Published in final edited form as:
*J Cell Physiol*. 2011 February ; 226(2): 469–476. doi:10.1002/jcp.22356.

# αVβ3 Integrin Regulates Macrophage Inflammatory Responses via PI3 Kinase/Akt-Dependent NF-κB Activation

**ALEXANDER S. ANTONOV**[1], **GALINA N. ANTONOVA**[1], **DAVID H. MUNN**[2], **NAHID MIVECHI**[3], **RUDOLF LUCAS**[1,4,5], **JOHN D. CATRAVAS**[1,4,5], and **ALEXANDER D. VERIN**[1,6,*]

[1]Vascular Biology Center, Medical College of Georgia, Augusta, Georgia

[2]Department of Pediatrics and Cancer Immunotherapy Program, Medical College of Georgia, Augusta, Georgia

[3]Center for Molecular Chaperone/Radiobiology and Cancer Virology, Medical College of Georgia, Augusta, Georgia

[4]Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, Georgia

[5]Cancer Center, Medical College of Georgia, Augusta, Georgia

[6]Department of Medicine, Medical College of Georgia, Augusta, Georgia

## Abstract

Controlling macrophage responses to pathogenic stimuli is critical for prevention of and recovery from the inflammatory state associated with the pathogenesis of many diseases. The adhesion receptor αVβ3 integrin is thought to be an important receptor that regulates macrophage differentiation and macrophage responses to external signaling, but it has not been previously identified as a contributor to macrophage-related inflammation. Using an in vitro model of human blood monocytes (Mo) and monocyte-derived macrophages (MDMs) we demonstrate that αVβ3 ligation results in sustained increases of the transcription factor NF-κB DNA-binding activity, as compared with control isotype-matched $IgG_1$. Activation of NF-κB parallels the increase of NF-κB-dependent pro-inflammatory cytokine mRNA expression in MDMs isolated from individual donors, for example, TNF-α (8- to 28-fold), IL-1β (15- to 30-fold), IL-6 (2- to 4-fold), and IL-8 (5- to 15-fold) whereas there is more than a 10-fold decrease in IL-10 mRNA level occurs. Upon ligation of the αVβ3 receptor, treatment with TNF-α (10 ng/ml) or LPS (200 ng/ml, 1,000 EU) results in the enhanced and synergistic activation of NF-κB and LPS-induced TNF-α secretion. As additional controls, an inhibitor of αVβ3 integrin, cyclic RGD (10 μg/ml; $IC_{50}$ = 7.6 μM), attenuates the effects of αVβ3 ligation, and the natural ligand of αVβ3 integrin, vitronectin, reproduces the effects of αVβ3 activation by an immobilizing anti-αVβ3 integrin mAb. We hypothesize that αVβ3 activation can maintain chronic inflammatory processes in pathological conditions and that the loss of αVβ3 ligation will allow macrophages to escape from the inflammatory state.

It is now widely recognized that macrophages are major components of the inflammatory and immunological reactions typically seen in inflammatory diseases. Macrophages can respond to environmental factors, account for the clearance of microparticles and microorganisms and serve as a first line of defense in the site of inflammation. This cell type is thought to orchestrate the progression of inflammation by producing pro- and anti-



*Correspondence to: CB-3210, Medical College of Georgia, 1120 15th Street, Augusta, GA 30912. averin@mcg.edu .

Additional Supporting Information may be found in the online version of this article.

inflammatory mediators and by controlling the expression of endothelial adhesion receptors. In addition, macrophages play an important role in inflammatory processes through the release of oxygen radicals and proteolytic enzymes.

αVβ3 integrin (vitronectin receptor, CD51/CD61) is a ubiquitous receptor that is expressed on a variety of cell types (Felding-Habermann and Cheresh, 1993; Byzova et al., 1998; Antonov et al., 2004; Cai and Chen, 2006), including differentiated macrophages. αVβ3 integrin interacts with the ligands present in the extracellular matrix or expressed on the cell surface in inflamed tissues. Importantly, αVβ3 integrin is highly expressed on activated or growing cells under pathological conditions, but its expression on quiescent cells in normal tissues is minimal (Laitinen et al., 2009). Therefore, αVβ3 integrin is thought to play a key role in the initiation and/or progression of several human diseases, including osteoporosis, rheumatoid arthritis, cancer, atherosclerosis, and ocular diseases (Byzova and Plow, 1998; Byzova et al., 1998; Eliceiri and Cheresh, 1999; Tucker, 2002; Antonov et al., 2004; Galliher and Schiemann, 2006; McCabe et al., 2007). However, to date αVβ3 integrin has received little attention as a potential macrophage activator and contributor to inflammation.

TNF-α is a key pro-inflammatory cytokine that is secreted in response to several risk factors (Lucas et al., 2009) and plays an important role in macrophage-related inflammatory processes. LPS is the principal component of the outer membrane of Gram-negative bacteria and induces the expression of many pro-inflammatory mediators in monocyte (Mo)/ macrophages, including TNF-α. Moreover, LPS may cause systemic inflammatory responses and local tissue injury (Parrillo, 1993; Xu et al., 2001; Leu et al., 2006; Jean-Baptiste, 2007). Although TNF-α is required for the regulation of normal inflammatory and immune responses, acutely elevated levels of the cytokine caused by an overreaction to the presence of LPS can lead to severe pathological conditions including sepsis, septic shock, or acute lung injury, while chronically elevated levels of TNF-α are associated with the inflammatory processes of several chronic diseases, including rheumatoid arthritis, osteoporosis, and atherosclerosis (Hamilton and Clair, 2000; Libby, 2002; Zhang et al., 2009a,b).

Recent evidence suggests that activation of the transcription factor NF-κB may be critical in linking diverse risk factors to the initiation and expansion of inflammation (Blackwell et al., 2000; Christman et al., 2000; Rajendrasozhan et al., 2010). The signaling pathways and the mechanisms of NF-κB activation/deactivation are well established for many cell types including macrophages (Liu and Malik, 2006). NF-κB is involved in the activation of an exceptionally large number of genes that are relevant to the pathophysiology of inflammation, including cytokines, chemokines, and leukocyte adhesion molecules, as well as genes that regulate cell proliferation and mediate cell survival. Important stimuli, with respect to inflammatory responses, that result in rapid and transient NF-κB activation include, among others, TNF-α, IL-1β, LPS, and oxidized LDL (Ohlsson et al., 1996; Thurberg and Collins, 1998; Zhang et al., 2009b). This beneficial NF-κB activation may be exaggerated in pathological situations that may occur either through the persistence of the stimulating agent(s) or through impairment of the mechanisms of NF-κB down-regulation (Parsons et al., 1989; Parrillo, 1993; Zhang et al., 2009a). This could result in persistent NF-κB activity, leading to inappropriate inflammatory responses. There is increasing evidence that the prolonged activation of NF-κB plays a critical role in maintaining the chronic inflammatory state associated with progression of many diseases by modulating genes involved in inflammatory responses in Mo/macrophages.

Signaling pathways involved in the activation of NF-κB in the response to external stimuli are specific for different cell types. NF-κB activation in macrophages can be regulated by PI3 kinase/Akt or p38 MAPK, dependent on external signals (Ardeshna et al., 2000).

