drug discovery.

## Experimental procedures

### Materials

Recombinant soluble  $\alpha$ v $\beta$ 3 was synthesized in Chinese hamster ovary K1 cells using the soluble  $\alpha$ v and  $\beta$ 3 expression constructs and purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography as described (26). CHO cells that express human  $\beta$ 3 have been described (22). Cyclic RGDfV was purchased from Enzo Life Sciences (Farmingdale, NY).

### Synthesis of IL-1 $\beta$ and IL1RN

The cDNA fragment of IL-1 $\beta$  was amplified using primers 5'-cgggatccgcacctgtacgatcactgaac-3' and 5'-ggaattccttaggaagacacaaattgc-3' with human IL-1 $\beta$  cDNA (Open Biosystems, Lafayette, CO) as a template, and subcloned into the BamHI/EcoRI site of pET28a expression vector. The cDNA fragment of

**Figure 4. The integrin-binding–defective Lys mutations reduce agonistic activity of IL-1 $\beta$ , and reintroduction of the missing disulfide linkage effectively suppresses remaining agonistic activity of the Lys mutants.** We studied whether integrin-binding–defective mutations affect agonistic activity of IL-1 $\beta$ . *a*, dose response of IL-1 $\beta$  mutants to induce NF- $\kappa$ B activation. We treated MCF7 cells that stably express NF- $\kappa$ B reporter gene with IL-1 $\beta$  for 4 h and measured the luciferase activity in cell lysates. Luciferase activity was normalized using that induced by 10 ng WT IL-1 $\beta$  as 100. *b* and *c*, summary of NF- $\kappa$ B activation by IL-1 $\beta$  mutants at 10 ng/ml (*b*) and at 1 ng/ml (*c*). The data are shown as means  $\pm$  S.E. of triplicate experiments. *d*, binding of IL-1 $\beta$  mutants to IL1R. Wells of 96-well microtiter plate were coated with IL-1 $\beta$  (His-tagged, WT and mutants of IL-1 $\beta$  and IL1RN, 20 or 40  $\mu$ g/ml) and blocked with BSA. Wells were incubated with soluble IL1R (10  $\mu$ g/ml), and bound IL1R was quantified using anti-IL1R antibody. The data are shown as means  $\pm$  S.E. of triplicate experiments. The columns represent 0, 20, and 40  $\mu$ g/ml IL-1 $\beta$ , in this order. *e*, CD spectra of WT or mutant IL-1 $\beta$ .



## Integrins in IL-1 $\beta$ signaling

IL1RN was amplified using primers 5'-cgggatcccggacctctgg-gagaaatccagc-3' and 5'-cgggaattcctactctctctctggaagtag-3' with human IL1RN cDNA (Open Biosystems) as a template, and subcloned into the BamHI/EcoRI site of pET28a AMP (which has ampicillin-resistant gene instead of kanamycin-resistant gene) expression vector. The IL-1 $\beta$  and IL1RN proteins were synthesized in BL21 induced by isopropyl 1-thio- $\beta$ -D-galactopyranoside (IPTG) as soluble proteins. The proteins were purified by Ni-NTA affinity chromatography as described (7). To remove endotoxin, affinity column was extensively washed with 1% Triton X-114 in PBS before protein elution.

### Synthesis of IL1R

The cDNA fragment of the domains 1–3 of IL1R was amplified using primers 5'-gaagatctgataatgcaaggaactggaag-3' and 5'-ggaattcctcaagtactgctgatatattaactg-3' with human IL1R cDNA (Open Biosystems) as a template, and subcloned into the BamHI/EcoRI site of pET28a AMP expression vector. The protein was synthesized in BL21 induced by isopropyl 1-thio- $\beta$ -D-galactopyranoside (IPTG) as an insoluble protein. The protein was solubilized in 8 M urea, purified by Ni-NTA affinity chromatography under denatured conditions and refolded as described (7). To remove endotoxin, the affinity column was extensively washed with 1% Triton X-114 in PBS before protein elution.

### Adhesion assays

Adhesion assays were performed as described previously (7). Briefly, to assess cell adhesion to immobilized IL-1 $\beta$ , 96-well Immulon 2 Microtiter Plates were coated with 100  $\mu$ l of 0.1 M NaHCO<sub>3</sub> containing IL-1 $\beta$  or its mutant and were incubated for 2 h at 37 °C. Remaining protein-binding sites were blocked by incubating with PBS/0.1% BSA for 30 min at room temperature. After washing with PBS, CHO cells in 100  $\mu$ l of DMEM/0.1% BSA were added to the wells and incubated at 37 °C for 1 h. After unbound cells were removed by rinsing the wells with the medium used for adhesion assays, bound cells were quantified by measuring endogenous phosphatase activity (7). To activate integrins, Hepes-Tyrode's buffer with 1 mM MgCl<sub>2</sub> was used instead of DMEM. To assess the effect of blocking antibodies and cyclic RGDfV, cells were pretreated with monoclonal antibodies or cyclic RGDfV at room temperature for 30 min before the assay.

### Surface plasmon resonance study

Recombinant soluble integrin  $\alpha$ v $\beta$ 3 was immobilized to Biacore Sensor Chip CM5 (Biacore, Piscataway, NJ) by the amine coupling method. 2-fold serially diluted IL-1 $\beta$  or its mutant in running buffer (HBS-P buffer containing 1 mM MnCl<sub>2</sub>) was injected for 3 min at the flow rate of 30  $\mu$ l/min. Then the sensor chip was washed with the running buffer alone at the same flow rate for another 15 min (the dissociation phase). 30-s injections of regeneration buffer (0.1 M NaOH, 1 M NaCl) at the same flow rate were used to regenerate the chip for another cycle of injection. The resonance unit elicited from the reference flow cell was subtracted from the resonance unit elicited from the integrin flow cell to eliminate the nonspecific protein–flow cell interaction and the bulk refractive index effect. The recorded

binding curves were analyzed using BIAevaluation Software version 4.

### IL-1 $\beta$ binding to IL1R

Wells of 96-well microtiter plates were coated with IL-1 $\beta$  (WT and mutants) and IL1RN, 20 or 40  $\mu$ g/ml in Hepes-Tyrode's buffer/1 mM EDTA, and blocked with BSA. Wells were incubated with soluble IL1R (10  $\mu$ g/ml) for 2 h in Hepes-Tyrode's buffer/1 mM EDTA, and bound IL1R was quantified using anti-IL1R antibody and anti-rabbit IgG conjugated with HRP.

### Other methods

We determined NF- $\kappa$ B activation as described (24). Treatment differences were tested using analysis of variance (ANOVA) and a Tukey multiple comparison test to control the global type I error using Prism 6.0 (GraphPad Software).

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*Author contributions*—Y.K.T., J.Y., M.F., J.S., and C.Y.W. performed the experiments. Y.T. conceived the experiments and wrote the manuscript.

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