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α V β 3 Integrin Regulates Macrophage Inflammatory Responses via PI3 Kinase/Akt-Dependent NF- κ B Activation

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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 α V β 3 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 α V β 3 ligation results in sustained increases of the transcription factor NF- κ B DNA-binding activity, as compared with control isotype-matched IgG₁. Activation of NF- κ B parallels the increase of NF- κ B-dependent pro-inflammatory cytokine mRNA expression in MDMs isolated from individual donors, for example, TNF- α (8- to 28-fold), IL-1 β (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 α V β 3 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- κ B and LPS-induced TNF- α secretion. As additional controls, an inhibitor of α V β 3 integrin, cyclic RGD (10 μ g/ml; IC₅₀ = 7.6 μ M), attenuates the effects of α V β 3 ligation, and the natural ligand of α V β 3 integrin, vitronectin, reproduces the effects of α V β 3 activation by an immobilizing anti- α V β 3 integrin mAb. We hypothesize that α V β 3 activation can maintain chronic inflammatory processes in pathological conditions and that the loss of α V β 3 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-

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.

α V β 3 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. α V β 3 integrin interacts with the ligands present in the extracellular matrix or expressed on the cell surface in inflamed tissues. Importantly, α V β 3 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, α V β 3 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 α V β 3 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- κ B 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- κ B activation/deactivation are well established for many cell types including macrophages (Liu and Malik, 2006). NF- κ B 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- κ B activation include, among others, TNF- α , IL-1 β , LPS, and oxidized LDL (Ohlsson et al., 1996; Thurberg and Collins, 1998; Zhang et al., 2009b). This beneficial NF- κ B 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- κ B down-regulation (Parsons et al., 1989; Parrillo, 1993; Zhang et al., 2009a). This could result in persistent NF- κ B activity, leading to inappropriate inflammatory responses. There is increasing evidence that the prolonged activation of NF- κ B 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- κ B in the response to external stimuli are specific for different cell types. NF- κ B activation in macrophages can be regulated by PI3 kinase/Akt or p38 MAPK, dependent on external signals (Ardeshtna et al., 2000).

Additionally, it was reported that these signaling pathways are involved in the transition of $\alpha V\beta 3$ -induced signals to the NF- κ B-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 $\alpha V\beta 3$ ligation, leading to inappropriate macrophage inflammatory responses. Little is known about the specific signaling pathways controlling persistent $\alpha V\beta 3$ -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 $\alpha V\beta 3$ 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 $\alpha V\beta 3$ integrin may also regulate macrophage-related inflammatory responses via PI3K/Akt-dependent activation of NF- κ B. We speculate that activation of macrophages by $\alpha V\beta 3$ 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 $\alpha V\beta 3$ (LM609) integrin were from Chemicon International (Temecula, CA); isotype control IgG₁ was from Pharmingen (San Diego, CA). Polyclonal antibodies to p50 and p65 subunits of NF- κ B 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 $\alpha V\beta 3$ ligation

Human Mo were obtained from 20 healthy donors of either sex by leukocytapheresis 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 $\alpha V\beta 3$ 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 $\alpha V\beta 3$ ligands. For $\alpha V\beta 3$ 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 μ g/ml of mAb in PBS for 18 h at 4°C. Vitronectin was immobilized on substrate by incubating