Additionally, it was reported that these signaling pathways are involved in the transition of αVβ3-induced signals to the NF-κB-dependent cellular responses in human chondrosarcoma cells (Yeh et al., 2008) and A549 lung cancer cells (Chetty et al., 2009), implying that PI3 kinase and p38 MAPK may be targeted by αVβ3 ligation, leading to inappropriate macrophage inflammatory responses. Little is known about the specific signaling pathways controlling persistent αVβ3-mediated macrophage activation.

Macrophage differentiation into a distinct phenotype is controlled by cytokines and adhesion molecules in response to external stimuli. We have recently demonstrated that αVβ3 integrin regulates macrophage differentiation into the foam cell phenotype that is directly associated with the pathogenesis of atherosclerosis (Antonov et al., 2004). We now hypothesize that αVβ3 integrin may also regulate macrophage-related inflammatory responses via PI3K/Akt-dependent activation of NF-κB. We speculate that activation of macrophages by αVβ3 integrin ligation results in amplification of secretion of pro-inflammatory mediators and sensitizes macrophages to synergistic responses to well-characterized pro-inflammatory stimuli.

## Methods

### Antibodies and reagents

Monoclonal antibodies (mAb) to αVβ3 (LM609) integrin were from Chemicon International (Temecula, CA); isotype control $IgG_1$ was from Pharmingen (San Diego, CA). Polyclonal antibodies to p50 and p65 subunits of NF-κB were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit Ab to phospho-Akt (Ser473) and phospho-p38 MAPK (Thr180/Tyr182) were from Cell Signaling Technology, Inc. (Beverly, MA). LY294002 and SB203580 were purchased from Biomol Research Laboratories (Plymouth, PA). Human recombinant TNF-α, LPS, rabbit anti-actin Ab, and vitronectin were from Sigma–Aldrich (St. Louis, MO). Human recombinant M-CSF was a gift from Genetics Institute, Inc. (Cambridge, MA). Cyclic RGD was a gift from Merck KGaA (Darmstadt, Germany). All other reagents, if not indicated, were from Sigma–Aldrich.

### Leukocyte isolation and in vitro model of αVβ3 ligation

Human Mo were obtained from 20 healthy donors of either sex by leukocytopheresis followed by counterflow centrifugation as described (Antonov et al., 1997) under a protocol approved by our Institutional Review Board. These cells (>95% Mo by morphology and cell surface markers) were either used immediately or cryopreserved in liquid nitrogen. Fresh and frozen Mo behaved indistinguishably in all assays. Mo and monocyte derived macrophages (MDMs) were used. To produce MDMs, Mo were differentiated in vitro for 5–7 days in the presence of M-CSF (200 U/ml) as described (Antonov et al., 2004). M-CSF is an important survival and growth factor for long-term Mo culture and Mo differentiation into MDMs (Wang et al., 2007; Way et al., 2009) and up-regulates αVβ3 integrin expression in cultured MDMs (Antonov et al., 2004). Previously, we demonstrated that in this model Mo can be cultured up to 24 days without detectable cell death or decreases in cell number (Antonov et al., 1997). In the current study, we monitored cell viability using cell counting after trypan blue staining. We did not observe cell death in the re-seeding model or in Mo when they were cultured on immobilized αVβ3 ligands. For αVβ3 integrin ligation, MDMs were harvested on ice and re-seeded on mAb or vitronectin immobilized on plastic dishes. Two hrs after re-seeding, nonadherent cells were aspirated and cells were cultured in the presence of M-CSF. More than 95% of re-seeded MDMs adhered to the substrate in 1 h. Most experiments with the re-seeding model were performed no longer than 24 h after re-seeding. Antibodies were immobilized onto the substrate by incubating dishes with 10 μg/ml of mAb in PBS for 18 h at 4°C. Vitronectin was immobilized on substrate by incubating

dishes with10 μg/ml of vitronectin in PBS for 2 h at 4°C according to the protocol from Sigma-Aldrich (St. Louis, MO). When the effects of αVβ3 ligation on Mo differentiation were of interest, Mo were seeded directly on immobilized mAb and cultured for 1–7 days as described (Antonov et al., 2004).

### Electrophoretic mobility shift assay (EMSA)

For detection of NF-κB-binding activities, nuclear extracts (NEs) were prepared from $10^7$ cells using NE-PER Reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. Protein concentration was measured with the BCA protein assay reagents (Pierce). NEs (5 μg protein) were assayed for DNA-binding activity in a total volume of 20 μl, 4 μl of 5×binding buffer (20% Ficoll-400, 20 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 0.25% NP-40, 0.5 mM DTT, and 0.5 mM PMSF), 200 ng/ml poly d(I-C). NEs were incubated for 20 min at room temperature with double-stranded labeled probes for NF-κB: 5′-GGGTTGTTACAA**GGGACTTTC**CGCTG**GGGACTTTC**CAGGGAGGCGTGG-3′ from the human immunodeficiency virus long terminal repeat (Gibco BRL, custom primers, Grand Island, NY). The binding sites responsible for NF-κB DNA binding are underlined and boldface. The samples were then loaded onto a nondenatured polyacrylamide gel and subjected to electrophoresis in TBE buffer. Gels were dried and exposed to Kodak XAR films at −80°C or were examined with PhosphoImager (Molecular Dynamics, Newark, DE). The specificity of binding was determined using competition assays with unlabeled oligonucleotide. Supershift experiments were performed by incubating NEs with Abs to the p50 and p65 subunits of NF-κB (20 μg/ml) for 20 min at 4°C or with the irrelevant $IgG_1$ Ab as a control before the adding of labeled oligonucleotide. These experiments confirmed that the mobility–shifted complex observed in EMSA is in fact comprised of NF-κB (Supplemental Fig. 1A). We also demonstrated that ligation of αVβ3 integrin results in nuclear translocation of both p50 and p65 subunits of NF-κB (Supplemental Fig. 1B).

### TNF-α mRNA expression and protein secretion

Total RNA was isolated using Qiagen RNeasy Mini Kit according to the manufacturer's instructions. For quantitative RT-PCR, total RNA was reverse transcribed and amplified using the LightCycler System (Roche Applied Science, Indianapolis, IN) with forward primer 5′-TCAACCTCCTCTCTGCCATCA AGA GC-3′and reverse primer 5′-GTCGAGATAGTCGGGCCGATTGAT C-3′ as described by Weghofer et al. (2001). The RT-PCR data were normalized to β-actin and cell number; data is presented as alterations in mRNA as fold of control $IgG_1$. The amount of TNF-α secreted was measured using an ELISA kit (R&D System, Minneapolis, MN). To prove that secreted TNF-α is biologically active we used a cell-based bioassay of TNF-α activity. Bioactive TNF-α levels in supernatants of treated cells were detected using the WEHI 164 subclone 13-cell line as previously described (Espevik and Nissen-Meyer, 1986). Briefly, cells were resuspended at $3\times10^5$ cells/ml in RPMI-1640 (Bio-Whittaker, Walkersville, MD) supplemented with 10% fetal calf serum, 2 mmol ʟ-glutamine, and 0.5 μg/ml actinomycin D, and dispensed into 96-well cell culture plates (Costar, Cambridge, MA). Samples were added to the cells in 8 twofold dilution steps, each step performed in triplicate. With each assay, a titration of recombinant human TNF-α (specific activity $2\times10^7$ IU/mg; kind gift from Dr. Daniela Maennel, University of Regensburg, Germany) was included as a standard. Samples were then incubated for 17 h at 37°C, after which 0.1% Triton-X was added for 1 h to the three negative control wells. Wells were incubated with 10 vol% of Alamar Blue solution (AbD Serotec, Oxford, UK). After 2 h of incubation, plates are read in the Paradigm Cytofluorimeter (Beckman-Coulter, Brea, CA) at an excitation wavelength of 530 nm and emission wavelength of 590 nm.

