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ABSTRACT

Erythroid progenitors differentiate in erythroblastic islands, bone marrow niches composed of erythroblasts surrounding a central macrophage. Evidence suggests that within islands adhesive interactions regulate erythropoiesis and apoptosis. We are exploring whether erythroid intercellular adhesion molecule-4 (ICAM-4), an immunoglobulin superfamily member, participates in island formation. Earlier, we identified α_V integrins as ICAM-4 counterreceptors. Since macrophages express α_V, ICAM-4 potentially mediates island attachments. To test this, we generated ICAM-4 knockout mice and developed quantitative, live cell techniques for harvesting intact islands and for reforming islands in vitro. We observed a 47% decrease in islands reconstituted from ICAM-4 null marrow compared to wild type. We also found a striking decrease in islands formed in vivo in knockout mice. Further, peptides that block ICAM-4/α_V adhesion produced a 53-57% decrease in reconstituted islands, strongly suggesting that ICAM-4 binding to macrophage α_V functions in island integrity. Importantly, we documented that α_V integrin is expressed in macrophages isolated from erythroblastic islands. Collectively, these data provide convincing evidence that ICAM-4 is critical in erythroblastic island formation via ICAM-4/α_V adhesion and also demonstrate that the novel experimental strategies we developed will be valuable in exploring molecular mechanisms of erythroblastic island formation and their functional role in regulating erythropoiesis.

INTRODUCTION

Erythroid progenitors proliferate, differentiate and enucleate within specialized bone marrow niches, termed erythroblastic islands.[1-4] These structural units are composed of developing erythroblasts surrounding a central macrophage. It is apparent from ultrastructural studies that extensive cell-cell interactions, both erythroblast-macrophage, as well as erythroblast-erythroblast, occur within these three dimensional structures. However, little is known regarding either the molecular nature or functional role of the specific adhesive interactions. We are exploring the potential function of erythroid ICAM-4, a recently characterized member of the immunoglobulin superfamily, in erythroblastic island formation. ICAM-4 expression is limited to erythroid tissue but, to date, there is no information on its role in erythropoiesis. We earlier identified $\alpha_4\beta_1$ and α_V-family integrins as ICAM-4 binding partners.[5] Since macrophages express α_V and erythroblasts exhibit $\alpha_4\beta_1$, ICAM-4 is an attractive candidate for mediating erythroblast-erythroblast interactions via ICAM-4/$\alpha_4\beta_1$ binding and regulating adhesion of erythroblasts to central macrophages via ICAM-4/α_V binding.

ICAM-4, which carries the Lansteiner Wiener (LW) blood group antigen system, has strong sequence homology with other members of the ICAM protein superfamily.[6,7] It is composed of two extracellular immunoglobulin-like domains, an N-terminal I set and a membrane proximal I2 set, and a single membrane spanning domain.[8,9] ICAM-4 is detected early during terminal differentiation, concordant with surface expression of glycophorin A and RhGP.[10] Hence, the timing of ICAM-4 expression during erythropoiesis is consistent with a functional role in erythroblastic islands.

To elucidate the structural basis of α_V integrin-ICAM-4 interaction we earlier performed targeted mutagenesis of ICAM-4 surface-exposed amino acid residues, using a molecular model of ICAM-4 derived from the crystal structure of closely related ICAM-2.[11] Employing adhesion assays with cells that bind ICAM-4 via $\alpha_V\beta_1$ and $\alpha_V\beta_5$, we identified a patch or "footprint" that mediates adhesion to α_V integrins composed of two series of residues on the N-terminal

extracellular domain: F18, W19, V20 and R92, A94, T95, S96, R97. In the protein structure these eight residues are close to one another, suggesting that this region is crucial for ICAM-4 attachment to α_V integrins. We also tested synthetic peptides comprised of sequences of ICAM-4 shown to be involved in adhesion to α_V integrins and found that they inhibited cell binding, providing independent support for the role of the proposed footprint in α_V integrin binding.[11]

To explore whether ICAM-4 participates in erythroblastic island formation we generated ICAM-4 null homozygous mice and studied whether islands were perturbed. For these investigations we established quantitative and reproducible live cell techniques for harvesting intact islands from mouse bone marrow or reforming islands in vitro from single cell suspensions of mouse marrow. Applying these methods, we observed a striking decrease in the number of islands formed in vivo or in vitro by ICAM-4 null erythroblasts. Collectively, the results of this phenotypic analysis provide convincing evidence for ICAM-4 in erythroblastic island formation. Further, we determined that synthetic peptides that block ICAM-4/α_V adhesion caused a marked concentration dependent decrease in islands reconstituted from single cell suspensions of wild type mouse marrow, thereby identifying erythroblast ICAM-4 binding to macrophage α_V integrins as functionally important attachments. We postulate that this newly identified erythroblast integrin counterreceptor may be crucial not only for adhesive integrity of island structure but also for initiating intracellular signaling essential for normal erythroid terminal differentiation.

