Structural insights into the function-modulating effects of nanobody binding to the integrin receptor αMβ2

Rasmus K. Jensen, Henrik Pedersen, Josefine Lorentzen, Nick Stub Laursen, Thomas Vorup-Jensen, and Gregers Rom Andersen

From the 1Department of Molecular Biology and Genetics, and 2Department of Biomedicine, Aarhus University, Denmark

Edited by Joseph Jez

The integrin receptor αMβ2 mediates phagocytosis of complement-opsonized objects, adhesion to the extracellular matrix, and transendothelial migration of leukocytes. However, the mechanistic aspects of αMβ2 signaling upon ligand binding are unclear. Here, we present the first atomic structure of the human αMβ2 headpiece fragment in complex with the nanobody (Nb) hCD11bNb1 at a resolution of 3.2 Å. We show that the receptor headpiece adopts the closed conformation expected to exhibit low ligand affinity. The crystal structure indicates that in the R77H αM variant, associated with systemic lupus erythematosus, the modified allosteric relationship between ligand binding and integrin outside–inside signaling is due to subtle conformational effects transmitted over a distance of 40 Å. Furthermore, we found the Nb binds to the αl domain of the αM subunit in an Mg2+-independent manner with low nanomolar affinity. Biochemical and biophysical experiments with purified proteins demonstrated that the Nb acts as a competitive inhibitor through steric hindrance exerted on the thioester domain of complement component iC3b attempting to bind the αM subunit. Surprisingly, we show that the Nb stimulates the interaction of cell-bound αMβ2 with iC3b, suggesting that it may represent a novel high-affinity proteinaceous αMβ2-specific agonist. Taken together, our data suggest that the iC3b–αMβ2 complex may be more dynamic than predicted from the crystal structure of the core complex. We propose a model based on the conformational spectrum of the receptor to reconcile these observations regarding the functional consequences of hCD11bNb1 binding to αMβ2.

Integrins are integral membrane proteins, which mediate cell–cell, cell–extracellular matrix, and cell–pathogen adhesion. Integrin αMβ2, also known as complement receptor 3, CD11b/CD18, and macrophage-1 antigen (Mac-1), participates in all three types of interactions. The noncovalently associated subunits αM (CD11b) and β2 (CD18) consist of a large N-terminal ectodomain, a single transmembrane helix, and a C-terminal cytoplasmic tail (1). Multiple crystal structures of the ectodomain or headpiece (HP) fragments of αMβ2 (lymphocyte function–associated antigen-1, CD11a/CD18) and αMβ2 (complement receptor 4, CD11c/CD18, and p150,95) form the foundation for the mechanistic understanding of β2-integrins (2–4). The ectodomain is divided into a HP consisting of the N-terminal domains, and the tailpiece consisting of the membrane-proximal C-terminal domains (Fig. 1A). αMβ2 contains a von Willebrand factor type A domain, known as the αl domain, in the α-chain (5). The αl domain harbors a Mg2+-binding site, known as the metal ion–dependent adhesion site (MIDAS) (6, 7). The MIDAS is directly involved in ligand recognition, where a glutamate or an aspartate of the ligand coordinates the MIDAS Mg2+ ion (7, 8). The αl domain adopts two major conformations, open and closed (7). Transition from the closed to the open conformation leads to a rearrangement of the C-terminal α7-helix within the αl domain and a geometry of the MIDAS that permits coordination of Mg2+ by the ligand aspartate/glutamate (9).

One primary function attributed to αMβ2 is phagocytosis of complement-opsonized cells and immune complexes. Proteolytic cleavage of component 3 (C3) by convertases deposits the osonin C3b on the activator (Fig. 1B). The C3b has a short half-life and is quickly converted to iC3b (10) and eventually C3dg. iC3b exhibits high affinity for αMβ2, whereas the smaller C3d and C3dg fragments bind the αMβ2 HP 20-fold weaker (8, 11). αMβ2 is highly expressed on the plasma membrane of myeloid cells, including macrophages, monocytes, dendritic cells, and neutrophil granulocytes, and upregulated from storage granules upon stimulation. αMβ2 is also highly expressed in microglia, the mononuclear phagocytes of the central nervous system, and αMβ2-mediated phagocytosis of iC3b-opsonized presynaptic termini of neurons is important for neural development and homeostasis (12–15). The αMβ2 also plays a key role in complement stimulation of the adaptive immune system. Immune complexes containing iC3b-opsonized antigens drain into the subcapsular sinus where complement-opsonized antigens are taken up by macrophages via αMβ2, and are carried across the subcapsular sinus floor (16). External stimuli, such as chemokines, cytokines, or foreign antigens, can lead to intracellular signaling, which, in turn, induces conformational changes in the integrin ectodomain to increase the ligand affinity (5) (Fig. 1A). In the low-affinity bent-closed state, the ectodomain conformation positions the αl close to the plasma membrane with the HP in the ligand-binding inactive conformation. In the intermediary extended-closed conformation, the integrin extends, leading to
the αI domain pointing away from the plasma membrane, but the HP remains in the closed conformation. In the high-affinity extended-open state, the β2 hybrid domain is swung away from the αM thigh domain opening the HP for ligand binding.

Within the β2 subunit, the βI domain is structurally homologous to the αI domain but contains two additional regulatory metal ion–binding sites (17). Structure–function studies of the αLβ2 and αXβ2 integrins revealed that the βI domain is responsible for relaying bidirectional signaling from or to the αI domain (18, 19). This has led to a model for the allosteric regulation of the αI domain affinity. Central to this model, the Mg2+ in the βI domain MIDAS may become coordinated by Glu320 (mature numbering) from the α7 helix of the αI subunit located at the C-terminal region of the α7 helix of αI. Its interaction with the βI domain exerts a pull on the helix, which forces the αI domain into the open conformation during inside–out signaling. Conversely, movement of the α7 helix induced by ligand binding to the αI domain induces Glu320 coordination of the βI domain MIDAS Mg2+ and shifts the βI domain into the open conformation. Crystal structures indicate that the transition to the open conformation of the βI domain translates into a 60°/C14 swing out of the β2 hybrid domain that moves the plexin–semaphorin–integrin (PSI) domain by 70 Å (20). This swing propagates into the open-extended conformation of the β2 subunit, which induces intracellular signaling. These major conformational changes enable the use
of allosteric antagonist of ligand to break the internal conformational signaling in β2 integrin ectodomains (21). It is not clear from available data if the conformational dynamics also permit functional regulation by agents affecting the steric freedom of integrins in the cell membrane environment.

Here, we present the first atomic structure of the αMβ2 HP fragment in complex with the nanobody (Nb) hCD11bNb1 obtained by crystallography. The receptor adopts the closed conformation with low ligand affinity. The Nb binds to the αl domain in the αM subunit in an Mg2+-independent manner, and biophysical experiments as well as a structural comparison suggest that it acts as a competitive inhibitor of iC3b binding. In assays with cell-bound αMβ2, however, the Nb stimulates interaction with iC3b. We propose a model that integrates the entire conformational spectrum of the receptor and the dynamic properties of ligand–αMβ2 complexes to reconcile these observations.

Results
Selection and characterization of hCD11bNb1
Nbs are single domain antibodies of typically 120 residues derived from the variable domain of heavy chain–only antibodies present in members of the Camelidae family. In addition to their potential for modulating the function of their antigen, Nbs often facilitate structure determination of bodies present in members of the Camelidae family. In the presence of the Nb, the αl domain (Fig. 1, E–G). Data analysis with a 1:1 binding model revealed that the Nb binds with a low nanomolar affinity to both the αMβ2 HP and the αl domain (Fig. 1G). Overall, our SEC and biophysical experiments demonstrated that hCD11bNb1 binds with nanomolar affinity to the αl domain and compete with the C3d ligand, the latter a prior expectation considering the selection strategy.

Structure determination of the αMβ2 complex with hCD11bNb1
Despite extensive screening of the αMβ2 HP alone and its complex with C3d or iC3b, we failed to obtain useful crystals or nonaggregated particles on cryo-EM grids; in the latter case, most likely because of the effects of the air–water interface (23). However, when bound to the Nb, the αMβ2 HP readily crystallized in a number of different organic salts capable of chelating Mg2+ ions. We obtained seven X-ray diffraction datasets with synchrotron radiation that extended to a maximum resolution of 3.2 Å, but all these suffered from strong anisotropy. From all these datasets, we were able to determine the structure by molecular replacement using the coordinates of the β-propeller of αMβ2 or the βl domain of αlβ2 as search models. The resulting electron density and comparison with the structures of αMβ2 and αlβ2 enabled us to place the αM thigh domain and the β2 domains hybrid, PSI, and integrin epidermal growth factor 1 (I-EGF1). We also identified electron density that could be manually fitted with a model of the Nb bound to its epitope in the αl domain. The electron density calculated from the resulting model and noncorrected diffraction data was of low quality in the receptor proximal part of the thigh domain, the PSI and I-EGF1, and at C-terminal pole of the Nb irrespective of the used dataset. Data were therefore scaled anisotropically with the STARANISO server (Global Phasing Ltd) (24), which led to a significant improvement of both 2mFo–Fo and density-modified electron density maps. The dataset exhibiting the best statistics after refinement of an initial model was selected for completion of the structure. To support the modeling of the αMβ2–Nb complex, we also determined the structure of hCD11bNb1 itself based on diffraction data extending to a resolution of 1.14 Å (Table 1 and Fig. S2, A–C). Using the anisotropy-corrected data, we refined the complex structure to an Rfree value of 0.295 (Table 1). Figure 2, A and B displays the resulting structure and an example of the electron density presenting the Ca2+ sites in the αM β-propeller. As judged from comparison with known structures of αMβ2 and αlβ2 together with our high-resolution structure of the Nb, large errors are unlikely in the model. The slightly elevated Rfree value is probably caused by data anisotropy. The refined temperature factors are comparatively high for the C-terminal end of the thigh domain, the C-terminal end of hCD11bNb1, and the PSI/EGF1 domains as compared with the rest of the structure, most likely because of a lack of crystal packing in those areas.
Structure of the αMβ2 headpiece in the closed conformation