### Statistical analysis

Experiments were repeated with Mo isolated from at least five different donors with consistent results. Values are reported as means ±SE. Comparisons between control and treated cells were performed utilizing unpaired *t*-test or one-way ANOVA, as appropriate, with the aid of GraphPad InStat 3.0 software. Differences of $P \leq 0.05$ were considered significant.

## Results

### Effect of αVβ3 integrin ligation on NF-κB activity and mRNA expression and major NF-κB-dependent cytokines

The binding of a Mo surface receptor to a specific mAb immobilized on plastic might mimic the effects of receptor engagement by its ligand on another cell or in the extracellular matrix (Antonov et al., 2004). Therefore, to test the hypothesis that αVβ3 integrin is involved in controlling NF-κB activation, we used MDMs differentiated in vitro for 5–7 days and then re-seeded them on an anti-αVβ3 integrin mAb immobilized on substrate, or on an immobilized control isotype-matched $IgG_1$ antibody. To analyze NF-κB activity, we measured the ability of NF-κB to bind to DNA by EMSA. The specificity of binding was demonstrated using supershift assay with Abs to the p50 and p65 subunits of NF-κB. We found that the mobility–shift complex observed in EMSA is in fact comprised of NF-κB (Supplemental Fig. 1A). We also demonstrated that ligation of αVβ3 integrin results in nuclear translocation of both p50 and p65 subunits of NF-κB (Supplemental Fig. 1B). Adhesion of MDMs to substrate resulted in a rapid but transient activation of NF-κB, returning to the initial level of NF-κB activity 4 h after re-seeding (Fig. 1A, lanes 2, 5, and 7), By contrast, MDMs that were re-seeded on an αVβ3 integrin antibody demonstrated an enhanced and prolonged (up to 24 h) NF-κB activation (Fig. 1A, lanes 4, 6, and 8). An NF-κB-dependent increase in TNF-α secretion is a well-characterized indicator of macrophage-related inflammatory responses. In order to verify that NF-κB activation by αVβ3 integrin ligation results in downstream inflammatory responses, we measured TNF-α secretion by MDMs. We found that the magnitude and duration of TNF-α secretion by MDMs that were re-seeded on mAb to αVβ3 integrin paralleled NF-κB activity (Fig. 1B). Next we examined the effect of αVβ3 integrin ligation on mRNA expression of several NF-κB-dependent cytokines. Using quantitative RT-PCR, we found that ligation of αVβ3 integrin on MDMs caused 8- to 28-fold increases in TNF-α mRNA expression (Fig. 1C), suggesting that the effects of αVβ3 integrin ligation on TNF-α secretion occur at the transcriptional level. Additionally, αVβ3 integrin ligation results in increases in IL-1β (15- to 30-fold), IL-6 (2- to 4-fold), and IL-8 (5- to 15-fold) mRNA expression. By contrast, in the same experiments, mRNA expression of the anti-inflammatory cytokine IL-10 was significantly suppressed. Finally, we demonstrated that the effect of αVβ3 ligation by antibodies can be reproduced by its physiological ligand vitronectin (Fig. 1D). Moreover, αVβ3 inhibition by the cRGD peptide abolished the effects of αVβ3 ligation by vitronectin and anti-αVβ3 mAb on the secretion of bioactive TNF-α by macrophages, as measured in the WEHI 164 bioassay (Fig. 1D). Taken together, these data suggest that the αVβ3 receptor is critically involved in the regulation of macrophage transition into a pro-inflammatory phenotype, as characterized by enhanced macrophage-related inflammatory responses.

### Effect of αVbβ3 integrin ligation on LPS- and TNF-α-indiced NF-κB activation

To test the hypothesis that αVβ3 integrin modulates inflammatory responses, we examined the effect of αVβ3 integrin ligation on NF-κB activation induced by LPS and TNF-α treatment. In these experiments, MDMs were re-seeded on the immobilized mAbs for 24 h, the medium was replaced and cells were stimulated with LPS (200 ng/ml, 1,000 EU) or TNF-α (10 ng/ml) for 1–24 h. We found that ligation of αVβ3 integrin on MDMs results in

sustained and enhanced NF-κB activation in response to LPS (Fig. 2A, lanes 5 and 7) and TNF-α (Fig. 2A, lanes 9, 11, and 13) stimulation, whereas MDMs re-seeded on the control $IgG_1$ demonstrate rapid but transient NF-κB activation induced by LPS (Fig. 2A, lanes 4 and 6) or TNF-α (Fig. 2A, lanes 8, 10, and 12). As predicted, MDMs that were re-seeded on anti-αVβ3 integrin mAb responded to LPS stimulation through significantly enhanced TNF-α secretion at all time intervals examined (Fig. 2B).

### Ligation of αVβ3 integrin on MDMs results in their transition into a sustained pro-inflammatory phenotype

Next, we examined the effect of LPS stimulation on NF-κB activity and TNF-α secretion in MDMs that had been re-seeded on the immobilized mAbs for prolonged times. Four days after re-seeding on the anti-αVβ3 integrin mAb, the MDMs demonstrated both enhanced basal (Fig. 3A, lane 2) and LPS-inducible (Fig. 3A, lane 4) NF-κB activity. At all time intervals examined (up to 7 days), LPS-induced TNF-α secretion by MDMs cultured on the anti-αVβ3 integrin mAb was significantly increased as compared with MDMs cultured on control $IgG_1$ (Fig. 3B), suggesting that MDMs over-respond to LPS exposure as long as αVβ3 integrin is ligated.

### Role of PI3 kinase/Akt signaling pathway

Depending on external signals, PI3 kinase/Akt and p38 MAPK can regulate NF-κB activity in macrophages (Ardeshna et al., 2000; Wang et al., 2007). We examined which signaling pathway is responsible for NF-κB activation induced by αVβ3 integrin ligation on MDMs and for enhanced responses to LPS stimulation. We examined the effect of αVβ3 ligation on activation of these signaling pathways in MDMs using Western blots with phospho-specific antibodies. MDMs that were re-seeded for 24 h on the immobilized anti-αVβ3 integrin mAb demonstrated significant increases in phospho-Akt and phospho-p38 MAPK signals (Fig. 4A). When MDMs were re-seeded on the immobilized anti-αVβ3 integrin mAb in the presence of a specific inhibitor of the PI3 kinase/Akt pathway, LY294002 (10 μM), activation and prolongation of NF-κB induced by αVβ3 integrin ligation was completely abolished (Fig. 4B, lanes 2 and 4) as revealed by EMSA, whereas an inhibitor of p38 MAPK, SB203580 (20 μM), has only a partial effect on NF-κB activity (Fig. 4B, lanes 2 and 6). It has been shown that the PI3 kinase/Akt pathway can regulate LPS-inducible NF-κB activation (Ardeshna et al., 2000). Treatment with LY294002 abolishes the effect of LPS stimulation on NF-κB activity in MDMs re-seeded on either $IgG_1$ or on the mAb to αVβ3 integrin (Fig. 4C, lanes 5 and 6). By contrast, the p38 MAPK inhibitor SB203580 has no effect on LPS-inducible NF-κB activation in MDMs re-seeded on $IgG_1$ or αVβ3 integrin (Fig. 4C, lanes 7 and 8).