MATERIALS AND METHODS

Generation of mice lacking ICAM-4. For targeted gene deletion, we designed a construct to delete 1.2kb of *ICAM-4* genomic sequence encoding exons 1-3, which encompasses the entire protein coding region[12] (Fig 1). To construct the targeting vector we ligated into the pPN2T-hGHterm vector[13,14] a 4.7 kb "long arm" of genomic DNA from the 5' end of the region to be deleted using EcoRI restriction site and a 1.3kb "short arm" from the 3'deletion boundary using

XhoI and NotI restriction sites. The two arms flank a PGKneo cassette modified with polyA-signal/terminator from the human growth hormone N-gene generating a 17kb targeting vector (Fig 1). After analysis with sequencing and restriction enzyme digestions to check for fidelity and correct orientation, the targeting vector was provided to Xenogen Biosciences (Cranberry, NJ) who generated the ICAM-4 null mice. Two ES cell lines with correct targeting were obtained. Injection of these lines into C57BL/6J (B6) blastocysts produced a number of chimeric males, ranging from 40-80% chimerism. The chimeric mice were mated to wild type B6 mice to generate heterozygous ICAM-4 knockout mice; breeding of heterozygous mice produced viable homozygous ICAM-4 null mice. Mice were maintained on a hybrid B6,129 background for all experiments.

Southern Blot Analysis. Neomycin-resistant colonies were screened by Southern blot analysis with a 0.5kb 3'probe derived from sequence just downstream of the 'short arm'. This probe hybridized to a 12.8kb *NsiI* fragment in normal mouse genomic DNA and a 5.2kb *NsiI* fragment in the targeted variant, respectively.

Polymerase Chain Reaction Analysis. Genomic DNA was prepared from 3.0mm-4.0mm tail samples with Dneasy tissue kit (Qiagen, Valencia, CA) and used to genotype progeny by polymerase chain reaction (PCR). To distinguish between wild type, heterozygote and ICAM-4 null mice, a multiplex PCR genotyping assay was developed using primers for the ICAM-4 gene and the neo gene. ICAM-4 primers generated a 528bp fragment while the neo primers produced a 381bp fragment. ICAM-4 forward primer located in exon 1: 5'-CAGCAAGAGTGGATGCAAAGTCC-3; reverse primer located in exon 2: 5'-CCAGGATCACCACCAAGAATC-3'. Neo forward primer: 5'TTGTCACTGAAGCGGG AAGG-3'; reverse primer: 5'-CACAGTCGATGAATCCAGAAAAGC-3'. PCR was setup using PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare, Piscataway, PA) with 0.5µM of each primer in a reaction volume of 100µl. PCR conditions: 32 cycles at 94°C for 30 seconds, 64°C for 30 seconds and 72°C for 60 seconds.

Western Blot Analysis. Blood was collected from ICAM-4 null and wild type mouse tails in potassium EDTA-treated microtubes and from an anonymous, existing normal human blood sample complying with the United Kingdom National Blood Service Policy under the Nuffield Council on Bioethics and the Medical Research Council operational and ethical guidelines. Red cell membranes were prepared by washing red cells 3 times in 15 volumes of 10 mM phosphate-buffered saline (PBS), pH 7.4, followed by 3 washes in 15 volumes of lysis buffer (Na_2HPO_4 6.8 mM, NaH_2PO_4 2.25 mM, pH 8.0). Membrane proteins (30μg per lane) were separated on a non-reducing 10% SDS-polyacrylamide gel, then transferred onto PVDF membrane (Hybond-P, Amersham, Bucks, UK) using a semidry electroblotter. After blocking for 1 hour in PBS, 0.1% Tween-20, 5% non-fat dry milk, blots were probed overnight at 4°C with polyclonal rabbit anti-mouse ICAM-4 (Pickwell Labs BV, Amsterdam, The Netherlands. Immunogen; mouse ICAM-4[12]) at 1:200 or anti-human ICAM-4 (BS56[15]; a gift from Dr. H Sonneborn, Biotest, Dreieich, Germany) at 50μg/ml, washed, and then incubated with either anti-rabbit (1:100) or anti-mouse (1:1000) IgGs coupled to horseradish peroxidase (DakoCytomation, Ely, Cambridgeshire, UK)**.** After several washes, blots were developed using DAB (3,3'-diaminobenzidine) method (Sigma-Aldrich, St. Louis, MO).