**Table 1**
Data collection and refinement statistics

| Structure                      | αMβ2–hCD11bNb1 (PDB ID: 7P2D) | hCD11bNb1 (PDB ID: 7NP9) |
|--------------------------------|--------------------------------|--------------------------|
| Wavelength                     | 0.9762                         | 0.9763                   |
| Resolution range               | 44.63–3.20 (3.35–3.20)         | 44.63–3.5 (3.625–3.5)    |
| Space group                    | P3,21                          | P3,21                    |
| Unit cell                      | 114.1 114.1 250.12 90 90 120   | 114.1 114.1 250.12 90 90 120 |
| Total reflections              | 1136,124 (151,037)             | 968,232 (139,390)        |
| Unique reflections             | 31,887 (4057)                  | 24,591 (3390)            |
| Multiplicity                   | 35.6 (37.2)                    | 35.2 (36.6)              |
| Completeness (%)               | 99.9 (99.9)                    | 99.9 (99.9)              |
| Mean I/sigma(I)               | 20.69 (1.26)                   | 26.53 (3.74)             |
| Wilson B-factor                | 128.3                          | 129.8                    |
| \(R_{\text{merge}}\)          | 0.114 (3.48)                   | 0.116 (3.53)             |
| \(R_{\text{free}}\)          | 0.096 (1.33)                   | 0.097 (1.34)             |
| CC1/2                          | 1 (0.865)                      | 1 (0.98)                 |
| Reflections refinement         | 26,420 (1303)                  | 23,536 (1559)            |
| Reflections \(R_{\text{rec}}\) | 1130 (55)                      | 1011 (70)                |
| \(R_{\text{work}}\)          | 0.2605 (0.3466)                | 0.2479 (0.3253)          |
| \(R_{\text{free}}\)          | 0.2950 (0.4033)                | 0.2888 (0.3335)          |
| CC (work)                      | 0.911 (0.583)                  | 0.911 (0.603)            |
| CC (free)                      | 0.851 (0.427)                  | 0.858 (0.651)            |
| Nonhydrogen atoms              | 10,419                         | 1175                     |
| RMS (bonds)                    | 0.006                          | 0.005                    |
| RMS (angles)                   | 1.16                           | 1.03                     |
| Ramachandran plot              | 95.62                          | 95.77                    |
| Favored (%)                    | 4.23                           | 4.15                     |
| Allowed (%)                    | 0.15                           | 0.08                     |
| Outliers (%)                   | 2.04                           | 1.77                     |
| Rotamer outliers (%)           | 4.56                           | 5.09                     |
| Clashscore                     | 127.62                         | 136.55                   |
| Average B-factor               | 3.48                           | 18.04                    |

Statistics for the highest-resolution shell are shown in parentheses. For the αMβ2–hCD11bNb1 complex, the data collection statistics were calculated by XSCE from the diffraction data not corrected for anisotropy. Refinement statistics were calculated from the data corrected for anisotropy by the STARANISO server. The deposited structure in PDB entry 7P2D was refined to a maximum resolution of 3.2 Å; statistics for a maximum resolution of 3.5 Å resolution is presented for comparison.

Overall structure of the αMβ2–hCD11bNb1 complex

The two subunits associate through an extensive intermolecular interface formed between the β-propeller in αM and the βI domain in the β2 subunit, with a buried surface area of 3650 Å². Superposition of the αMβ2 onto structures of αββ2 and αβIβ2 revealed that the β-propeller and the βI domains interact in an almost identical manner across the three β2 receptors (Fig. S3A). The orientation of the thigh domain relative to the β-propeller is also quite similar in αMβ2 and αMβ2 (Fig. S3B). In the αM β-propeller, two calcium ions organize coordinating loop regions that together with the first residue of αM forms the interface with the thigh domain (Fig. 2B). In contrast, the metal–ion–binding sites in both the αI and βI domains are empty, and the internal ligand region at the C-terminal end of the αI αI helix is not interacting with the βI MIDAS site (Fig. 2C). This is an important notion, as ligand binding to the αI domain induces movement of the αI helix. In turn, αM Glu320 may coordinate the metal ion within the βI MIDAS site and induce the ligand-bound open conformation of the βI domain that propagates into the extended-open conformation of αMβ2 (Fig. 1A). For this reason, the region around Glu320 is known as the internal ligand (Fig. 3B). The overall conformation of the αMβ2 HP is closed with the hybrid, PSI, and I-EGF1 domain in the β2 subunit located toward the αM thigh domain, in contrast to the open conformation of the β2 subunit known from a structure of ligand-bound αMβ2 integrin (Fig. 2D). Within the β2 subunit, the arrangement of the four domains in the closed conformation of αMβ2 is also highly similar to those observed for αMβ2 and αIβ2 (Fig. S3C).

Structural basis for the altered allosteric coupling in the αM R77H variant

A single nucleotide polymorphism resulting in the substitution of Arg77 to histidine in the αM subunit β-propeller predisposes the carrier for systemic lupus erythematosus (SLE) (25). The R77H mutation neither does change the surface expression of αMβ2 on neutrophils and monocytes nor does effect the inside–out signaling (26, 27). However, the mutation interferes with outside–in signaling since it significantly decrease phagocytosis of iC3b-opsonized red blood cells by macrophages and cell lines expressing αMβ2 (26, 27). In addition, the monocytes carrying the mutated αMβ2 adhere less efficiently to surfaces coated with iC3b, fibronectin, intercellular adhesion molecule 1 (ICAM-1), ICAM-2, and DC-SIGN (dendritic cell–specific intercellular adhesion molecule-3-grabbing nonintegrin). Furthermore, R77H monocytes stimulated with a Toll-like receptor 7/8 agonist exhibit a significantly smaller decrease in cytokine secretion upon binding of iC3b-opsonized red blood cells compared with WT monocytes (26). Biomembrane force probe experiments revealed that the αM R77H variant of the αMβ2 receptor fails to respond to force with formation of catch bonds normally induced when cells adhering through αMβ2 to an immobilized ligand are exposed to an external force (28).

In our structure, Arg61 (the mature numbering of Arg77) after release of the propeptide) is exposed on the edge of the αM R77H variant.

In the αMβ2 (the mature numbering of Arg77 after release of the propeptide) is exposed on the edge of the αM β-propeller (Fig. 2F). The side chain of Arg61 only appears to interact with the nearby loop Gly111–Pro118 by nonspecific van der Waals interactions. The arginine side chain does not engage in specific hydrogen bonds or electrostatic interactions.
that could directly explain the altered allosteric coupling in 
$\alpha_{M}\beta_2$ containing the R77H variant. In our crystal structure, Arg61 is located $\sim$40 Å from both the α7 helix in the αI domain and the MIDAS site in the βI domain. Hence, a direct interaction of Arg61 with residues in the αM–β2 interface involved in the allosteric coupling between ligand binding and transition to the extended-open conformation cannot explain the observed functional defects. Furthermore, based on the structure of the closed-bent conformation $\alpha_X\beta_2$ (2, 3), we also predict that Arg61 does not interact with other domains in either of the two subunits in the bent-closed conformation of $\alpha_{M}\beta_2$.

However, a possible consequence of a histidine at position 61 is that the neighboring 111 to 118 loop (Fig. 2F) changes conformation and dynamic properties because of perturbation of the $\alpha_M$–β2 interface. Alternatively, a histidine side chain at position 61 may engage in hydrogen bonds with the 111 to 118 loop. In support of an altered conformation of the 111 to 118 loop, we notice that in $\alpha_X$ with a glycine residue corresponding to $\alpha_M$ Arg61, the equivalent loop adopts a different conformation and is not in contact with the region containing the glycine. An altered conformation of the 111 to 118 loop could propagate and influence the dynamic properties of the N-terminal linkage between the β-propeller domain and the αI domain in residues 123 to 129. Alternatively, such conformational changes could propagate to $\alpha_M$ residues located at the interface to the βI domain such as the loop region Thr96–Thr101. Transmission of force is crucially dependent on a stable $\alpha_M$–β2 interface; even a small perturbation may give rise to the observed abnormal outside–in signaling in the R77H $\alpha_M$ variant.