### αVβ3 integrin ligation in blood Mo results in their differentiation into a sustained pro-inflammatory phenotype

Recently, using quantitative flow cytometry, we demonstrated that αVβ3 integrin is expressed not only in differentiated macrophages (Antonov et al., 2004) but also in circulating blood Mo. We reported that expression of αVβ3 integrin in Mo can be up-regulated by stressful signals such as M-CSF and oxidized LDL (Antonov et al., 2004). We therefore examined whether differentiation of Mo on immobilized anti-αVβ3 integrin mAb results in their transition into a pro-inflammatory phenotype. Freshly isolated Mo were seeded on the immobilized anti-αVβ3 integrin mAb or the control $IgG_1$ and differentiated in culture for the indicated period of time. Ligation of the αVβ3 integrin in blood Mo for 24 h increases the activity of NF-κB (Fig. 5A, left part, lanes 1 and 2) and results in enhanced responses to LPS stimulation (Fig. 5A, left part, lanes 3 and 4). When the cells are further differentiated in vitro for 5 days, Mo that are cultured on the anti-αVβ3 integrin mAb showed increased basal NF-κB activity (Fig. 5B, right part, lanes 1 and 2) and an enhanced

NF-κB activation induced by LPS (Fig. 5B, right part, lanes 3 and 4). As predicted, maturation of Mo parallels sustained increases in TNF-α secretion after LPS stimulation (Fig. 5C).

## Discussion

Transient macrophage activation induced by risk factors is considered to be an important defense mechanism of physiologically relevant cellular responses to intruder stimuli. However, when macrophages differentiate into a persistent pro-inflammatory phenotype, this can drive the inflammatory events to a pathological state associated with macrophage dysfunction. In the present study we demonstrate for the first time that signaling pathways initiated by αVβ3 integrin ligation modulate TNF-α and LPS-induced transient NF-κB activation towards an enhanced and sustained NF-κB activation, that primes macrophages to an over-reaction to LPS or TNF-α exposure. We have identified activation of the PI3 kinase/Akt signaling pathway as a possible mechanism leading from αVβ3 receptor to NF-κB-dependent pro-inflammatory gene activation. We thus postulate that the αVβ3 receptor plays a crucial role in controlling local and systemic macrophage-related inflammation. Indeed, αVβ3 ligation sensitizes macrophages for enhanced responses to LPS and TNF-α stimulation, implying that αVβ3 ligation results in a pathological loop formation that maintains abnormal macrophage responses to exogenous stimulation. Thus, the macrophage phenotype, characterized by transient and physiologically relevant inflammatory responses, can be transmitted by αVβ3 integrin ligation into an established pathologic pro-inflammatory phenotype (Fig. 6). As such, our data provide a rationale for understanding the mechanisms controlling persistent, macrophage-related inflammation as well as the mechanisms leading to impaired responses to TNF-α and LPS exposure. These findings uncover a novel role for the αVβ3 integrin in controlling macrophage inflammatory responses, which may be important for the development of therapeutic anti-inflammatory strategies.

Previously we demonstrated (Antonov et al., 2004) that αVβ3 integrin is expressed not only on differentiated macrophages but also on peripheral blood Mo and that its expression can be up-regulated by M-CSF and oxidized LDL. It thus appears that αVβ3 integrin expression on blood Mo may be up-regulated in response to certain conditions (such as atherogenic risk factors or M-CSF). As blood Mo migrate through the endothelium into the site of inflammation, αVβ3 receptor can be activated by its ligands exposed on the endothelium or extracellular matrix. Current data demonstrate that peripheral blood Mo can be differentiated into a pro-inflammatory phenotype capable of secreting inflammatory cytokines such as TNF-α, when αVβ3 integrin is ligated. If this hypothesis is correct, interruption of the signaling induced by αVβ3 ligation will allow macrophages to escape from the sustained pro-inflammatory state (Fig. 6).

αVβ3 integrin expression in macrophages located in different tissue compartments depends on the balance between normal and pathological conditions in the sites where macrophages accumulate (Byzova et al., 1998). We have previously demonstrated that macrophages located in the tonsils or spleen do not express αVβ3 integrin, whereas macrophages present in atherosclerotic lesions demonstrate a high level of αVβ3 expression (Antonov et al., 2004). Since both αVβ3 ligand and the cells expressing this receptor are present in the same milieu, in this scenario, αVβ3 integrin should be persistently activated. However, αVβ3 integrin ligation activates macrophages only under specific conditions. The co-existence of both ligands and receptor in normal tissue is probably not sufficient for macrophage activation, and other alternative mechanisms, for example, the mechanisms of NF-κB deactivation, are involved to ensure that responses induced by αVβ3 integrin are physiologically relevant. However, in inflamed tissue, the crosstalk between αVβ3 integrin

and other activated receptors and signaling pathways, as well as cell–cell interactions, may be important to initiate a cascade of αVβ3-dependent events ultimately leading to macrophage differentiation into a sustained and irreversible pro-inflammatory phenotype. Also, it is possible that αVβ3 activation in macrophages initiates a currently unknown crosstalk between activated macrophages and other cells, involved in inflammatory reactions, such as polymorphonuclear leukocytes and T-lymphocytes (Fig. 6).

The in vivo studies investigating the involvement of αVβ3 integrin in the regulation of inflammation are controversial. As such, it was reported that macrophage β3 integrin suppresses hyperlipidemia-induced inflammation in an apoE-null and LDL receptor-null mouse model of atherosclerosis (Schneider et al., 2007). It was also demonstrated that β3 integrin deficiency promotes atherosclerosis in the same mouse model (Weng et al., 2003). By contrast, in vivo studies demonstrate anti-inflammatory effects of αV antagonism in a model of acute kidney allograft rejection (Bedke et al., 2007) and in a model of heart transplant rejection using β3 integrin-deficient mice (Lacy-Hulbert et al., 2007). Additionally, studies of lung inflammation induced by intratracheal LPS administration show that αVβ3 inhibitors suppress lung inflammation (Lee et al., 2007; Moon et al., 2009). These latter reports support our hypothesis that αVβ3 integrin plays a pro-inflammatory role.