Reconstitution of erythroblastic islands. Bone marrow was gently flushed from both tibias and femurs of 3-5 month old adult mice by inserting the end of bones into flexible tubing attached to a 1ml syringe. Extracted marrow was resuspended in 3 ml Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Carlsbad, CA) containing 3.5% sodium citrate (BD Pharmingen, San Diego, CA) and 20% fetal calf serum (FCS; Invitrogen, Carlsbad, CA) by pipetting 20 times with a Pasteur pipette; bone and tissue fragments were removed by passage through a 70 μm cell strainer. A single cell suspension was obtained by reaspirating cells through a 26 gauge needle five times and the cells viewed and counted using bright field microscopy. We normally obtain ~90 million nucleated cells/mouse. Cells were aliquoted (1×10^6 /tube), incubated on ice for 15 minutes in activation buffer (IMDM, 3.5% sodium citrate, 20%FCS, 2mM Mn^{+2}, 2mM EGTA),

in the presence or absence of specific synthetic peptides, and subsequently incubated for 2 hours at room temperature with erythroid (Ter119-PE, Ebiosciences, San Diego, CA), Macrophage (F4/80-FITC, Ebiosciences, San Diego, CA) and DNA (Hoeschst 33342, Sigma-Aldrich, Saint Louis, MO) specific probes. 1 x 10^5 cells were then transferred into 8-well chambered coverglass containing 400 μl of IMDM, 20% FCS and allowed to settle for 15 minutes. The timing was carefully controlled throughout the experiments so that each aliquot of cells was analyzed after the same amount of time from introduction into Mn^{+2} containing buffer. Labeled live cell samples were analyzed by a blinded observer at room temperature using conventional fluorescence microscopy on a Nikon TE2000 (Nikon Instruments, Inc., Melville, NY) with a 10x0.5 NA S Fluor S objective equipped with a Q-imaging RetigaEX CCD camera (QIMAGING, Burnaby, BC Canada). The number of islands contained in 20 random fields covering 12% of the chamber were counted per concentration of peptide. Images were acquired/processed by Image Pro 4.5 (MediaCybernetics, Silver Spring, MD) and Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Bone marrow was obtained from MacGreen mice[16] for peptide inhibition studies or from B6,129 ICAM-4 null and wild type littermates. Macrophages from MacGreen mice express macrophage colony-stimulating factor (M-CSF) receptor-green fluorescent protein transgene, thereby providing a useful macrophage identifier.

Harvesting intact erythroblastic islands. To harvest erythroblastic islands formed in vivo, bone marrow was gently flushed from both tibias and femurs of 3-5 month old adult B6,129 ICAM-4 null and wild type littermates by inserting the end of bones into flexible tubing attached to a 1ml syringe. Extracted marrow was gently resuspended in 5 ml IMDM containing 3.5% sodium citrate, and 20% FCS by pipetting 20 times with a Pasteur pipette; bone and tissue fragments were removed by passage through a 70 μm cell separator. To obtain a cell count, a single cell suspension was prepared from a small aliquot, as described above, the cell count determined, and then a volume of the filtered, island-containing suspension equivalent to the volume containing 1

x 10^6 cells was diluted to a final volume of 200 μl, labeled, as above, with erythroid, macrophage, and DNA specific probes and incubated undisturbed for 2 hours at room temperature. 20 μl of labeled cells were transferred into 8-well chambered coverglass containing 400 μl of IMDM, 20% FCS and analyzed by a blinded observer at room temperature using conventional fluorescence microscopy on a Nikon TE2000 with a 10x0.5 NA S Fluor S objective equipped with a Q-imaging RetigaEX CCD camera. The number of islands contained in 20 random fields covering 12% of the chamber were counted per experiment. Images were acquired/processed by Image Pro 4.5 and Adobe Photoshop.