The αI domain helix 7 adopts the closed conformation

Prior structures of $\alpha_X\beta_2$ and $\alpha_L\beta_2$ revealed that the α-subunit β-propeller and the βI domain form a platform above which the αI domain has considerable freedom to orientate in response to crystal packing and the βI coordination state.
of the $\alpha_M$ internal ligand region (2–4). Confirming this idea, and in contrast to the highly conserved arrangement of the remaining domains discussed previously, the orientation of the $\alpha_{I}$ domain is unique. Compared with structures of $\alpha_X\beta_2$ with the $\alpha_X$ internal ligand region interacting with the $\beta_2$ MIDAS, the $\alpha_{I}$ domain is rotated by 180° (Fig. 2E). When comparing our $\alpha_M\beta_2$ structure to $\alpha_X\beta_2$, where the internal ligand region is not contacting the $\beta_2$ MIDAS, the $\alpha_{I}$ domain is rotated by 125° and 42°, respectively (Fig. 2E). The $\alpha_M\beta_2$-specific orientation of the $\alpha_{I}$ domain may well be a result of crystal packing, since the $\alpha_{I}$-hCD11bNb1 part of the complex firmly contacts three symmetry-related complexes (Fig. S3D). Supporting this, there are no specific interaction between the N-terminal linker region ($\alpha_M$ residues 123–131) and the platform. At the C-terminal linker region (residues 321–328), only a putative hydrogen bond between Thr322 and a sugar residue from the glycan attached to Asn375 appears to be specific for the $\alpha_M\beta_2$ structure.

The $\alpha_{I}$ domain has no clear density for a Mg$^{2+}$ ion although it was available during crystallization. Also, the electron density suggests that the main-chain conformation of residues Asp242–Glu244 may not be fixed. As this region differs between the open and closed forms of the $\alpha_{I}$ domain (7), the conformation of the MIDAS site itself cannot be defined (Fig. 3A). Hence, the Nb does not appear to depend on a particular MIDAS conformation, and SEC analysis confirms that its binding to the $\alpha_{I}$ domain is Mg$^{2+}$ independent (Fig. 1, C and D). This is also consistent with that the conformation of the epitope described later does not differ significantly in structures representing the open and closed states of the $\alpha_{I}$ domain.
Another signature of the α domain conformational state is the length and position of the α7 helix (7). In the Nb complex, the αM Phe302–Glu320 region is in a helical conformation (Figs. 3, A and B, S3E) meaning that this region adopts the closed conformation that prevents Glu320 from interacting with the βl MIDAS. Since hCD11bNb1 and the α7 helix are located oppositely on the α domain (Fig. 3A) and the Nb apparently does not induce a specific conformation of the α MIDAS, the Nb is unlikely to influence the conformation of the α7 helix significantly. Its closed conformation is more likely to be a result of the crystal packing that favors the overall closed conformation of the β2 subunit in the β domain incompatible with binding of the αM internal ligand region. In summary, both the α domain α7 helix and the overall conformation of the αMβ2 HP signify the closed conformation; however, this appears not to be a consequence of the Nb. In solution, the αMβ2 HP contains a mixture of the open and closed conformations (11) that are likely to bind the Nb with very similar affinities.

The Nb epitope on the α domain is proximal to the C3d-binding site

The quality of the electron density for the Nb–α interface is overall good considering the resolution and data anisotropy (Fig. S3D). Furthermore, known structures of the α domain and our own 1.14 Å-resolution structure of hCD11bNb1 itself (Fig. S2A) considerably facilitated modeling of the intermolecular interface. The buried surface area of the interface is 1330 Å², which is low, but not unusual, when compared with most other Nb–antigen complexes (29). The interface is dominated by polar interactions (Fig. 3, C and D). Extensive burial of hydrophobic side chains at the Nb–α interface plays a prominent role in our complexes of Nbs with complement C3b, C4b, and C1q (29–31) but is not observed in this case, although the hCD11bNb1 Phe54 stacks with the guanidinium group of α Arg181 (Fig. 3D). The epitope of the Nb comprises two distinct regions in the α domain. First, Nb complementarity-determining regions (CDRs) 1 and 3 recognize α residues Pro201–Leu206 with hydrogen bonds and van der Waals interactions (Figs. 3C and S2D). Second, α residues in the region Glu179–His183 encompassing β-strand C interact with hCD11bNb1 CDR1 and CDR2 through van der Waals interactions and hydrogen bonds (Figs. 3D and S2D).

Interestingly, a comparison of the α–CD11bNb1 and the α–C3d (8) complexes revealed a small, but significant, overlap between the Nb and C3d suggesting that their binding is mutually exclusive (Fig. 3, E and F). Specifically, the Nb CDR3 residues 103 to 107 are predicted to exert steric hindrance on C3d residues Ala1214–Lys1217 in a loop region at the end of a C3d α-helix. Since the thioester domain of iC3b is expected to bind αMβ2 in the same manner (8, 11), this predicts that the Nb interferes with both αMβ2–iC3b and αMβ2–C3d interactions. In summary, our structural analysis defined the paratope and epitope and their interactions in details and predicted that hCD11bNb1 acts as a competitive inhibitor of iC3b and C3d binding to αMβ2 by exerting steric hindrance on the thioester domain of the ligand.

Biophysical analysis of the iC3b–αMβ2 complex confirms the crystal structure

To test the prediction that hCD11bNb1 acts as a competitive inhibitor for the αMβ2–iC3b interaction, we took advantage of the high-affinity monovalent interaction occurring between iC3b and the αMβ2 HP (11). We biotinylated the free cysteine appearing in nascent C3b upon thioester cleavage using a maleimide–biotin reagent, converted the C3b to iC3b, and coupled the biotinylated iC3b to a streptavidin-loaded surface plasmon resonance (SPR) sensor. This strategy presents iC3b in the geometry that it would have on an activator after C3b deposition and factor I degradation as outlined in Figure 1B. We next flowed recombinant αMβ2 HP over the iC3b-coated sensor in the presence or the absence of a 1.5-fold molar excess of hCD11bNb1. As previously reported (11), we observed a KD value of 30 nM in the absence of the Nb (Fig. 4A). In the presence of hCD11bNb1, the signal decreased to 32 to 55% of the signal obtained in the absence of the Nb (Fig. 4, B and C), demonstrating that the Nb acts as an inhibitor of iC3b for binding to the αMβ2 HP.

To validate the interface between hCD11bNb1 and the αMβ2 HP observed in the crystal structure, we mutated Nb residues in direct contact with the receptor (Asn30, Phe54, and Asn101) or in the vicinity (Ile29 and Phe32) likely to support the conformation of the directly interacting residues. We next coupled biotinylated WT hCD11bNb1 to streptavidin-coated BLI sensors and carried out a competition assay where variants of hCD11bNb1 were present in the fluid phase in 10-fold molar excess to the αMβ2 HP. As expected, the presence of the parental hCD11bNb1 in the fluid phase reduced the binding to approximately 22% of the signal obtained with the αMβ2 HP only (Fig. 4D). The Ile29Ala variant competed almost as well as the parental Nb, whereas the remaining variants more or less lost the ability to inhibit binding of the αMβ2 HP to the immobilized hCD11bNb1 (Fig. 4D). Overall, our experiments with mutated Nb variants validated the paratope–epitope interaction deduced from the crystal structure.

Since hCD11bNb1 appeared to modulate the function of αMβ2, we investigated whether the Nb could bind to the recombinant α domain from the murine αM subunit. If hCD11bNb1 also modulates the activity of murine αMβ2, it may be an attractive reagent for in vivo murine models of pathogenesis where the receptor plays a role as discussed later. We charged anti-His BLI sensors with His-tagged hCD11bNb1 and compared the binding of the recombinant murine α domain to the human α domain. While we observed a strong signal for the human domain, the murine α domain bound much weaker (Fig. 4E), and the data could not be fitted to obtain rate constants or the KD value. To understand why the Nb binds the murine α domain much weaker, we constructed a homology model of the murine domain. Inspection of this model suggested two reasons for our observations. First, a
were transferred into 20 nM present. Loaded SPR chip. Next, 6.25, or 3.125 nM. BLI, biolayer interferometry; CDR, complementarity-determining region; HP, headpiece; SPR, surface plasmon resonance.

binds to the murine domain. Second, L206 in the human domain. This is likely to lead to steric hindrance when the Nb the polar N222 in murine domain making contact with CDR3 of the Nb is replaced by N101A, or WT hCD11bNb1.

immobilized on anti-

(11). In the absence of iC3b, the αβ2 HP revealed molecular weights in good agreement with their predicted values (Fig. 5). As expected, the receptor and iC3b ligand to αβ2 was evaluated in two types of cell-based assays. The first assay was based on the binding of fluorescent-labeled iC3b (iC3b*) incubated with K562 cells that express αβ2. Here, the long incubation time permitted the reaction to reach equilibrium (11), and the fluorescent signal from cell-bound iC3b* was quantitated by flow cytometry. The signal obtained for incubations with iC3b* alone or together with hCD11bNb1 was subtracted. Autofluorescence (Δ mean fluorescence intensity) was measured in cells with no addition of iC3b*, either for conditions without integrin activation (Fig. 6A) or with Mn2+ added to activate ligand binding (Fig. 6B). In this assay, the influence of hCD11bNb1 showed a dose-dependent increase of *iC3b both under conditions with and without integrin activation (Fig. 6, C and D). Importantly,
an unrelated control Nb did not increase the binding quantitatively. When this binding was normalized to the signal with no hCD11bNb1 (Fig. 6, E and F), it became clear that the observed increase in iC3b* binding was independent of integrin activation: in both cases, the stimulation was approximately twofold higher in the presence of hCD11bNb1. Especially for nonactivating conditions (Fig. 6 E), the binding signal followed the hCD11bNb1 concentration with lower signal for a concentration of 1 μg/ml (Fig. 6 C) compared with 5 or 10 μg/ml, which showed signs of saturation.