Research efforts to elucidate the mechanisms of pathogenesis of atherosclerosis are tremendously important because this inflammatory disease has the highest mortality rate in the Western population. Even with recent progress in treatments of atherosclerosis, studies of the pathogenic mechanisms may be important for the development of new therapeutic strategies against this inflammatory state. Many studies have confirmed that macrophages play a critical role in orchestrating the inflammatory processes characteristically seen in atherosclerotic plaques (Schaffner et al., 1980; Brown and Goldstein, 1990; Ross, 1993, 1999). The prevailing view of atherosclerosis also implicates lipid accumulation in the arterial wall, particularly in macrophages, which is seen as critical to the initiation and development of these inflammatory events (Gerrity, 1981; Brown and Goldstein, 1990; Ross, 1993, 1999; Huh et al., 1996; Silverstein and Febbraio, 2000). We have recently shown that αVβ3 ligation prevents differentiation of human Mo/macrophages into a foam cell phenotype in vitro (Antonov et al., 2004). There is a consensus that a balance between pro- and anti-atherogenic mechanisms is crucial for lesion progression or regression. There are no data demonstrating that one physiological mechanism or pharmacological treatment can control both foam cell development and inflammatory processes in the lesion. Based on our previous (Antonov et al., 2004) and our current studies of the regulation of macrophage inflammatory responses by αVβ3 integrin, our hypothesis potentially links foam cell formation and macrophage-related inflammation and may provide new insights into the mechanisms controlling pro- and anti-atherogenic processes in the lesion. As such, the αVβ3 integrin emerges as a potentially important mediator regulating macrophage responses to inflammatory signals, suggesting it to be a single cell surface receptor which can simultaneously regulate two processes critical to the progression of atherosclerosis. Our data suggest that antagonists of αVβ3 integrin, such as cyclic RGD, that have shown positive results in clinical trials for several pathologies (Miller et al., 2000; Tucker, 2002) could potentially be useful in the treatment of inflammatory diseases in which activated macrophages exert pathogenic effects.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The authors gratefully acknowledge the late Dr. Ross Gerrity for scientific discussions and advice. We thank J. Waller, R. Eisenstein, and M. Curuk for technical assistance; N. Freeman for help with manuscript preparation and Dr. Rhea Markowitz for critical reading of manuscript. This work was supported by a grant from the Department of Pathology, MCG to A.S. Antonov and the start up fund to A.D. Verin.

Contract grant sponsor: Department of Pathology, MCG.

## Literature Cited

Antonov AS, Munn DH, Kolodgie FD, Virmani R, Gerrity RG. Aortic endothelial cells regulate proliferation of human monocytes in vitro via a mechanism synergistic with macrophage colony-stimulating factor. Convergence at the cyclin E/p27(Kip1) regulatory checkpoint. J Clin Invest. 1997; 99:2867–2876. [PubMed: 9185509]

Antonov AS, Kolodgie FD, Munn DH, Gerrity RG. Regulation of macrophage foam cell formation by alphaVbeta3 integrin: Potential role in human atherosclerosis. Am J Pathol. 2004; 165:247–258. [PubMed: 15215180]

Ardeshna KM, Pizzey AR, Devereux S, Khwaja A. The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. Blood. 2000; 96:1039–1046. [PubMed: 10910920]

Bedke J, Kiss E, Behnes CL, Popovic ZV, Heuser M, Stojanovic T, Sijmonsma T, Huber P, Domhan S, Muschal S, Abdollahi A, Gretz N, Grone HJ. Anti-inflammatory effects of alphav integrin antagonism in acute kidney allograft rejection. Am J Pathol. 2007; 171:1127–1139. [PubMed: 17702892]

Blackwell TS, Yull FE, Chen CL, Venkatakrishnan A, Blackwell TR, Hicks DJ, Lancaster LH, Christman JW, Kerr LD. Multiorgan nuclear factor kappa B activation in a transgenic mouse model of systemic inflammation. Am J Respir Crit Care Med. 2000; 162:1095–1101. [PubMed: 10988136]

Brown MS, Goldstein JL. Atherosclerosis. Scavenging for receptors. Nature. 1990; 343:508–509. [PubMed: 2300201]

Byzova TV, Plow EF. Activation of alphaVbeta3 on vascular cells controls recognition of prothrombin. J Cell Biol. 1998; 143:2081–2092. [PubMed: 9864377]

Byzova TV, Rabbani R, D'Souza SE, Plow EF. Role of integrin alpha(v)beta3 in vascular biology. Thromb Haemost. 1998; 80:726–734. [PubMed: 9843163]

Cai W, Chen X. Anti-angiogenic cancer therapy based on integrin alphavbeta3 antagonism. Anticancer Agents Med Chem. 2006; 6:407–428. [PubMed: 17017851]

Chetty C, Lakka SS, Bhoopathi P, Rao JS. MMP-2 alters VEGF expression via alphaVbeta3 integrin-mediated PI3K/AKT signaling in A549 lung cancer cells. Int J Cancer. 2009; 124:2468–2477. [PubMed: 19165865]

Christman JW, Sadikot RT, Blackwell TS. The role of nuclear factor-kappa B in pulmonary diseases. Chest. 2000; 117:1482–1487. [PubMed: 10807839]

Eliceiri BP, Cheresh DA. The role of alphav integrins during angiogenesis: Insights into potential mechanisms of action and clinical development. J Clin Invest. 1999; 103:1227–1230. [PubMed: 10225964]

Espevik T, Nissen-Meyer J. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J Immunol Methods. 1986; 95:99–105. [PubMed: 3782828]

Felding-Habermann B, Cheresh DA. Vitronectin and its receptors. Curr Opin Cell Biol. 1993; 5:864–868. [PubMed: 7694604]

Galliher AJ, Schiemann WP. Beta3 integrin and Src facilitate transforming growth factor-beta mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res. 2006; 8:R42. [PubMed: 16859511]

Gerrity RG. The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am J Pathol. 1981; 103:181–190. [PubMed: 7234961]

Hamilton K, Clair EW. Tumour necrosis factor-alpha blockade: A new era for effective management of rheumatoid arthritis. Expert Opin Pharmacother. 2000; 1:1041–1052. [PubMed: 11249494]

Huh HY, Pearce SF, Yesner LM, Schindler JL, Silverstein RL. Regulated expression of CD36 during monocyte-to-macrophage differentiation: Potential role of CD36 in foam cell formation. Blood. 1996; 87:2020–2028. [PubMed: 8634453]

Jean-Baptiste E. Cellular mechanisms in sepsis. J Intensive Care Med. 2007; 22:63–72. [PubMed: 17456726]

Lacy-Hulbert A, Ueno T, Ito T, Jurewicz M, Izawa A, Smith RN, Chase CM, Tanaka K, Fiorina P, Russell PS, Auchincloss H Jr. Sayegh MH, Hynes RO, Abdi R. Beta 3 integrins regulate lymphocyte migration and cytokine responses in heart transplant rejection. Am J Transplant. 2007; 7:1080–1090. [PubMed: 17359504]

Laitinen I, Saraste A, Weidl E, Poethko T, Weber AW, Nekolla SG, Leppanen P, Yla-Herttuala S, Holzlwimmer G, Walch A, Esposito I, Wester HJ, Knuuti J, Schwaiger M. Evaluation of alphavbeta3 integrin-targeted positron emission tomography tracer 18F-galacto-RGD for imaging of vascular inflammation in atherosclerotic mice. Circulation. 2009; 2:331–338. [PubMed: 19808614]

Lee HS, Moon C, Lee HW, Park EM, Cho MS, Kang JL. Src tyrosine kinases mediate activations of NF-kappaB and integrin signal during lipopolysaccharide-induced acute lung injury. J Immunol. 2007; 179:7001–7011. [PubMed: 17982091]

Leu TH, Charoenfuprasert S, Yen CK, Fan CW, Maa MC. Lipopolysaccharide-induced c-Src expression plays a role in nitric oxide and TNFalpha secretion in macrophages. Mol Immunol. 2006; 43:308–316. [PubMed: 15869794]

Libby P. Inflammation in atherosclerosis. Nature. 2002; 420:868–874. [PubMed: 12490960]

Liu SF, Malik AB. NF-kappa B activation as a pathological mechanism of septic shock and inflammation. Am J Physiol. 2006; 290:L622–L645.