Peptide inhibition studies. Synthetic peptides V(16)P**FWV**RMS **(FWV)** and T(91)RW**ATSR**I **(ATSR)** were reconstituted with activation buffer to a concentration of 5mM, vortexed for 30 minutes, ultracentrifuged at 313,000 x g for 30 minutes at 4°C, and peptide concentrations determined on centrifuged supernatants. FWV and ATSR peptides (0.5mM-3.0mM) were then used in erythroblastic island reconstitution assays.

Central macrophage α_V phenotyping. To obtain central macrophages from erythroblastic islands for α_V integrin phenotyping, intact erythroblastic islands were harvested as described above and islands separated from single cells by velocity sedimentation on a 4 ml discontinuous gradient of 0.5% Bovine Serum Albumin (BSA; Chemicon Temecula, CA), 20% FBS-IMDM overlaying 1%BSA, 20% FBS-IMDM at 4°C. After 1.0 hour, fractions were collected and those fractions highly enriched for islands and depleted of single cells were pooled. The islands were then dispersed into a single cell suspension by aspirating through a 25 gauge needle and erythroid cells removed from the single cell suspension using Ter119 microbeads (Miltenyi Biotech, Auburn, CA). Central macrophages were settled onto cover glass for 4 hours, fixed with 4% paraformaldehyde for 10 minutes, washed 6 times with 1% BSA-PBS and then blocked overnight at 4°C with 10μg/ml SeroBloc (Serotec, Raleigh, NC), 20μg/ml Goat IgG (Jackson Immuno Research, West Grove, PA) and 10% normal goat serum (Jackson Immuno Research, West Grove, PA). Following blocking, cells were stained with 25μg/ml monoclonal anti-mouse

CD51, which recognizes mouse α_V integrin, (Biolegend, San Diego, CA), and F4/80 for 1 hour at room temperature. After washing 6 times with 1% BSA-PBS, cells were stained with Rhodamine Red-X-conjugated F(ab')2 fragment Goat Anti-Rat IgG (Jackson Immuno Research, West Grove, PA) and Hoeschst DNA dye for 1 hour at room temperature and washed 6 times with 1% BSA-PBS. Cells on cover glasses were mounted onto slides with Vectashield Mounting Medium (Vector Labs, Burlingame, CA) and analyzed using conventional fluorescence microscopy on a Nikon TE2000 with a 60x1.40 NA plan Apochromat oil objective equipped with a Q-imaging RetigaEX CCD camera. Images were acquired/processed by Image Pro 4.5 and Adobe Photoshop.

RESULTS

Generation of knockout mice.

To study the physiological role of ICAM-4 in erythroblastic islands we targeted *ICAM-4* for germ line deletion in mice. The mouse erythrocyte *ICAM-4* gene of 1.2kb is composed of 3 exons (Fig 1).[12] Exons 1-3, encompassing the entire protein coding domain, were targeted for replacement with the vector pPN2T-hGHterm, containing a PGKneomycin resistance cassette modified with polyA-signal/terminator from the human growth hormone N-gene (Fig 1). As summarized under Materials and Methods, embryonic stem cells derived from inbred strain 129 were transfected and neomycin-resistant colonies screened by Southern blot analysis. Two ES cell lines with correct targeting were obtained. Injection into B6 blastocysts produced a number of chimeric males, ranging from 40-80% chimerism. Chimeric mice were mated to wild type B6 mice to generate heterozygous ICAM-4 knockout mice and breeding of heterozygous mice produced viable homozygous ICAM-4 null mice. Correct targeting of ICAM-4 in heterozygous and homozygous mice was documented by Southern blot analysis (Fig 2A). Deletion of ICAM-4 DNA and protein was further confirmed by PCR analysis of tail genomic DNA and Western blotting of erythrocyte membranes. Employing a multiplex PCR genotyping assay using primers

for the ICAM-4 and neo genes, ICAM-4 primers generated a 528bp fragment while neo primers produced a 381bp fragment (Fig 2B). This assay allowed us to readily distinguish between wild type, heterozygous and ICAM-4 null mice, thereby enabling the determination of the genotype of each mouse that was used experimentally. When Western blots of equivalent amounts of erythrocyte membranes from wild type and knockout mice were probed with antibody recognizing mouse ICAM-4, a band of appropriate size for ICAM-4 was present in wild type membranes and lacking in knockout membranes (Fig 2C). No reactivity was observed using pre-immune rabbit control serum (data not shown). As a positive control, human erythrocyte membranes were probed with BS56, a well-characterized antibody against an epitope on the LW blood group active extracellular region of ICAM-4[15] (Fig 2C). The immunoreactive band observed in human membranes migrated at a similar molecular weight as the band observed in wild type mouse erythrocyte membranes. Together, these data confirm the targeted deletion of the *ICAM-4* gene.