Our second cell-based assay involved force exerted through centrifugation of V-shaped microtiter wells coated with iC3b. In this way, adherent cells transmit a force load onto the ligand-bound αMβ2 mimicking physiological conditions, where shear stress acts similarly (33). Adhesion of K562 cell-expressing αMβ2 to the iC3b-coated wells increased with addition of hCD11bNb1; however, with no sign of titration of the signal in the hCD11bNb1 concentration range was investigated. Interestingly, in this case, the increase only occurred under integrin-activating conditions in an applied force regime from 30 to 100 g (Fig. 6, G and H). As a control, we tested αxβ2-expressing K562 cells, which also bind iC3b in the centrifugation assay. In this case, the hCD11bNb1 had no influence on the cell adhesion, consistent with the absence of the αM chain (Fig. 6, I and J). Overall, the cell-based assays demonstrated that hCD11bNb1, through its binding to the αI domain, can both stimulate the interaction of αMβ2 with fluid phase monovalent iC3b as well as the multivalent interaction between αMβ2-presenting cells and an iC3b-coated surface. Hence, with the receptor presented on the cell surface, hCD11bNb1 acted as new protein-based αMβ2-specific agonist that promotes interaction with iC3b.

Figure 5. Characterization of αMβ2 HP, iC3b, and the influence of hCD11bNb1 in solution measured by mass photometry. A, mass distribution of 10 nM αMβ2 HP. The signal below 50 kDa is due to noise and present in all measured curves. The small peak at 285 kDa is likely to correspond to a small amount of αMβ2 HP dimers as described (11). B, 10 nM αMβ2 HP with 100 nM hCD11bNb1. The nanobody does not induce oligomerization of the αMβ2 HP. C, overlay of the two MP distributions in A and B. D, 10 nM αMβ2 HP mixed with 10 nM iC3b gives rise to a peak for complex at 323 kDa. The percentage of complex formed corresponds to the area of the complex peak compared with the total area under the curve including the area under the curve below 100 kDa as calculated by the DiscoverMP software. E, as in D with 7.4 nM hCD11bN1. Partial dissociation of the complex is evident, F, as in D with 100 nM hCD11bN1. Partial dissociation of the complex is evident. G, mass distribution of 10 nM iC3b. H, bar chart displaying the percentage of αMβ2 HP–iC3b complex as a function of the molar ratio between αMβ2 HP and the nanobody at a fixed 10 nM concentration of αMβ2 HP and iC3b. The ratios are based on the average of two experiments. For some ratios, the percent complex reported as an integer number was identical. I, table of molecular masses observed that extracted from the mass distributions. HP, headpiece.
A model for hCD11bNb1 stimulation of iC3b binding

As noted previously, the hCD11bNb1 showed an inhibitory effect on ligand binding to the \( \alpha_M \beta_2 \) HP and the isolated \( \alpha_I \) domain in solution-based assays. In this case, the apparent affinity (\( K_D \)) of hCD11bNb1 was 1 to 6 nM. By contrast, the cell-expressed \( \alpha_M \beta_2 \) responded to hCD11bNb1 by increased iC3b* binding. One of the major differences between the fluid phase assays with pure components and those involving the cell-expressed \( \alpha_M \beta_2 \) was the presence of the cell membrane in the latter experiments. Adair et al. (34) previously reported a low-resolution EM structure of the \( \alpha_M \beta_2 \) with an HP almost parallel to the cell membrane, extending the bent form of the integrin sufficiently at least for smaller ligand to gain access to the \( \alpha_M \) MIDAS. Modeling of the bent conformation of \( \alpha_M \beta_2 \) based on the structure of the \( \alpha_X \beta_2 \) ectodomain (2, 3) also supports that the \( \alpha_I \) domain in bent-closed \( \alpha_M \beta_2 \) must be close to the cell membrane (Fig. 6). This membrane proximity may contribute to the unexpected effect of the Nb on cell-bound \( \alpha_M \beta_2 \). Binding of the Nb possibly promotes a transition to a more extended conformation (Figs. 1A and 6K). If this influence of the Nb affects a rate-limiting step with respect to presenting a high-affinity binding site for iC3b, it may explain...
the agonist behavior of the Nb. This would also agree with the somewhat higher dosage requirement for inducing the agonistic effect (i.e., ~75 nM) compared with expectations from the formal affinity of hCD11bNb1 for isolated HP ($K_D$ ~1 nM). In the solution-based protein interaction assays with our $\alpha_M\beta_2$ HP, this putative membrane-dependent unbending effect on $\alpha_M\beta_2$ conformation is, of course, not a factor. Here, hCD11bNb1 may either be neutral or act as a weak stabilizer of the $\alpha_M$I closed conformation, which together with steric hindrance exerted on the C3d or the iC3b thioester domain explains the observed blocking of iC3b and C3d binding. Nevertheless, if induction of $\alpha_M\beta_2$ unbending accounts for the agonistic effect of hCD11bNb1, it is surprising that the effect also manifests itself in the presence of Mn$^{2+}$, which conformationally activates integrins. However, in agreement with our proposal, the influence of the Mn$^{2+}$ is mainly through binding to a metal-binding site adjacent to the $\beta$I MIDAS, and occupation of this site with Mn$^{2+}$ may not lead to a fully extended integrin unless high concentrations of ligand are present as was noted for $\alpha_I\beta_2$ (35). Hence, in the cell adhesion assays, repulsion from the membrane may still be able to promote a transition to the high-affinity state of $\alpha_M\beta_2$.

Despite the possible Nb-induced conformational changes in cell-bound $\alpha_M\beta_2$, our comparison of crystal structures still predicts that binding of iC3b and hCD11bNb1 is mutually exclusive (Fig. 3E). The discrepancy between this prediction and the cell-based assay suggests that iC3b binding to the receptor is more dynamic than apparent from our crystal structure of the C3d–$\alpha$ core complex (8). Considering that the overlap between the Nb and the iC3b thioester domain only involves a few residues, it is feasible that this overlap can be reduced if the iC3b thioester domain undergoes small internal conformational changes in the region involved in the overlap or the two $\alpha$I binders reorient slightly relative to each other. In the light of the well-established conformational dynamics of $\beta_2$-integrin receptors, it is plausible that the interaction of iC3b and hCD11bNb1 with the $\alpha$ domain could be somewhat dynamic on the cell-bound receptor compared with our crystal structures of hCD11bNb1–$\alpha_M\beta_2$ and C3d–$\alpha$I domains (8). Hence, complexes where the predicted overlap is reduced or eliminated may actually occur on $\alpha_M\beta_2$-presenting cells. This agrees with that we and others have demonstrated that there are additional interactions outside the core complex (11) that may support a spectrum of conformations of the iC3b–$\alpha_M\beta_2$ complex rather than a single rigid complex as captured in the structure of the core complex (8).

Discussion

The structure of $\alpha_M\beta_2$ in the closed conformation

Here, we present the first atomic structure of $\alpha_M\beta_2$ featuring its HP in the closed conformation characterized by the proximity of the $\beta_2$ hybrid domains to the $\alpha_M$ $\beta$-propeller and the approach of the $\beta_2$ PSI and I-EGF1 domains to the $\alpha_M$ thigh domain. Except for the $\alpha$I domain, this conformation is strikingly similar to closed conformations known from structures of $\alpha_I\beta_2$ and $\alpha_I\beta_2$ (2, 4). Our structure of the closed conformation of $\alpha_M\beta_2$ is the first step toward establishing the mechanism of outside–in signaling in this receptor. Strikingly, structures of $\beta_2$-integrins in the open-extended conformation of high-ligand affinity are still lacking although low-resolution negative-stain EM micrographs confirm the presence of the open conformation (2, 11). Comprehensive prior studies of other integrins, such as $\alpha_2\beta_2$ lacking an $\alpha$I domain, has defined in details the conformational rearrangements occurring in the $\beta$I domain upon binding of an external ligand to the $\beta$I domain. Ligand binding and modulation of the metal-binding sites in the $\beta$I domain propagates into swing out of the hybrid domain and presumably favors extension of the $\beta_2$ subunits and its associated $\alpha$-subunit and culminates in outside–in signaling (36). The $\beta_2$ integrins, including $\alpha_M\beta_2$, are expected to react in a similar manner to $\alpha$-subunit ligand binding and binding of the internal ligand to the $\beta$I domain.