Lucas R, Verin AD, Black SM, Catravas JD. Regulators of endothelial and epithelial barrier integrity and function in acute lung injury. Biochem Pharmacol. 2009; 77:1763–1772. [PubMed: 19428331]

McCabe NP, De S, Vasanji A, Brainard J, Byzova TV. Prostate cancer specific integrin alphavbeta3 modulates bone metastatic growth and tissue remodeling. Oncogene. 2007; 26:6238–6243. [PubMed: 17369840]

Miller WH, Keenan RM, Willette RN, Lark MW. Identification and in vivo efficacy of small-molecule antagonists of integrin alphavbeta3 (the vitronectin receptor). Drug Discov Today. 2000; 5:397–408. [PubMed: 10931657]

Moon C, Han JR, Park HJ, Hah JS, Kang JL. Synthetic RGDS peptide attenuates lipopolysaccharide-induced pulmonary inflammation by inhibiting integrin signaled MAP kinase pathways. Respir Res. 2009; 10:18. [PubMed: 19272161]

Ohlsson BG, Englund MC, Karlsson AL, Knutsen E, Erixon C, Skribeck H, Liu Y, Bondjers G, Wiklund O. Oxidized low density lipoprotein inhibits lipopolysaccharide-induced binding of nuclear factor-kappaB to DNA and the subsequent expression of tumor necrosis factor-alpha and interleukin-1beta in macrophages. J Clin Invest. 1996; 98:78–89. [PubMed: 8690807]

Parrillo JE. Pathogenetic mechanisms of septic shock. N Engl J Med. 1993; 328:1471–1477. [PubMed: 8479467]

Parsons PE, Worthen GS, Moore EE, Tate RM, Henson PM. The association of circulating endotoxin with the development of the adult respiratory distress syndrome. Am Rev Respir Dis. 1989; 140:294–301. [PubMed: 2764364]

Rajendrasozhan S, Hwang JW, Yao H, Kishore N, Rahman I. Anti-inflammatory effect of a selective IkappaB kinase-beta inhibitor in rat lung in response to LPS and cigarette smoke. Pulm Pharmacol Ther. 2010; 23:172–181. [PubMed: 20080200]

Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990s. Nature. 1993; 362:801–809. [PubMed: 8479518]

Ross R. Atherosclerosis—An inflammatory disease. N Engl J Med. 1999; 340:115–126. [PubMed: 9887164]

Schaffner T, Taylor K, Bartucci EJ, Fischer-Dzoga K, Beeson JH, Glagov S, Wissler RW. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. Am J Pathol. 1980; 100:57–80. [PubMed: 6772035]

Schneider JG, Zhu Y, Coleman T, Semenkovich CF. Macrophage beta3 integrin suppresses hyperlipidemia-induced inflammation by modulating TNFalpha expression. Arterioscler Thromb Vasc Biol. 2007; 27:2699–2706. [PubMed: 17951320]

Silverstein RL, Febbraio M. CD36 and atherosclerosis. Curr Opin Lipidol. 2000; 11:483–491. [PubMed: 11048891]

Thurberg BL, Collins T. The nuclear factor-kappa B/inhibitor of kappa B autoregulatory system and atherosclerosis. Curr Opin Lipidol. 1998; 9:387–396. [PubMed: 9812192]

Tucker GC. Inhibitors of integrins. Curr Opin Pharmacol. 2002; 2:394–402. [PubMed: 12127872]

Wang Y, Zeigler MM, Lam GK, Hunter MG, Eubank TD, Khramtsov VV, Tridandapani S, Sen CK, Marsh CB. The role of the NADPH oxidase complex, p38 MAPK, and Akt in regulating human monocyte/macrophage survival. Am J Respir Cell Mol Biol. 2007; 36:68–77. [PubMed: 16931806]

Way KJ, Dinh H, Keene MR, White KE, Clanchy FI, Lusby P, Roiniotis J, Cook AD, Cassady AI, Curtis DJ, Hamilton JA. The generation and properties of human macrophage populations from hemopoietic stem cells. J Leukoc Biol. 2009; 85:766–778. [PubMed: 19181863]

Weghofer M, Karlic H, Haslberger A. Quantitative analysis of immune-mediated stimulation of tumor necrosis factor-alpha in macrophages measured at the level of mRNA and protein synthesis. Ann Hematol. 2001; 80:733–736. [PubMed: 11797114]

Weng S, Zemany L, Standley KN, Novack DV, La Regina M, Bernal-Mizrachi C, Coleman T, Semenkovich CF. Beta3 integrin deficiency promotes atherosclerosis and pulmonary inflammation in high-fat-fed, hyperlipidemic mice. Proc Natl Acad Sci USA. 2003; 100:6730–6735. [PubMed: 12746502]

Xu XH, Shah PK, Faure E, Equils O, Thomas L, Fishbein MC, Luthringer D, Xu XP, Rajavashisth TB, Yano J, Kaul S, Arditi M. Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. Circulation. 2001; 104:3103–3108. [PubMed: 11748108]

Yeh YY, Chiao CC, Kuo WY, Hsiao YC, Chen YJ, Wei YY, Lai TH, Fong YC, Tang CH. TGF-beta1 increases motility and alphavbeta3 integrin up-regulation via PI3K, Akt and NF-kappaB-dependent pathway in human chondrosarcoma cells. Biochem Pharmacol. 2008; 75:1292–1301. [PubMed: 18191107]

Zhang H, Park Y, Wu J, Chen X, Lee S, Yang J, Dellsperger KC, Zhang C. Role of TNF-alpha in vascular dysfunction. Clin Sci (Lond). 2009a; 116:219–230. [PubMed: 19118493]

Zhang H, Zhang J, Ungvari Z, Zhang C. Resveratrol improves endothelial function: Role of TNF{alpha} and vascular oxidative stress. Arterioscler Thromb Vasc Biol. 2009b; 29:1164–1171. [PubMed: 19478208]