Reconstituted erythroblastic islands.

To begin to test whether ICAM-4 has a functional role in erythroblastic islands we developed a quantitative live cell assay for reforming islands from single cell suspensions of freshly harvested mouse bone marrow. Adult mice 3-5 months of age were used and all females were virgins. A single cell suspension was prepared, then cells were incubated for carefully controlled times in media containing Manganese. We determined that we could identify islands and their cellular components by three color immunofluorescent microscopy employing fluoresceinated erythroid-specific Ter119 antibody[17], macrophage-specific F4/80 antibody[18,19] and a DNA probe (Fig 3A-C). We observed that the number of cells per island varied as did their stage of differentiation, consistent with observations by others of erythroblastic islands formed in vivo.[20-24] Since surface expression of Glycophorin A increases during terminal differentiation, the intensity of Ter119 staining served as an effective indicator of erythroblast stage. A faint blush of Ter119 fluorescence was present in early erythroblasts and increasing degrees of staining were

observed in progressively more differentiated cells. We also found that the fluorescence intensity of Ter119 label varied among erythroblasts in an individual island, indicating that islands were composed of erythroblasts at various stages of differentiation. Young, multilobulated reticulocytes were present in many islands, again consistent with prior descriptions of erythroblastic islands formed in vivo. To determine the amount of variation in total number of islands that reformed from a single cell suspension of 1 x 10^5 cells, we counted islands containing 6 or more erythroblasts in experiments on 10 different mice. Total islands were counted at the beginning (i.e. end of the incubation period) and conclusion of each experiment. We found that the number of reconstituted islands in control mice was highly reproducible (918 +/- 148) and did not vary substantially from experiment to experiment using different mice (Fig 3D).

Erythroblastic island formation is decreased in ICAM-4 null mice.

To test for functional ICAM-4 mediated adhesion in erythroblastic islands, we analyzed ICAM-4 knockout mice, comparing the capacity of single cell suspensions from ICAM-4 null and wild type bone marrow to form erythroblastic islands in vitro. We observed a marked decrease in the percentage of islands formed from bone marrow of ICAM-4 null mice compared to wild type littermates. Strikingly, we found a 47% decrease in the total number of islands formed from 1 x 10^5 bone marrow cells from ICAM-4 null mice compared to wild type littermates. Control wild type cells reformed 953 +/- 141 islands while ICAM-4 null cells reformed 504 +/- 88 islands (Fig 4A).

Erythroblastic islands formed in vivo.

In addition to analyzing erythroblastic islands reconstituted in vitro, we wanted to develop the capability to study islands that had been formed in vivo. Hence, we performed experiments to establish methodology for reproducibly harvesting intact erythroblastic islands from mouse bone marrow. We determined that by gently extracting marrow and removing bone and tissue fragments we were able to retain erythroblastic island structures. As with the reconstituted

islands, we identified islands formed in vivo and their cellular components by three color immunofluorescent microscopy employing fluoresceinated erythroid-specific Ter119 antibody, macrophage-specific F4/80 antibody and a DNA probe. We found that the number of harvested islands in normal mice was very reproducible and equaled 898 +/- 246 in volume equivalent to 1 x 10^5 bone marrow cells (Fig 4B).

Erythroblastic islands formed in vivo are decreased in ICAM-4 null mice.

To determine the ability of ICAM-4 null erythroblasts to form islands in vivo we collected and quantitated intact islands from freshly harvested mouse bone marrow. Similar to the in vitro data, we found a marked decrease in the percentage of islands from bone marrow of ICAM-4 null mice compared to wild type littermates. We observed a 64% decrease in the total number of islands from ICAM-4 null mice compared to wild type littermates. Control wild type cells reformed 898 +/- 246 islands, while ICAM-4 null cells reformed 327 +/- 97 islands (Fig 4B). Taken together, the results of this phenotypic analysis provide convincing evidence that ICAM-4 is critical in erythroblastic island formation.