dishes with 10 μ g/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 α V β 3 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- κ B-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 μ g protein) were assayed for DNA-binding activity in a total volume of 20 μ l, 4 μ l of 5 $\times$ binding buffer (20% Ficoll-400, 20 mM HEPES, 50 mM KCl, 5 mM MgCl₂, 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- κ B: 5'-GGGTTGTTACAAGGGACTTTCCGCTGGGACTTTCCAGGGAGGCGTGG-3' from the human immunodeficiency virus long terminal repeat (Gibco BRL, custom primers, Grand Island, NY). The binding sites responsible for NF- κ B 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 PhosphorImager (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- κ B (20 μ g/ml) for 20 min at 4°C or with the irrelevant IgG₁ 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- κ B (Supplemental Fig. 1A). We also demonstrated that ligation of α V β 3 integrin results in nuclear translocation of both p50 and p65 subunits of NF- κ B (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'-GTTCGAGATAGTCGGGCCGATTGAT 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 IgG₁. 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 3×10^5 cells/ml in RPMI-1640 (Bio-Whittaker, Walkersville, MD) supplemented with 10% fetal calf serum, 2 mmol L-glutamine, and 0.5 μ g/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 2×10^7 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 $\pm$ 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 $\alpha V\beta 3$ integrin ligation on NF- κ B activity and mRNA expression and major NF- κ B-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 $\alpha V\beta 3$ integrin is involved in controlling NF- κ B activation, we used MDMs differentiated in vitro for 5–7 days and then re-seeded them on an anti- $\alpha V\beta 3$ integrin mAb immobilized on substrate, or on an immobilized control isotype-matched IgG₁ antibody. To analyze NF- κ B activity, we measured the ability of NF- κ B 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- κ B. We found that the mobility-shift complex observed in EMSA is in fact comprised of NF- κ B (Supplemental Fig. 1A). We also demonstrated that ligation of $\alpha V\beta 3$ integrin results in nuclear translocation of both p50 and p65 subunits of NF- κ B (Supplemental Fig. 1B). Adhesion of MDMs to substrate resulted in a rapid but transient activation of NF- κ B, returning to the initial level of NF- κ B activity 4 h after re-seeding (Fig. 1A, lanes 2, 5, and 7). By contrast, MDMs that were re-seeded on an $\alpha V\beta 3$ integrin antibody demonstrated an enhanced and prolonged (up to 24 h) NF- κ B activation (Fig. 1A, lanes 4, 6, and 8). An NF- κ B-dependent increase in TNF- α secretion is a well-characterized indicator of macrophage-related inflammatory responses. In order to verify that NF- κ B activation by $\alpha V\beta 3$ 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 $\alpha V\beta 3$ integrin paralleled NF- κ B activity (Fig. 1B). Next we examined the effect of $\alpha V\beta 3$ integrin ligation on mRNA expression of several NF- κ B-dependent cytokines. Using quantitative RT-PCR, we found that ligation of $\alpha V\beta 3$ integrin on MDMs caused 8- to 28-fold increases in TNF- α mRNA expression (Fig. 1C), suggesting that the effects of $\alpha V\beta 3$ integrin ligation on TNF- α secretion occur at the transcriptional level. Additionally, $\alpha V\beta 3$ integrin ligation results in increases in IL-1 β (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 $\alpha V\beta 3$ ligation by antibodies can be reproduced by its physiological ligand vitronectin (Fig. 1D). Moreover, $\alpha V\beta 3$ inhibition by the cRGD peptide abolished the effects of $\alpha V\beta 3$ ligation by vitronectin and anti- $\alpha V\beta 3$ 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 $\alpha V\beta 3$ 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 $\alpha V\beta 3$ integrin ligation on LPS- and TNF- α -induced NF- κ B activation

To test the hypothesis that $\alpha V\beta 3$ integrin modulates inflammatory responses, we examined the effect of $\alpha V\beta 3$ integrin ligation on NF- κ B 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 $\alpha V\beta 3$ integrin on MDMs results in

sustained and enhanced NF- κ B 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 IgG₁ demonstrate rapid but transient NF- κ B 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- α V β 3 integrin mAb responded to LPS stimulation through significantly enhanced TNF- α secretion at all time intervals examined (Fig. 2B).

Ligation of α V β 3 integrin on MDMs results in their transition into a sustained pro-inflammatory phenotype

Next, we examined the effect of LPS stimulation on NF- κ B 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- α V β 3 integrin mAb, the MDMs demonstrated both enhanced basal (Fig. 3A, lane 2) and LPS-inducible (Fig. 3A, lane 4) NF- κ B activity. At all time intervals examined (up to 7 days), LPS-induced TNF- α secretion by MDMs cultured on the anti- α V β 3 integrin mAb was significantly increased as compared with MDMs cultured on control IgG₁ (Fig. 3B), suggesting that MDMs over-respond to LPS exposure as long as α V β 3 integrin is ligated.

Role of PI3 kinase/Akt signaling pathway

Depending on external signals, PI3 kinase/Akt and p38 MAPK can regulate NF- κ B activity in macrophages (Aradeshna et al., 2000; Wang et al., 2007). We examined which signaling pathway is responsible for NF- κ B activation induced by α V β 3 integrin ligation on MDMs and for enhanced responses to LPS stimulation. We examined the effect of α V β 3 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- α V β 3 integrin mAb demonstrated significant increases in phospho-Akt and phospho-p38 MAPK signals (Fig. 4A). When MDMs were re-seeded on the immobilized anti- α V β 3 integrin mAb in the presence of a specific inhibitor of the PI3 kinase/Akt pathway, LY294002 (10 μ M), activation and prolongation of NF- κ B induced by α V β 3 integrin ligation was completely abolished (Fig. 4B, lanes 2 and 4) as revealed by EMSA, whereas an inhibitor of p38 MAPK, SB203580 (20 μ M), has only a partial effect on NF- κ B activity (Fig. 4B, lanes 2 and 6). It has been shown that the PI3 kinase/Akt pathway can regulate LPS-inducible NF- κ B activation (Aradeshna et al., 2000). Treatment with LY294002 abolishes the effect of LPS stimulation on NF- κ B activity in MDMs re-seeded on either IgG₁ or on the mAb to α V β 3 integrin (Fig. 4C, lanes 5 and 6). By contrast, the p38 MAPK inhibitor SB203580 has no effect on LPS-inducible NF- κ B activation in MDMs re-seeded on IgG₁ or α V β 3 integrin (Fig. 4C, lanes 7 and 8).