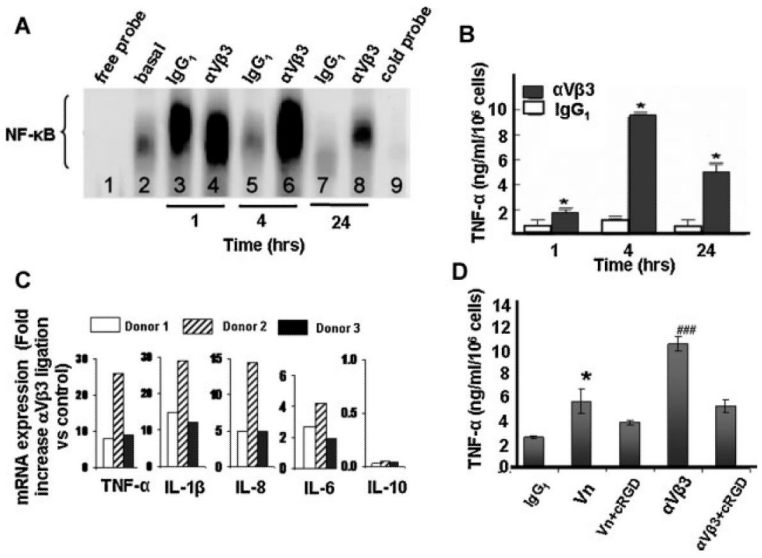


**Fig. 1.**

Ligation of αVβ3 integrin on MDMs results in NF-κB activation and increases in pro-inflammatory cytokines mRNA expression and enhanced TNF-α secretion. MDMs differentiated in vitro for 5–7 days in the presence of M-CSF were re-seeded on immobilized anti-αVβ3 integrin mAb, isotype-matched control $IgG_1$, or vitronectin (Vn) for the indicated time intervals. A: NF-κB DNA-binding activity was analyzed by EMSA in nuclear extracts as described in the Methods Section. Lane 2 shows basal NF-κB activity in MDMs prior to re-seeding; lanes 1 and 9 indicate free and excess unlabeled probes, respectively. B: In parallel with part A, TNF-α secretion by MDMs was measured by ELISA (n = 4; $^{*}P < 0.01$ vs. $IgG_1$, *t*-test). C: MDMs from three donors were re-seeded on immobilized anti-αVβ3 mAb or $IgG_1$ for 24 h, mRNA amounts were analyzed by RT-PCR as described in the Methods Section. D: MDMs were re-seeded on immobilized anti-αVβ3 mAb, $IgG_1$, or vitronectin (Vn) in the presence or absence of cRGD (10 μg/ml) for 24 h and TNF-α secretion into medium was determined using the WEHI164 fibrosarcoma cell bioassay as described in the Methods Section (n=6; $^{*}P < 0.03$, $^{###}P < 0.001$ vs. $IgG_1$, ANOVA: Kruskal–Wallis test with Dunn's post-test).

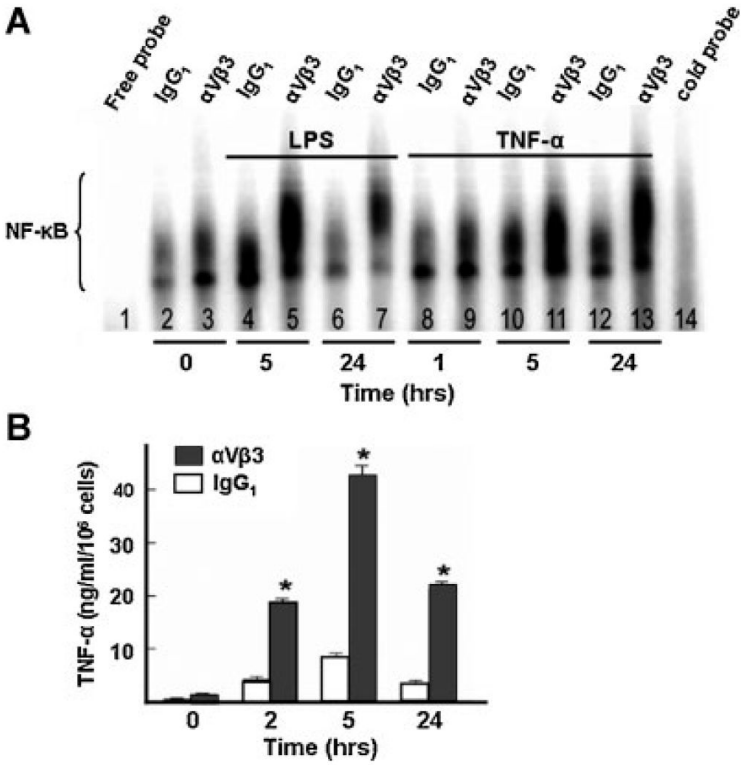


**Fig. 2.**

Ligation of αVβ3 integrin on MDMs results in enhanced responsiveness to LPS and TNF-α stimulation. A: MDMs were re-seeded on the anti-αVβ3 integrin immobilized mAb or control $IgG_1$ for 24 h, and MDMs were exposed to LPS (200 ng/ml, 1,000 EU) or TNF-α (10 ng/ml) for 1–24 h. NF-κB-binding activity was analyzed using EMSA as described in the Methods Section. Lanes 2 and 3 show basal NF-κB activity prior to stimulation. Lanes 1 and 14 represent free and excess unlabeled probes, respectively. B: MDMs prepared as in A were stimulated with LPS (200 ng/ml, 1,000 EU) and TNF-α secretion into medium was measured by ELISA (n=4; *$P < 0.0$ vs. $IgG_1$, *t*-test).

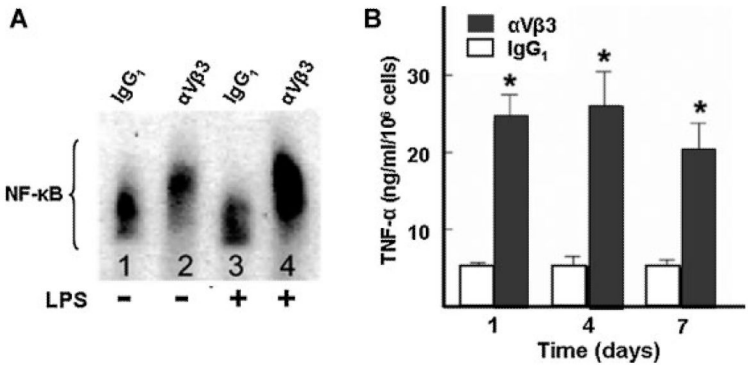


**Fig. 3.**

Ligation of αVβ3 integrin on MDMs results in a sustained hyperresponsiveness to LPS stimulation. A: MDMs were re-seeded on anti-αVβ3 integrin immobilized mAbs or $IgG_1$ and were cultured for an additional 4 days after re-seeding and then stimulated with LPS (200 ng/ml, 1,000 EU) for 5 h (lanes 3 and 4).Lanes 1 and 2 show basal NF-κB activity prior to stimulation. B:MDMs,re-seeded on the anti-αVβ3 integrin mAb or the control $IgG_1$, were stimulated with LPS (200 ng/ml) for 5 h on different days after re-seeding, and secretion of TNF-α was measured using ELISA (n=4, $*P < 0.01$ vs. $IgG_1$, *t*-test).