Erythroblastic island formation is inhibited by peptides that block adhesion of ICAM-4 to α_V integrins.

We next tested the effects on erythroblastic island formation of two synthetic peptides that we have previously shown block ICAM-4/α_V adhesion.[11] Peptides V(16)P**FWV**RMS **(FWV)** and T(91)RW**ATSR**I **(ATSR),** correspond to sequences within the α_V binding region located on the A and G strands of ICAM-4 domain 1, respectively (Fig 5A). A single cell suspension was prepared from freshly harvested normal mouse marrow and then cells were incubated for carefully controlled times in media containing Manganese and then in media containing various peptides or media alone. Both peptides caused a marked, concentration dependent decrease in the percentage of islands formed. 0-1.0mM, 1.0-2.0mM, and 2.0-3.0mM ATSR inhibited island formation 17%, 28%, and 53% respectively (Fig 5B). While 0-1.0mM, 1.0-2.0mM, and 2.0-3.0mM FWV inhibited island formation 20%, 37%, and 57% respectively (Fig 5C). These data

strongly suggest that erythroblast ICAM-4 binding to macrophage α_V integrins is critical for erythroblastic island formation.

Central macrophages isolated from erythroblastic islands express the α_V integrin subunit.

Macrophages vary phenotypically and although macrophages from various tissues have been shown to express the α_V integrin subunit CD51, it was crucial to confirm its presence on the surface of erythroblastic island central macrophages. Therefore, we performed α_V integrin phenotyping on central macrophages isolated from erythroblastic islands. For these investigations, intact islands were separated from single cells by velocity sedimentation, the islands were disrupted and then erythroblasts removed from the single cell suspension using Ter119 microbeads. The remaining cells were analyzed by immunofluorescent microscopy using macrophage-specific probe F4/80 and anti-CD51. In double label experiments we observed co-localization of the two markers (Fig 6), clearly showing that central macrophages isolated from erythroblastic islands express the α_V integrin subunit.

DISCUSSION

Terminally differentiating erythroblasts express a variety of cell adhesion molecules on their surfaces.[10,25-34] These proteins mediate interactions between erythroblasts and stromal cells and between erythroblasts and extracellular matrix components, such as fibronectin and laminin. Prior to the current investigations the function of erythroblast adhesion molecule ICAM-4 during differentiation was unknown.

As a definitive exploration of whether ICAM-4 mediated adhesive interactions function in erythroblastic island integrity, we generated ICAM-4 null mice and quantitated these marrow substructures. In novel island reconstitution experiments we observed a 47% decrease in the total number of islands formed from bone marrow cells from ICAM-4 null mice compared to wild type littermates. We felt that it was crucial to also obtain data on in vivo island formation in these null mice. To achieve this objective we developed techniques for harvesting and analyzing

intact erythroblastic islands that were both reproducible and quantitative. Applying these novel methods, we found a 64% decrease in islands harvested from ICAM-4 null mice compared to wild type littermates. This striking decrease in islands formed both in vivo and in vitro by ICAM-4 null erythroblasts clearly shows that ICAM-4 protein, that is only expressed in erythroid tissue, is critical in erythroblastic island formation.

A major finding of the current study is that adhesive interactions between erythroblast ICAM-4 and its α_V integrin counterreceptor on central macrophages is critical for erythroblastic island integrity. An important aspect of the present study is our unequivocal demonstration by live cell microscopy that central macrophages of native bone marrow erythroblastic islands indeed express α_V integrin. We consider this finding critical for making a definitive conclusion regarding a role for macrophage α_V integrin in erythroblastic island formation, in view of the marked heterogeneity of macrophage phenotypes. Using the quantitative and reproducible live cell technique that we developed for reforming islands in vitro, we observed that synthetic peptides ATSR and FWV, that block ICAM-4/α_V adhesion[11], caused a marked concentration dependent decrease in the percentage of islands reconstituted from bone marrow single cell suspensions. ATSR and FWV inhibited island formation 53% and 57%, respectively, at the highest peptide concentrations tested. Significant similarity in the inhibiting effects of the two peptides was also observed at lower peptide concentrations. Our findings that two different peptides, each comprised of amino acid residues within the α_V integrin binding region on ICAM-4, blocked island reconstitution to similar degrees, strongly argues for the importance of ICAM-4/α_V attachments in three dimensional erythroblastic islands.