The $\alpha$I domain in $\alpha_M\beta_2$

Our comparison of the $\alpha_M\beta_2$ structure with structures of $\alpha_I\beta_2$ and $\alpha_2\beta_2$ demonstrated a unique orientation of the $\alpha$I domain relative to the platform in the closed conformation. Furthermore, two crystal structures of a bent $\alpha_I\beta_2$ in which the internal ligand interacts with the $\beta$I MIDAS site demonstrated a slight variation in the orientation of the $\alpha$I domain adopting the open conformation (3). The internal ligand region in these two structures is highly extended, and overall, these structures indicate that the distance between the MIDAS sites in the $\alpha$I and $\beta$I domains as well as the orientation of the $\alpha$I domain relative to the platform is not necessarily fixed (3). In crystal structures of $\beta_2$ integrins, including our $\alpha_M\beta_2$ structure, lattice packing appears to play a major role in stabilizing the position of the $\alpha$I domain. Thus, it is possible that the $\alpha$I domain is never locked relative to the platform in a cell-bound $\beta_2$ integrin. One striking example supporting this notion is the complex between $\alpha_I\beta_2$ and its iC3b ligand, where negative-stain 2D classes revealed two opposite orientations of the ligand compared with the platform. This implies that in the ligand-bound state, two orientations of the $\alpha$I domain differing by up to 180° were present in the sample (37). Nevertheless, other studies of $\alpha_M\beta_2$ by negative-stain EM featured a more defined orientation of the $\alpha$I domain relative to the platform (34). Our own 3D reconstructions of the $\alpha_M\beta_2$ HP also offered evidence that the $\alpha$I domain is at least somewhat restricted with respect to rotation relative to the platform (11). However, because of the resolution in negative stain and the roughly spherical shape of the $\alpha$I domain, it is difficult to quantitate the variability in domain orientation from such data.

At present, the only ligand-bound structures involving $\alpha$I domains from $\beta_2$ integrins are our $\alpha_M\beta_2$ $\alpha$I–C3d complex (8) and the complexes of $\alpha_I\beta_2$ $\alpha$I with ICAM-1/3/5 (9, 38, 39). In addition, a model for the $\alpha_M\beta_2$ $\alpha$I–GP1bN complex based on NMR restraints and the crystal structure of the murine glycoprotein Ibx N-terminal domain featured the interaction of an aspartate from a ligand $\alpha$-helix (40). Detailed structures of multiple integrin–ligand complexes with intact ectodomain or their HP fragments are required to establish the relationship
between high-affinity ligand binding, the conformational freedom of the α1 domain, and the structural events underlying outside-in signaling in αMβ2 and other β2 integrin–ligand complexes. To avoid crystal-packing effects on the α1 location and the conformation of the rest of the receptor, single-particle analysis by cryo-EM is likely to be the best approach for establishing the detailed molecular mechanism of outside-in and inside-out signaling of the β2-integrins.

A very recent crystal structure of iC3b in complex with the α1 domain was reported, which confirmed the core interaction between α1 and the iC3b thioester domain (41) previously captured in the α1–C3d complex (8). Two different crystal-packing interactions between the α1 domain and domains in iC3b far from the thioester domain were suggested to mirror cell-bound α1β2 interaction with iC3b on an opsonized surface. Additional experimental evidence is needed to confirm the suggested in vivo relevance of these α1 interactions with regions outside the iC3b thioester domains.

Function-modulating molecules targeting αMβ2

In vivo studies leave no doubt about the importance of the αMβ2 as a protective agent against infection (42) and as an aggravating factor in diseases with a poorly regulated inflammatory response, for instance, as observed in animal models of multiple sclerosis and Alzheimer’s disease (43). For multiple sclerosis, there is evidence from the pharmacological mode of action of drugs and animal models that αMβ2 may also play in this case an aggravating role, at least in the relapsing-remitting form of the disease (44, 45). With respect to stroke, blocking of αMβ2 by the use of the hookworm-derived neutrophil inhibitory factor improved the outcome in animal models (46). Later trials in humans were however compromised, by pre-existing antibodies to this parasite protein. SLE is an autoimmune disease where complement plays a central role. It is a long-standing observation that αMβ2 expression increases in neutrophils and scales with the severity of the disease (47). αMβ2 has recently been implicated in SLE and lupus nephritis, a kidney disease that is a common complication of SLE (48).

Three missense mutations in the gene coding for αM have shown a strong association with both SLE and lupus nephritis in genome-wide association studies (25, 49, 50). The negative impact on αMβ2 function and strong association with SLE for the αM R77H variant has been difficult to explain, but our structural data now suggest that the arginine to histidine mutation could affect the structural dynamics of the ectodomain through long-range effects on conformation and dynamic properties of residues in the αMβ2 subunit interface.

The examples of aforementioned αMβ2-linked diseases demonstrate that pharmacological regulation of αMβ2 activity is clinically relevant. The complications with respect to therapeutic modulation of the receptor and the repertoire of natural and man-made molecules targeting αMβ2 has recently been extensively reviewed (51). Currently, the most advanced drug candidate is the αMβ2 agonist leukadherin-1 (LA-1), a small molecule that stimulates leukocyte αMβ2 interaction with ICAM-1 and iC3b-presenting cells (52). Mechanistically, LA-1 suppresses leukocyte infiltration into tissues by increasing αM/CD11b-dependent cell adhesion to ICAM-1 on the endothelium, preventing subsequent extravasation (53, 54). Modeling suggests that LA-1 binds at the interface between the α1 and β1 domains and involves the C-terminal end of the α1 a7 helix carrying the internal ligand. Such a binding pocket is difficult to reconcile with considerable rotational freedom of the α1 domain in the ligand-bound state, so in LA-1-bound αMβ2, the α1 domain may have significantly less rotational freedom compared with αMβ2 not binding this small-molecule drug. Our crystal structure provides a valuable scaffold for accurate modeling of αMβ2 complexes with existing and future function-modulating molecules.

The mechanism and application of the hCD11bNb1 Nb

In our report, we characterize an Nb-based αMβ2 agonist with previously unappreciated mode of action. With an epitope on the α1 domain, hCD11bNb1 represents a highly specific reagent compared with conventional monoclonal antibodies, which stimulate ligand interaction to αMβ2 by manipulating the conformation of the β2 subunit (55). The Nb stimulated binding of iC3b to cell-bound αMβ2 similar to the agonist LA-1, but with an epitope quite far from the putative LA-1-binding site at the αM–β2 interface. The mechanism of hCD11bNb1 stimulation of iC3b binding to αMβ2 on cells appears to be unique and complex in the light of the inhibition of iC3b–αMβ2 HP and C3d–α1 interaction observed in binding experiments with the pure components.

In general, a Nb is a versatile module that is easily humanized and targeted to specific tissues and cell types by fusion to other proteins. Fusion may also increase the short circulation time of unmodified Nbs (reviewed in Ref. (56)). Animal experiments could investigate the in vivo utility of properly modified hCD11bNb1 as a highly specific αMβ2 agonist, but such studies are complicated by the lack of crossreactivity with the murine α1 domain. Another major complication with respect to the in vivo effects of our Nb is the large number of proteins reported to interact with the α1 domain besides iC3b (51), with ICAM-1, fibrinogen, RAGE, JAM-C, and glycoprotein b as prominent examples. For other ligands, steric hindrance exerted by hCD11bNb1 could be larger than for the C3d/iC3b–α1 interactions investigated here. In such cases, rather than functioning as an agonist, the Nb may function as an antagonist. In contrast, if steric hindrance with the Nb does not occur for other αMβ2–ligand pairs, an even stronger stimulation of ligand binding by hCD11bNb1 may be experienced.

Experimental procedures

Nb selection

The hCD11bNb1 Nb was selected as previously described (29). Briefly, a Lama glama was immunized with the αMβ2 HP by Capralogics (www.capralogics.com), and the peripheral blood lymphocytes were isolated from a blood sample. The RNA was purified from these lymphocytes and used to prepare a complementary DNA library. The region corresponding to
the variable domain of heavy-chain only antibodies were cloned into a phagemid vector by PCR, and phage display was used to select Nbs specific toward the α1 domain of αMβ2. *Escherichia coli* TG1 cells harboring the phagemid vectors were coinfected with the VCMS13 helper phages and grown for 16 h at 30 °C to generate Nb-presenting phages. Meanwhile, one well in a microtiter plate was coated with 1 μg of αMβ2 α1 domain in 100 μl PBS and 3 mM MgCl2. The well of the microtiter plate was subsequently blocked by addition of PBS and 3 mM MgCl2 supplemented with 2% (w/w) bovine serum albumin (BSA). Next, 3 × 1012 Nb-presenting M13 phages were added to the well, and the plate was incubated for 1 h at room temperature to allow binding of phages to the α1 domain. Next, the well was washed 15 times in PBS, 3 mM MgCl2, 0.1% Tween-20, and 15 times in PBS and 3 mM MgCl2 to remove unbound phages. The α1 domain–binding phages were liberated through competitive elution by addition of a 100-fold molar excess, to αMβ2 α1 domain, of recombinant C3d in PBS and 3 mM MgCl2. The eluted phages were amplified in the ER2748/TG1 strain *E. coli* and provided the basis for the second round of selection, performed similarly, however only using 0.1 μg αMβ2 α1 domain for coating. ELISA was used to identify Nbs binding the αMβ2 α1 domain. To this end, an ELISA plate was coated with 100 μl of 0.1 μg/ml αMβ2 α1 domain in PBS and 3 mM MgCl2. Meanwhile, in a 96-well format, single phage–infected colonies were inoculated LB and grown at 37 °C for 6 h followed by induction of Nb expression by addition of isopropyl-β-D-thiogalactopyranoside side to a final concentration of 0.8 mM. The cells were grown for 16 h at 30 °C, then pelleted, and the Nb-enriched supernatant was transferred to the ELISA plate. The plate was incubated for 1 h followed by six washes in PBS, 3 mM MgCl2, and 0.1% Tween-20. Then, 1:10,000 diluted E-tag-horseradish peroxidase antibody (Bethyl) was added, and the plate was incubated for 1 h. The plate was washed and developed using 3,3′,5,5′-tetramethylbenzidine, until a clear signal was obtained. About 1 M HCl was added to stop further development, and the absorbance at 450 nm was measured. Unique Nbs were identified by sequencing and subsequently cloned into the bacterial expression vector pET-22b(+).