α V β 3 integrin ligation in blood Mo results in their differentiation into a sustained pro-inflammatory phenotype

Recently, using quantitative flow cytometry, we demonstrated that α V β 3 integrin is expressed not only in differentiated macrophages (Antonov et al., 2004) but also in circulating blood Mo. We reported that expression of α V β 3 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- α V β 3 integrin mAb results in their transition into a pro-inflammatory phenotype. Freshly isolated Mo were seeded on the immobilized anti- α V β 3 integrin mAb or the control IgG₁ and differentiated in culture for the indicated period of time. Ligation of the α V β 3 integrin in blood Mo for 24 h increases the activity of NF- κ B (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- α V β 3 integrin mAb showed increased basal NF- κ B activity (Fig. 5B, right part, lanes 1 and 2) and an enhanced

NF- κ B 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 α V β 3 integrin ligation modulate TNF- α and LPS-induced transient NF- κ B activation towards an enhanced and sustained NF- κ B 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 α V β 3 receptor to NF- κ B-dependent pro-inflammatory gene activation. We thus postulate that the α V β 3 receptor plays a crucial role in controlling local and systemic macrophage-related inflammation. Indeed, α V β 3 ligation sensitizes macrophages for enhanced responses to LPS and TNF- α stimulation, implying that α V β 3 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 α V β 3 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 α V β 3 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 α V β 3 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 α V β 3 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, α V β 3 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 α V β 3 integrin is ligated. If this hypothesis is correct, interruption of the signaling induced by α V β 3 ligation will allow macrophages to escape from the sustained pro-inflammatory state (Fig. 6).

α V β 3 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 α V β 3 integrin, whereas macrophages present in atherosclerotic lesions demonstrate a high level of α V β 3 expression (Antonov et al., 2004). Since both α V β 3 ligand and the cells expressing this receptor are present in the same milieu, in this scenario, α V β 3 integrin should be persistently activated. However, α V β 3 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- κ B deactivation, are involved to ensure that responses induced by α V β 3 integrin are physiologically relevant. However, in inflamed tissue, the crosstalk between α V β 3 integrin

and other activated receptors and signaling pathways, as well as cell–cell interactions, may be important to initiate a cascade of $\alpha V\beta 3$ -dependent events ultimately leading to macrophage differentiation into a sustained and irreversible pro-inflammatory phenotype. Also, it is possible that $\alpha V\beta 3$ 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 $\alpha V\beta 3$ integrin in the regulation of inflammation are controversial. As such, it was reported that macrophage $\beta 3$ 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 $\beta 3$ 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 $\beta 3$ integrin-deficient mice (Lacy-Hulbert et al., 2007). Additionally, studies of lung inflammation induced by intratracheal LPS administration show that $\alpha V\beta 3$ inhibitors suppress lung inflammation (Lee et al., 2007; Moon et al., 2009). These latter reports support our hypothesis that $\alpha V\beta 3$ 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 $\alpha V\beta 3$ 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 $\alpha V\beta 3$ 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 $\alpha V\beta 3$ 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 $\alpha V\beta 3$ 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

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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 IgG₁, 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. IgG₁, t-test). C: MDMs from three donors were re-seeded on immobilized anti-αVβ3 mAb or IgG₁ 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, IgG₁, 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. IgG₁, ANOVA: Kruskal-Wallis test with Dunn's post-test).

Fig. 2.

Ligation of $\alpha V\beta 3$ integrin on MDMs results in enhanced responsiveness to LPS and TNF- α stimulation. A: MDMs were re-seeded on the anti- $\alpha V\beta 3$ integrin immobilized mAb or control IgG₁ 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- κ B-binding activity was analyzed using EMSA as described in the Methods Section. Lanes 2 and 3 show basal NF- κ B 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. IgG₁, t -test).

Fig. 3.

Ligation of $\alpha V\beta 3$ integrin on MDMs results in a sustained hyperresponsiveness to LPS stimulation. A: MDMs were re-seeded on anti- $\alpha V\beta 3$ integrin immobilized mAbs or IgG₁ 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- κ B activity prior to stimulation. B: MDMs, re-seeded on the anti- $\alpha V\beta 3$ integrin mAb or the control IgG₁, 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. IgG₁, 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 IgG₁, 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 IgG₁ 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 IgG₁ 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.

α V β 3 integrin ligation on blood Mo results in their differentiation into sustained pro-inflammatory phenotype. A: Mo were seeded directly on the immobilized anti- α V β 3 integrin mAb or the control IgG₁ 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- κ B activities (lanes 3 and 4) were measured using EMSA. B: In parallel with part A experiments, Mo were seeded on immobilized mAbs or IgG₁; 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 IgG₁, *t*-test).

Fig. 6.

Hypothetical model of the regulation of macrophage-related inflammation by $\alpha V\beta 3$ integrin ligation. Macrophages as a part of the host defense system respond to external pro-inflammatory stimuli (e.g., $TNF-\alpha$, 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 $\alpha V\beta 3$ 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 $\alpha V\beta 3$ 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]