Earlier we reported marked similarities between mouse and human ICAM-4 protein with 68% overall identity.[12] Critical cysteine residues and other key residues within the two extracellular IgSF domains are conserved, suggesting that these disulfide-bonded domains are similarly folded in human and murine proteins and may have analogous functional properties. In support of this we have determined that the α_V integrin-binding properties of ICAM-4 are conserved across

species.[12] These data strongly suggest that our current findings regarding the adhesive role of ICAM-4 in mouse erythroblastic island integrity may be equally pertinent to human erythropoiesis.

Growing evidence supports the concept of erythroblastic islands as microenvironmental niches within bone marrow where cell-cell attachments, in concert with cytokines, are crucial for terminal erythroid differentiation and regulation of apoptosis. To date, only a few receptor-counterreceptor interactions have been described, but the data regarding their impact on erythropoiesis is tantalizing. Previous studies identified a transmembrane protein Emp (erythroblast macrophage protein) present in both erythroblasts and macrophages, that appears to mediate erythroblast-erythroblast and erythroblast-macrophage attachments via homophilic binding.[3,35] Another identified attachment within erythroblastic islands occurs between erythroblast $\alpha_4\beta_1$ integrin and its counterreceptor, VCAM-1, in central macrophages.[36] Island integrity is perturbed by antibodies to either VCAM-1 or $\alpha_4\beta_1$. Finally, a recent report appears to demonstrate the importance of intercellular signaling between erythroblasts in regulating GATA-1 activity.[37] We postulate that the various linkages within erythroblastic islands are dynamic during erythroid development and that the signaling pathways stimulated by these attachments could influence differentiation and enucleation.

Interestingly, Fas/Fas ligand-related regulation of apoptosis also appears to occur within erythroblastic islands.[38] Orthochromatic erythroblasts expressing Fas ligand demonstrate a Fas-based cytotoxicity against immature erythroblasts expressing Fas, that is abolished by high levels of erythropoietin. Fas-independent regulation of apoptosis may also occur within islands. Bone marrow macrophages secret soluble RCAS1 (receptor binding cancer antigen expressed in SiSo cells) that permeabilizes mitochondrial membranes and activates caspases 8 and 3 in immature erythroblasts that express RASC1 receptor.[39] In sum, these findings delineate potential mechanisms for negative regulatory feedback between mature and immature erythroblasts.

In earlier investigations we discovered a novel secreted isoform of mouse ICAM-4, termed ICAM-4S.[12] We found that ICAM-4S mRNA is upregulated late in terminal differentiation, suggesting a regulatory role in late erythropoiesis. Secreted ICAM-4S may compete with cellular ICAM-4 for integrin counterreceptors, thereby interfering with adhesion between membrane ICAM-4 and its binding partners. This potential repressive function of ICAM-4S could enable young reticulocytes to detach from erythroblastic islands in preparation for their egress into the peripheral circulation.

We postulate that the novel receptor-counterreceptor interaction between erythroblast ICAM-4 and macrophage α_V integrin identified in the current report may be important not only for adhesive integrity of erythroblastic island structures but also for initiating intracellular signaling essential for normal erythroid terminal differentiation. In addition, the novel quantitative island reconstitution assays we developed are likely to be extremely valuable in furthering our understanding of the molecular basis for cell-cell interactions in erythroblastic islands and in delineating the functional sequeli of these interactions.

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FIGURE LEGENDS

Figure 1: Deletion of *ICAM-4* gene. Targeting strategy for homologous recombination in ES cells. Restriction map of wildtype *ICAM-4* allele (top), targeting vector (middle), and targeted allele (bottom). In the wildtype *ICAM-4* allele the black boxes represent the three exons. In the targeting vector: hGh-N, human growth hormone-N gene; PGK, phosphoglycerate kinase; Neo, bacterial neomycin resistance gene.