**Protein production**

αMβ2 HP was produced as previously described (11). In short, the supernatant of stable human embryonic kidney 293S cells expressing αMβ2 HP was recovered and purified by immobilized ion-affinity chromatography using a 5 ml HisTrap Excel column (GE Healthcare). The protein was subsequently applied to a 1 ml StrepTactin column (GE Healthcare) yielding pure αMβ2 HP. The affinity tags and coiled coil domains were removed by addition of the 3C protease, and a final polishing step was performed using SEC into 20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM MgCl2, and 1 mM CaCl2. Recombinant α1 domain was purified as described (11).

C3 was purified and cleaved to C3b as described (29). C3b was cleaved to iC3b by addition of 1% (w/w) factor H (Complement Tech) and 0.2% (w/w) factor I (Complement Tech), and the reaction was incubated for 16 h at 4 °C. The cleavage was assessed by SDS-PAGE and stopped by addition of 0.5 mM Pefabloc SC. To remove C3c or C3b, the sample was loaded on a 1 ml MonoQ column (GE Healthcare) equilibrated in 20 mM Hepes (pH 7.5) and 200 mM NaCl. The protein was eluted by a 30 ml linear gradient from 200 to 350 mM NaCl. C3d was purified as described (11). The hCD11bNb1 point mutants I29A, N30A, F32A, F54A, and N101A were generated by site-directed mutagenesis using the QuickChange Lightning kit (Agilent). hCD11bNb1, hCD11bNb1 mutants, and avi-tagged hCD11bNb1 were purified and generated as described for hC3Nb1 and avi-tagged hC3Nb1 (29). Endotoxin removal from Nbs used for flow cytometry was performed as described (57). Endotoxin levels were quantified using LAL chromogenic endotoxin quantification kit (Thermo Fisher Scientific) performed as described by the manufacturer. Nbs with endotoxin levels below 2 EU/mg Nb were considered to be endotoxin free.

**SEC assays**

For analysis of hCD11bNb1: αMβ2–α1 interaction, 40 μg αMβ2 α1 domain was incubated in the presence or the absence of a 1.5-fold molar excess of hCD11bNb1 in 20 mM Hepes (pH 7.5), and 150 mM NaCl. The mix was incubated for 30 min on ice and then applied to a 24 ml Superdex 75 increase (GE Healthcare) column equilibrated in 20 mM Hepes (pH 7.5) and 150 mM NaCl. For analysis of the inhibition, 170 μg α1 domain and an equimolar amount of C3d were incubated for >5 min on ice in the presence or the absence of a twofold molar excess of hCD11bNb1 in a reaction buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl, and 2 mM MgCl2. The mix was next applied to a 24 ml Superdex 75 increase column equilibrated in 20 mM Hepes (pH 7.5), 150 mM NaCl, and 2 mM MgCl2.

**Structure determination**

Crystals of hCD11bNb1 were grown by vapor diffusion at 4 °C by mixing an hCD11bNb1 solution at 35 mg/ml 1:1 with reservoir solution containing 1.5 M AmSO4 and 0.1 M Bis–Tris (pH 6.5). The crystals were soaked in reservoir solution supplemented with 30% glycerol before being flash frozen in liquid nitrogen. The data were collected at BioMAX (MAX IV) at 100 K and processed with XDS (58). A search model was prepared for molecular replacement using Phenix.sculpt (59), and the structure was solved with Phaser (60). Missing residues and side chains were built using Coot (61). In an iterative manner, the structure was rebuilt in Coot and refined with Phenix.refine using positional refinement, individual B-factors, and TLS groups. In the last round of refinement, anisotropic B-factors were refined for the sulfur atoms.

Prior to crystallization of the Nb complex, αMβ2 HP in 20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM MgCl2, and 1 mM CaCl2 was mixed with a 1.5-fold molar excess of hCD11bNb1 to a final complex concentration of 9 mg/ml. Crystals were grown at 19 °C by vapor diffusion in sitting
Structure of the αMB2 headpiece in the closed conformation

drops made by mixing the complex in a 1:1 ratio with reservoir containing 1.25 M sodium malonate, 76 mM Hepes (pH 8.0), 24 mM Hepes (pH 6.5), and 0.5% Jefﬁeamine ED2001 (pH 7.0). The crystals were soaked in a saturated sodium malonate solution before being flash frozen in liquid nitrogen. The data were collected at BioMAX at 100 K and processed with XDS (58). The structure was determined using the coordinates of the β-propeller from αβ2 (Protein Data Bank [PDB] entry: 4NEH) and βI domain of αβ2 (PDB entry: 5E6S) in Phaser (60). The remaining domains were placed manually in COOT (61). The resulting model was reﬁned with rigid body reﬁnement in Phenix.refine (59). At this stage, it had become apparent that the data suffered from anisotropic diﬀraction. The data were therefore scaled anisotropically using the STARANIISO server (24). Following this, the structure was manually rebuilt in COOT and reﬁned with Phenix.refine using positional reﬁnement, grouped B-factors, and TLS groups in an iterative manner. In the ﬁnal round of reﬁnement, individual B-factor reﬁnement was conducted.

BLI

All BLI experiments were performed on an Octet Red96 (ForteBio) at 30 °C and shaking at 1000 rpm. The running and wash buffer was 20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM MgCl2, and 1 mM CaCl2 unless otherwise stated. For assessing the binding of hCD11bNb1 to αMB2 HP, anti–penta-HIS sensors (ForteBio) were ﬁrst washed for 2 min, followed by a 5 min loading step where hCD11bNb1 at 5 μg/ml was loaded on the sensors. Subsequently, the sensors were washed for 30 s and then baselined for 2 min. Association to αMB2 HP (50, 25, 12.5, 6.25, and 0 nM) was followed for 3 min, followed by a 5 min dissociation step. The binding assay with the αMB2 αI domain was performed in the same manner, except that the association was followed for 300 s, and that the concentrations used were 25, 12.5, 6.25, 3.135, 1.6, 0.8, and 0 nM. All experiments were performed in triplicates. The 0 nM measurements were subtracted from all data series before ﬁtting to a 1:1 Langmuir binding model. The association was modeled as:

\[ R(t) = R_{\text{max}} \left( \frac{[\alpha\beta_2]/([\alpha\beta_2] + K_D)(1-\exp(-t \cdot (k_{\text{on}}[\alpha\beta_2] - k_{\text{off}})))}{K_D = k_{\text{on}}/k_{\text{off}}} \right) \]

For the competition assay assessing the ability of different hCD11bNb1 mutants to compete with WT hCD11bNb1 for αMB2 binding, the streptavidin biosensors (ForteBio) were ﬁrst washed for 2 min in running buffer supplemented with 1 mg/ml BSA, before biotinylated avi-tagged hCD11bNb1 at 5 μg/ml were loaded on the sensors for 5 min. The sensors were then washed for 2 min in running buffer supplemented with 1 mg/ml BSA, before being baselined for 2 min. Thereafter, association between 20 nM αMB2 HP alone or 20 nM αMB2 HP preincubated with 10-fold molar excess of hCD11bNb1 mutants I29A, N30A, F32A, F54A, N101A, or WT hCD11bNb1 was followed for 5 min. Subsequently, the dissociation was followed for 5 min.

For analysis of the interaction between hCD11bNb1 and murine αMB2 αI, anti–penta-HIS sensors were washed for 5 min in 20 mM Hepes (pH 7.5) and 150 mM NaCl. The hCD11bNb1 at 5 μg/ml was loaded onto the sensors, followed by a 2 min wash step and a 2 min baselined step. The association of hCD11bNb1 to murine αI (50, 25, 12.5, 6.25, 3.135, and 0 nM) or 50 nM human αI was followed for 5 min followed by a 5 min dissociation step. The 0 nM measurement was subtracted from all data series. This experiment was performed in duplicates.

SPR

The SPR experiment was performed on a Biacore T200 (GE Healthcare) instrument as described (11). The system was equilibrated in running buffer 20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM MgCl2, and 1 mM CaCl2. Streptavidin was immobilized on a CMD500M chip (XanTec bioanalytics GmbH) to 200 response units. Next, biotinylated iC3b was injected on one ﬂow cell in excess, saturating the chip surface. For competition experiment, αMB2 at 1.25 to 100 nM was injected in either the presence or the absence of a 1.5-fold molar excess of hCD11bNb1. Upon the competition experiment, 50 mM EDTA, 1 M NaCl, and 100 mM Hepes (pH 7.5) were injected over the chip to regenerate the surface.