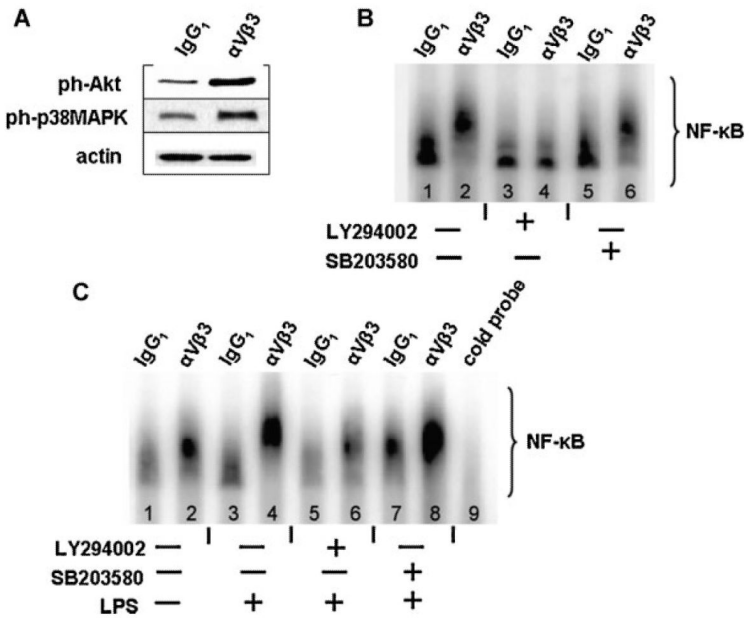


**Fig. 4.**

NF-κB activation induced by αVβ3 integrin ligation and enhanced responsiveness to LPS stimulation is PI3 kinase/Akt-dependent and p38 MAPK-independent. A: MDMs were re-seeded on the immobilized anti-αVβ3 integrin mAb or control $IgG_1$, and equal amounts of whole cell extracts were analyzed by Western blot 24 h after re-seeding using phosphorylation-specific antibodies. B: MDMs were re-seeded on the immobilized anti-αVβ3 integrin mAb or control $IgG_1$ in the presence of the specific inhibitor of PI3 kinase, LY294002 (10 μM) (lanes 3 and 4), inhibitor of p38 MAPK, SB203580 (20 μM) (lanes 5 and 6), or vehicle (DMSO) (lanes 1 and 2). NF-κB-binding activity was examined 24 h after re-seeding using EMSA as described in the Methods Section. C: MDMs were re-seeded on the anti-αVβ3 integrin or the $IgG_1$ mAbs for 24 h in the presence of LY294002 (10 μM) (lanes 5 and 6), SB203580 (20 μM) (lanes 7 and 8), or vehicle (lanes 3 and 4), then stimulated with LPS (200 ng/ml, 1,000 EU)for 5 h NF-κB binding was measured using EMSA. Lanes 1 and 2 show basal NF-κB activity prior to LPS stimulation. Lane 9 shows an excess of unlabeled probe.

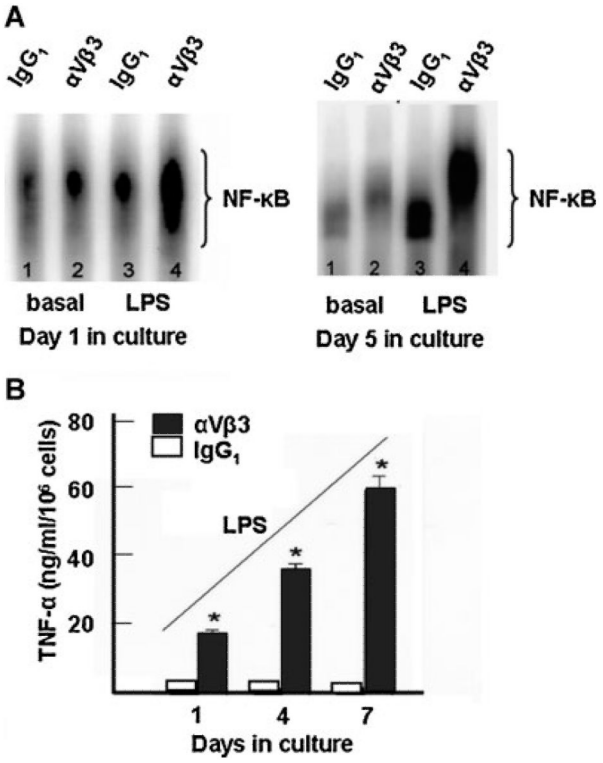


**Fig. 5.**

αVβ3 integrin ligation on blood Mo results in their differentiation into sustained pro-inflammatory phenotype. A: Mo were seeded directly on the immobilized anti-αVβ3 integrin mAb or the control $IgG_1$ and were differentiated in vitro for 1 day (left part) or 5 days (right part). Basal (lanes 1 and 2) and LPS-induced (200 ng/ml, 1,000 EU for 5 h) NF-κB activities (lanes 3 and 4) were measured using EMSA. B: In parallel with part A experiments, Mo were seeded on immobilized mAbs or $IgG_1$; LPS-induced (200 ng/ml for 5 h) TNF-α secretion by Mo was assessed at different stages of their maturation by ELISA (n=4; $*P < 0.01$ vs. control $IgG_1$, *t*-test).

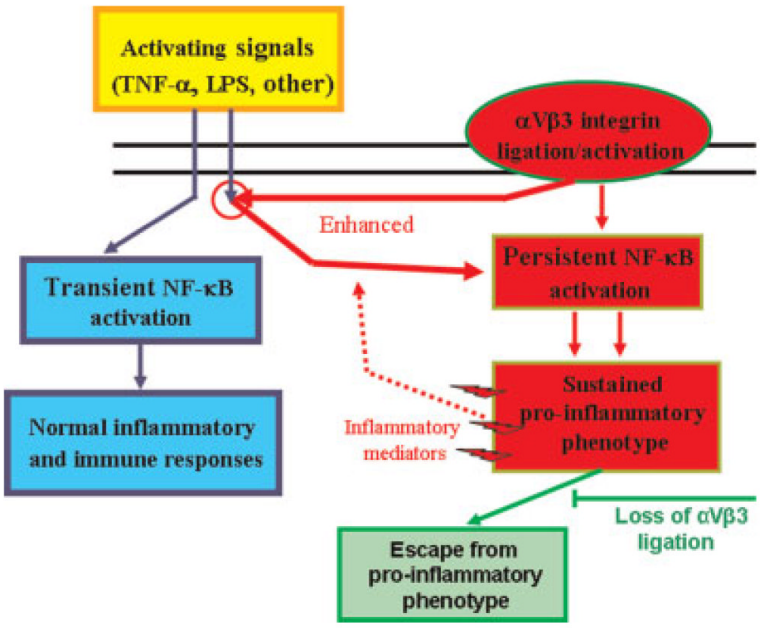


**Fig. 6.**

Hypothetical model of the regulation of macrophage-related inflammation by αVβ3 integrin ligation. Macrophages as a part of the host defense system respond to external pro-inflammatory stimuli (e.g., TNF-α, LPS) by transient NF-κB activation which leads to physiologically relevant and transient inflammatory responses (blue arrows). The feedback mechanisms of NF-κB deactivation prevent progression of macrophage-related inflammation into a pathological state. We hypothesize that if αVβ3 integrin is ligated (red arrows), under the same conditions this ligation results in sustained activation of NF-κB and the transition of macrophages into a pro-inflammatory phenotype. Moreover, the increased release of pro-inflammatory mediators (dotted arrows) potentially may lead to formation of a pathogenic loop maintaining chronic inflammation in the lesions. We speculate that loss of αVβ3 ligation allows macrophages to escape from the sustained pro-inflammatory phenotype (green arrows). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]