Figure 2: Targeted Disruption of *ICAM-4*. (A) Southern blot analysis of NsiI-digested DNA derived from tail vein samples of offspring from heterozygous mating pair. Blot probed with 3' probe (shown in Figure 1) depicts homozygous animals containing only a 5.2kb band derived from the targeted allele and the neomycin cassette (lanes 1 and 2). Heterozygote possesses both the 5.2kb band and the endogenous DNA migrating at 12.8kb (lane 3). Wildtype animal contains only the endogenous 12.8kb band. (lane 4). (B) PCR analysis of tail genomic DNA. Primers binding to *ICAM-4* exon 1 and exon 2 amplified a 528bp fragment; primers binding to the Neo gene amplified a 381bp fragment. Molecular weight markers (lane 1); genomic DNA from wildtype mouse generated a 528bp fragment (lane 2); genomic DNA from heterozygote generated 528bp and 381bp fragments (lane 3); genomic DNA from homozygous mouse

generated a 381bp fragment (lane 4). (C) Western blot analysis of erythrocyte membranes. Equivalent amounts of erythrocyte membranes from wild type and knockout mice probed with antibody recognizing mouse ICAM-4 produced a band of appropriate size for ICAM-4 in wild type membranes which was absent in knockout membranes. As a positive control, human erythrocyte membranes were probed with BS56, a well-characterized antibody to ICAM-4 and produced an immunoreactive band migrating at a similar molecular weight as the band observed in wild type mouse erythrocyte membranes.

Figure 3: Reconstituted erythroblastic islands. Bright field (A) and immunofluorescent standard (B) and confocal (C) micrographs of typical erythroblastic islands formed from single cell suspensions of mouse bone marrow. Immunofluorescent micrographs of islands show cells stained for erythroid-specific marker GPA (Ter119 -*red*), macrophage marker M-CSF receptor GFP transgene expression (*green*) and DNA (Hoescht 33342 - *blue*). In the confocal image some of the cells appear blurred because they are not in the plane of focus. However, macrophage staining is apparent in various regions of the island. Reticulocytes, arrowheads; macrophage, arrows; *bars, 10 μm*. Histogram of number of erythroblastic islands formed from $1x10^5$ single cells. n=10 (D).

Figure 4: Erythroblastic islands from ICAM-4 null and wild type mouse bone marrow. (A) Islands reconstituted from ICAM-4 null and wild type mouse bone marrow cells. Histogram of number of erythroblastic islands formed from $1x10^5$ single cells obtained from wild type (n=10) and ICAM-4 null (n=10). ❋ p=0.0000003 when compared to islands formed from wild type marrow. (B) Erythroblastic islands formed in vivo in ICAM-4 null and wild type mice. Histogram of number of erythroblastic islands obtained from wild type (n=6) and ICAM-4 null (n=6). ❋ p=0.0001 when compared to islands formed from wild type marrow.

Figure 5: Erythroblastic island formation in the presence and absence of peptides blocking adhesion of ICAM-4 to α_V integrins. Model of extracellular domain of ICAM-4 is shown revealing its solvent exposed surface in three orientations rotated 120^o to each other. The region

of ICAM-4 involved in adhesion to α_V integrins is shown in yellow and green. Yellow designates area of FVW peptide sequence and green depicts location of ATSR peptide sequence (A). Histogram of percentage of islands formed in the presence of 0.5mM-1.0mM FWV peptide (n=11), 1.0-2.0mM FWV peptide (n=16), and 2.0-3.0mM FWV peptide (n=8) compared to islands formed in media alone. ❋ p=0.02, ❋❋ p=0.000004, ❋❋❋ p=0.000004 when compared to islands formed in media alone (B). Histogram of percentage of islands formed in the presence of 0.5mM-1.0mM ATSR peptide (n=3), 1.0-2.0mM ATSR peptide (n=8), and 2.0-3.0mM ATSR peptide (n=4) compared to islands formed in media alone. ❋ p=0.1, ❋❋ p=0.004 ,❋❋❋ p=0.00007 when compared to islands formed in media alone (C).

Figure 6: Expression of F4/80 and α_V integrin on central macrophages. Central macrophages isolated from erythroblastic islands were analyzed by immunofluorescent microscopy in double antibody label experiments using macrophage-specific probe antibody F4/80 (*green*), anti-CD51 (*red)* and DNA (Hoescht 33342 - *blue*). Two representative cells are shown, one in the top three panels and the other in the bottom three panels. Merged image signals showed co-localization (*yellow*) of the two antibody markers indicating that central macrophages isolated from erythroblastic islands express the α_V integrin subunit. *Bar, 10 μm.*