Mass photometry

The measurements were performed on glass coverslips and recorded on a mass photometer (MP_T2WG; Refeyn Ltd) (32) for 60 to 120 s. Each measurement was repeated at least twice. The αMB2 HP and iC3b were immediately prior to measurements in 20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM MgCl2, and 1 mM CaCl2. For 1:1 complex formation, αMB2 HP and iC3b were mixed in a 1:1 M ratio, whereas αMB2 HP and hCD11bNb1 were mixed in a 1:10 M ratio. The inﬂuence of hCD11bNb1 on αMB2 HP–iC3b complex formation was measured using molar ratios of hCD11bNb1 to αMB2 HP (0.49, 0.74, 1.1, 1.6, 2.5, 5, and 10). The recorded videos were analyzed using DiscoverMP (Refeyn Ltd; version 2.5.0) to quantify protein-binding events. The molecular weight was obtained by contrast comparison with known mass standard calibrants measured on the same day.

Cell-expressed αMB2–ligand interaction with iC3b

The binding of iC3b by cell-expressed αMB2 was investigated by use of K562 cells with a recombinant expression of αMB2, or, as a control, αXβ2. For binding under conditions approaching equilibrium, αMB2/K562 cells were cultured and treated with ﬂuorescence-conjugated iC3b as described (11). Brieﬂy, the cells were kept in buffer with 1 mM Ca2+ and 1 mM Mg2+ or as further supplemented with 1 mM Mn2+ to activate integrin ligand binding. Following 45 min of incubation with 10 μg/ml Alexa Fluor 488-conjugated (iC3b*) together with 0, 1, 5, or 10 μg/ml of either hCD11bNb1 or an unrelated control (Ctrl) Nb speciﬁc for complement C4 called
To investigate the influence of hCD11bNb1 under cell adhesion with a mimic of the shear stress—implicating cells under physiological conditions, adhesion of αMβ2/K562 or αXβ2/K562 cells was tested in a centrifugation-based assay described earlier (63). Briefly, V-shaped microtiter wells were coated with 1 μg/ml iC3b (A115; Complement Tech) or not coated as reference, and blocked in PBS with 0.05% (v/v) Tween-20. Cells were applied either in buffer with Ca²⁺ and Mg²⁺ or with a further addition of 1 mM Mn⁵⁺ to activate integrin ligand binding. Nbs were added in concentrations of 0, 1, 5, or 10 μg/ml for either buffer condition. Following incubation for 10 min at 37 °C, the cells were centrifuged at 10g for 5 min and read in a fluorescence plate reader. The centrifugation and plate reading were repeated at 30g, 50g, and 100g.

Data availability

Coordinates and structure factor for the hCD11Nb–αMβ2 complex and hCD11Nb1 are available at the PDB as entries 7P2D and 7NP9.

Supporting information—This article contains supporting information.

Acknowledgements—We are grateful to the excellent technical assistance from Karen Margrethe Nielsen and Bettina Grumsen and the staff at BioMAX and Petra P13 for assistance with data collection. We appreciate mentoring by Dr Goran Bajic in the early phase of our work with αMβ2. This work was supported by the Lundbeck Foundation, Denmark (BRAINSTRUC; grant no.: R155-2015-2666), the Danish Foundation for Independent Research (grant no: 4181-00137), and the Novo Nordisk Foundation, Denmark (grant no.: NNF18OC0052105).

Author contributions—R. K. J., J. L., T. V.-J., and G. R. A. methodology; R. K. J., J. L., T. V.-J., and G. R. A. formal analysis; R. K. J., H. P., and J. L. investigation; R. K. J., H. P., T. V.-J., and G. R. A. writing—original draft.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: BLI, biolayer interferometry; BSA, bovine serum albumin; C3, component 3; CDR, complementarity-determining region; HP, headpiece; ICAM, intercellular adhesion molecule; I-EGF1, integrin epidermal growth factor 1; LA-1, leukadherin-1; MFI, mean fluorescence intensity; MIDAS, metal ion—dependent adhesion site; Nb, nanobody; PDB, Protein Data Bank; PSI, plexin–semaphorin–integrin; SEC, size-exclusion chromatography; SLE, systemic lupus erythematosus; SPR, surface plasmon resonance.

References

1. Carman, C. V., and Springer, T. A. (2003) Integrin avidity regulation: are changes in affinity and conformation underemphasized? Curr. Opin. Cell Biol. 15, 547–556
2. Xie, C., Zhu, J., Chen, X., Mi, L., Nishida, N., and Springer, T. A. (2010) Structure of an integrin with an alphaR domain, complement receptor type 4. EMBO J. 29, 666–679
3. Sen, M., Yuki, K., and Springer, T. A. (2013) An internal ligand-bound, metastable state of a leukocyte integrin, alphaXBeta2. J. Cell Biol. 203, 629–642
4. Sen, M., and Springer, T. A. (2016) Leukocyte integrin alphaLbeta2 headpiece structures: the alphaR domain, the pocket for the internal ligand, and concerted movements of its loops. Proc. Natl. Acad. Sci. U. S. A. 113, 2940–2945
5. Luo, B. H., Carman, C. V., and Springer, T. A. (2007) Structural basis of integrin regulation and signaling. Annu. Rev. Immunol. 25, 619–647
6. Michishita, M., Videv, V., and Arnaout, M. A. (1993) A novel divalent cation-binding site in the A domain of the beta 2 integrin CR3 (CD11b/CD18) is essential for ligand binding. Cell 72, 857–867
7. Lee, J. O., Rieu, P., Arnaout, M. A., and Liddington, R. (1995) Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). Cell 80, 631–639
8. Bajic, G., Yatime, L., Sim, R. B., Vorup-Jensen, T., and Andersen, G. R. (2013) Structural insight on the recognition of surface-bound opsonins by the integrin I domain of complement receptor 3. Proc. Natl. Acad. Sci. U. S. A. 110, 16426–16431
9. Shimaoka, M., Xiao, T., Liu, J. H., Yang, Y., Dong, Y., Jun, C. D., et al. (2003) Structures of the alpha I I domain and its complex with ICAM-1 reveal a shape-shifting pathway for integrin regulation. Cell 112, 99–111
10. Zipfel, P. F., and Skerka, C. (2009) Complement regulators and inhibitory proteins. Nat. Rev. Immunol. 9, 729–740
11. Jensen, R. K., Bajic, G., Sen, M., Springer, T. A., Vorup-Jensen, T., and Andersen, G. R. (2021) Complement receptor 3 forms a compact high-affinity complex with iC3b. J. Immunol. 206, 3032–3042
12. Stevens, B., Allen, N. J., Vazquez, L. E., Howell, G. R., Christopherson, K. S., Nouri, N., et al. (2007) The classical complement cascade mediates CNS synapse elimination. Cell 131, 1164–1178
13. Schafer, D. P., Lehman, E. K., Kutzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., et al. (2012) Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neurosci. 74, 691–705
14. Waksman, S., Bechade, C., Roumier, A., Bernard, D., Triller, A., and Bessis, A. (2008) Developmental neuronal death in hippocampus requires the microglial CD11b integrin and DAP12 immunoreceptor. J. Neurosci. 28, 8138–8143
15. Jiang, L., Chen, S. H., Chu, C. H., Wang, S. J., Oyarzabal, E., Wilson, B., et al. (2015) A novel role of microglial NADPH oxidase in mediating extra-synaptic function of norepinephrine in regulating brain immune homeostasis. Glia 63, 1057–1072
16. Heesters, B. A., van der Poel, C. E., Das, A., and Carroll, M. C. (2016) Antigen presentation to B cells. Trends Immunol. 37, 844–854
17. Chen, J., Salas, A., and Springer, T. A. (2003) Bistable regulation of integrin adhesiveness by a bipolar metal ion cluster. Nat. Struct. Biol. 10, 995–1001
18. Huth, J. R., Olejniczak, E. T., Mendoza, R., Liang, H., Harris, E. A., Luperhe, M. L., Jr., et al. (2000) NMR and mutagenesis evidence for an I domain allosteric site that regulates lymphocyte function-associated antigen-1 ligand binding. Proc. Natl. Acad. Sci. U. S. A. 97, 5231–5236
19. Alonso, J. L., Essafi, M., Xiong, J. P., Stehle, T., and Arnaout, M. A. (2002) Does the integrin alphaA domain act as a ligand for its betaR domain? Curr. Biol. 12, R340–R342
20. Xiao, T., Takagi, J., Coller, B. S., Wang, J. H., and Springer, T. A. (2004) Structural basis for allostericity in integrins and binding to fibrinogen-mimetic therapeutics. Nature 432, 59–67
Structure of the $\alpha_{\text{M}}\beta_2$ headpiece in the closed conformation

21. Moore, T. I., Aaron, J., Chew, T. L., and Springer, T. A. (2018) Measuring integrin conformational change on the cell surface with super-resolution microscopy. Cell Rep. 22, 1903–1912

22. Tereshko, V., Uysal, S., Koide, A., Margalef, K., Koide, S., and Kossiakoff, A. A. (2008) Toward chaperone-assisted cryo-electron microscopy: protein engineering enhancement of crystal packing and X-ray phasing capabilities of a cameldil single-domain antibody (VHH) scaffold. Protein Sci. 17, 1175–1187

23. D’Imprima, E., Floris, D., Joppe, M., Sanchez, R., Grininger, M., and Kuhlbrandt, W. (2019) Protein denaturation at the air-water interface and how to prevent it. Elife 8, e42747

24. Tickle, I. J., Flensburg, C., Keller, P., Paciorek, W., Sharff, A., Vonrhein, C., et al. (2018) STARANISO Global Phasing Ltd, Cambridge, United Kingdom

25. Nath, S. K., Han, S., Kim-Howard, X., Kelly, J. A., Viswanathan, P., Gilkeson, G. S., et al. (2008) A nonsynonymous functional variant in integrin-alphaM (encoded by ITGAM) is associated with systemic lupus erythematosus. Nat. Genet. 40, 152–154

26. Rhodes, B., Furnrohr, B. G., Roberts, A. L., Tzircotis, G., Schett, G., Tpector, T. D., et al. (2012) The rs1143679 (R77H) lupus associated variant of ITGAM (CD11b) impairs complement receptor 3 mediated functions in human monocytes. Ann. Rheum. Dis. 71, 2028–2034

27. MacPherson, M., Lek, H. S., Prescott, A., and Fagerholm, S. C. (2011) A systemic lupus erythematosus-associated R77H substitution in the CD11b chain of the Mac-1 integrin compromises leukocyte adhesion and phagocytosis. J. Biol. Chem. 286, 17303–17310

28. Rosetti, F., Chen, Y., Sen, M., Thayer, E., Azcutia, V., Herter, J. M., et al. (2015) A lupus-associated Mac-1 variant has defects in integrin allostery and interaction with ligands under force. Cell Rep. 10, 1655–1664

29. Jensen, M. R., Bajic, G., Zhang, X., Laustsen, A. K., Koldso, H., Skeby, K., et al. (2016) Structural basis for simvastatin competitive antagonism of the leukocyte integrin conformational change on the cell surface with super-resolution microscopy. J. Biol. Chem. 291, 1955–1965

30. Saratavazo, A., Presumey, I., Simon, I., Yalcin, E., Fox, R., Hansen, A., et al. (2020) An ultra-high-affinity complement C4b-specific nanobody inhibits in vivo assembly of the classical pathway proconvertase. J. Immunol. 205, 1678–1694

31. Laursen, N. S., Pedersen, D. V., Gytz, H., Saratavazo, A., Berth Jensen, J. M., Hansen, A. G., et al. (2020) Functional and structural characterization of a potent Clq inhibitor targeting the classical pathway of the complement system. Front. Immunol. 11, 1504

32. Young, G., Hundt, N., Cole, D., Fineberg, A., Andrecke, J., Tyler, A., et al. (2018) Quantitative mass imaging of single biological macromolecules. Science 360, 423–427

33. Jensen, M. R., Bajic, G., Zhang, X., Laustsen, A. K., Koldso, H., Skeby, K. K., et al. (2016) Structural basis for simvastatin competitive antagonism of complement receptor 3. J. Biol. Chem. 291, 16963–16976

34. Adair, B. D., Xiong, J., P. W., Alonso, J. L., Hyman, B. T., and Arnaout, M. A. (2013) EM structure of the ectodomain of integrin CD11b/CD18 and localization of its ligand-binding site relative to the plasma membrane. Proc. Natl. Acad. Sci. U. S. A. 110, 4586–4591

35. Sens, M., Koksik, A. C., Yuki, K., Wang, J., and Springer, T. A. (2018) Ligand- and cation-induced structural alterations of the leukocyte integrin LFA-1. J. Biol. Chem. 293, 6565–6577

36. Zhu, J., Zhu, J., and Springer, T. A. (2013) Complete integrin headpiece opening in eight steps. J. Cell Biol. 201, 1053–1068

37. Chen, X., Yu, Y., Mi, L. Z., Walz, T., and Springer, T. A. (2012) Molecular basis for complement recognition by integrin alphaXbeta2. Proc. Natl. Acad. Sci. U. S. A. 109, 4586–4591

38. Zhang, H., Casasnovas, J. M., Jin, M., Liu, J. H., Gahmberg, C. G., Springer, T. A., et al. (2008) An unusual allosteric mobility of the C-terminal helix of a high-affinity alphaL integrin I domain variant bound to ICAM-5. Mol. Cell 31, 432–437

39. Song, G., Yang, Y., Liu, J. H., Casasnovas, J. M., Shimaoka, M., Springer, T. A., et al. (2005) An atomic resolution view of ICAM recognition in a complex between the binding domains of ICAM-3 and integrin alphabeta2. Proc. Natl. Acad. Sci. U. S. A. 102, 3366–3371

40. Morgan, J., Saleem, M., Ng, R., Armstrong, C., Wong, S. S., Caulton, S. G., et al. (2019) Structural basis of the leukocyte integrin Mac-1 I-domain interactions with the platelet glycoprotein Ib. Blood Adv. 3, 1450–1459

41. Fernandez, F. J., Santos-Lopez, J., Martinez-Barricarte, R., Querol-Garcia, J., Martin-Merino, H., Navas-Yuste, S., et al. (2022) The crystal structure of iC3b-CR3 alphal reveals a modular recognition of the main opsonin iC3b by the CR3 integrin receptor. Nat. Commun. 13, 1955

42. Kadioglu, A., De Filippo, K., Bangert, M., Fernandes, V. E., Richards, L., Jones, K., et al. (2011) The integrins Mac-1 and alpha4beta1 perform crucial roles in neutrophil and T cell recruitment to lungs during Streptococcus pneumoniae infection. J. Immunol. 186, 5907–5915

43. Hong, S., Beja-Glasser, V. F., Nilooyini, B. M., Frouin, A., Li, S., Ramakrishnan, S., et al. (2016) Complement and microglia mediate early synapse loss in Alzheimer mouse models. Science 352, 712–716

44. Stapollini, R., Oliveira, C. L., Gjelstrup, M. C., Pedersen, J. S., Høkland, M. E., Hoffmann, S. V., et al. (2008) Structural insight into the function of myelin basic protein as a ligand for integrin alphaM beta2. J. Immunol. 180, 3946–3956

45. Jalilian, B., Einarsson, H. B., and Vorup-Jensen, T. (2012) Glatiramer acetate in treatment of multiple sclerosis: a toolbox of random copolymers for targeting inflammatory mechanisms of both the innate and adaptive immune system? Int. J. Mol. Sci. 13, 14579–14605

46. Krams, M., Lees, K. R., Hacke, W., Greive, A. P., Orpogozo, J. M., Ford, G. A., et al. (2003) Acute stroke therapy by inhibition of neutrophils (ASTIN): an adaptive dose-response study of UK-279,276 in acute ischemic stroke. Stroke 34, 2543–2548

47. Buyon, J. P., Shadick, N., Berkman, R., Hopkins, P., Dalton, J., Weissmann, G., et al. (1988) Surface expression of Gp 165/95, the complement receptor CR3, as a marker of disease activity in systemic Lupus erythematosus. Clin. Immunol. Immunopathol. 46, 141–149

48. Khan, S. Q., Khan, L., and Gupta, V. (2018) CD11b activity modulates pathogenesis of lupus nephritis. Front. Med. (Lausanne) 5, 52

49. International Consortium for Systemic Lupus Erythematosus, G., Harley, J. B., Alarcón-Riquelme, M. E., Criswell, L. A., Jacob, C. O., Kimberly, R. P., et al. (2008) Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. Nat. Genet. 40, 204–210

50. Hom, G., Graham, R. M., Modrek, B., Taylor, K. E., Ortmann, W., Garnier, S., et al. (2008) Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. N. Engl. J. Med. 358, 900–909

51. Lamers, C., Pluss, C. J., and Ricklin, D. (2021) The promiscuous profile of complement receptor 3 in ligand binding, immune modulation, and pathophysiology. Front. Immunol. 12, 662164

52. Maiguel, D., Faridi, M. H., Wei, C., Kuwano, Y., Balla, K. M., Hernandez, S., et al. (2019) Agonism of CD11b reprograms innate immunity to inflammatory disease. J. Immunol. 202, 2581–2588
58. Kabsch, W. (2010) Integration, scaling, space-group assignment and post-refinement. *Acta Crystallographica. Section D, Biol. Crystallogr.* **66**, 133–144

59. Afonine, P. V., Grosse-Kunstleve, R. W., Echols, N., Headd, J. J., Moriarty, N. W., Mustyakimov, M., *et al.* (2012) Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallographica. Section D, Biol. Crystallogr.* **68**, 352–367

60. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* **40**, 658–674

61. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallographica. Section D, Biol. Crystallogr.* **66**, 486–501

62. Malhotra, V., Hogg, N., and Sim, R. B. (1986) Ligand binding by the p150, 95 antigen of U937 monocytic cells: properties in common with complement receptor type 3 (CR3). *Eur. J. Immunol.* **16**, 1117–1123

63. Juul-Madsen, K., Qvist, P., Bendtsen, K. L., Langkilde, A. E., Vestergaard, B., Howard, K. A., *et al.* (2020) Size-selective phagocytic clearance of fibrillar alpha-synuclein through conformational activation of complement receptor 4. *J. Immunol.* **204**, 1345–1361