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Fine mapping of the *Bmgr5* quantitative trait locus for allogeneic bone marrow engraftment in mice

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Abstract

To identify novel mechanisms regulating allogeneic hematopoietic cell engraftment, we used forward genetics and previously described identification, in mice, of a bone marrow (BM) engraftment quantitative trait locus (QTL), termed *Bmgr5*. This QTL confers dominant and large allele effects for engraftment susceptibility. It was localized to chromosome 16 by quantitative genetic techniques in a segregating backcross bred from susceptible BALB.K and resistant B10.BR mice. We now report verification of the *Bmgr5* QTL using reciprocal chromosome 16 consomic strains. The BM engraftment phenotype in these consomic mice shows that *Bmgr5* susceptibility alleles are not only sufficient but also indispensable for conferring permissiveness for allogeneic BM engraftment. Using panels of congenic mice, we resolved the *Bmgr5* QTL into two separate subloci, termed *Bmgr5a* (Chr16:14.6–15.8 Mb) and *Bmgr5b* (Chr16:15.8–17.6 Mb), each conferring permissiveness for the engraftment phenotype and both fine mapped to an interval amenable to positional cloning. Candidate *Bmgr5* genes were then prioritized using whole exome DNA sequencing and microarray gene expression data. Further studies are warranted to elucidate the genetic interaction between the *Bmgr5a* and *Bmgr5b* QTL and identify causative genes and underlying gene variants. This may lead to new approaches for overcoming the problem of graft rejection in clinical hematopoietic cell transplantation.

Keywords

Bone marrow; engraftment; quantitative trait locus; consomic mouse; congenic mouse

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AUTHORSHIP CONTRIBUTIONS

Conceived and designed the experiments: TMC, JAS, AT, and ST. Performed the experiments: YW, TMC and ST. Analyzed the data: YW, TMC, XC, and ST. Wrote the paper: YW, ST and TMC.

CONFLICTS OF INTEREST DISCLOSURES

The authors declare no competing financial interests.

INTRODUCTION

Transplantation of allogeneic bone marrow (BM) and peripheral blood stem cells is established therapy for many hematologic malignancies and BM failure syndromes (Gluckman 2009; Thomas and Blume 1999). The transplantation procedure requires recipient conditioning with chemotherapy or irradiation to prevent graft rejection (Barrett 2000). Preparative regimens used in the clinical setting are effective for promoting donor engraftment, but are associated with toxicities that contribute to post-transplant morbidity and mortality (Bacher et al. 2009). Thus, understanding barriers that resist engraftment of donor cells is key to successful allogeneic hematopoietic cell transplantation.

We previously identified a BM engraftment quantitative trait locus (QTL), termed *Bmgr5* (Cao et al. 2009b), in a major histocompatibility complex (MHC)-matched mouse model of allogeneic BM transplantation. In this model, BALB.K mice are susceptible whereas B10.BR mice are resistant to engraftment when conditioned with non-myeloablative total body irradiation (TBI) and transplanted with AKR/J BM. An experimental backcross bred from parental BALB.K and B10.BR mice was used to localize the *Bmgr5* QTL to proximal chromosome 16 by linkage analysis. This QTL conferred large effects for engraftment and susceptibility was mediated by dominant permissive alleles derived from the BALB.K strain.

Using reciprocal consomic strains, we now report verification of *Bmgr5* QTL localization to chromosome 16. The consomic strains further showed that, in this model, *Bmgr5* susceptibility alleles are not only sufficient but also absolutely required for conferring permissiveness for BM engraftment. The consomic mice were then used as parental strains for constructing panels of congenic and phenotype-selected subcongenic mice for fine mapping the *Bmgr5* QTL. Using these mouse mapping panels we resolved the *Bmgr5* QTL into two separate subloci, termed *Bmgr5a* and *Bmgr5b*. Each sublocus individually conferred permissiveness for the engraftment phenotype, although the genetic interaction between *Bmgr5a* and *Bmgr5b* could not be determined. Both *Bmgr5a* and *Bmgr5b* have been fine mapped to an interval amenable to positional cloning, however, and we prioritized *Bmgr5* candidate genes using whole exome DNA sequencing and microarray gene expression profiling. Further studies are necessary to elucidate the genetic interaction between the *Bmgr5a* and *Bmgr5b* QTLs and to identify causative genes and underlying gene variants.

MATERIALS AND METHODS

Animal facilities and equipment

All work with mice was conducted using protocols approved by the University of Utah Institutional Animal Care and Use Committee. The mice were bred and maintained at the Animal Resource Center at the University of Utah, Salt Lake City, Utah. TBI was delivered using an X-RAD 320 Biological Irradiator (Precision X-Ray, North Branford, CT).

Inbred mice and BM transplantation

AKR/J ($H2^k$, Thy1.1) mice served as BM donors. BALB.K ($H2^k$, Thy1.2) and B10.BR ($H2^k$, Thy1.2) mice were used as BM transplant recipients and as parental strains used to construct consomic and congenic mouse lines. BALB.K is a MHC congenic mouse line created by Dr. Hugh McDevitt and was imported from Stanford University into our mouse colony in 2004. The B10.BR line was purchased from the Jackson Laboratory (Stock #004804, Bar Harbor, MN). Donor BM harvesting, transplantation, and evaluation for post-transplant donor blood chimerism was performed as previously described (Cao et al. 2009b).

Genotyping

Genotyping for chromosome 16 single nucleotide polymorphism (SNP) markers was used to select for desired recombinants during consomic and congenic mouse line production. SNP genotyping was performed by PCR of tail tip DNA for high-resolution melt curve analysis on a LightCycler 480 Instrument (Roche Applied Science, Indianapolis, IN) as previously described (Cao et al. 2009b). In 23 of the 43 chromosome 16 SNP markers used in this study, SNP genotyping was also performed by high-resolution melt curve analysis using unlabeled 3'-phosphorylated oligonucleotide probes as described elsewhere (Bramwell et al. 2012; Montgomery et al. 2007). A list of chromosome 16 SNP markers, primers and probes used for genotyping is provided as Supplemental Table 1. Marker map position has been updated using the Standard Genetic Map for the Laboratory Mouse (Cox et al. 2009). An additional 117 SNP and 86 simple sequence length polymorphism (SSLP) markers evenly distributed across the remaining autosomal chromosomes, used before and listed previously (Cao et al. 2009a; Cao et al. 2009b), were used to select against residual background heterozygosity. Verification of consomic line construction was performed using the high-density 620K Affymetrix Mouse Diversity Genotyping Array (Affymetrix, Santa Clara, CA) via a service provided by the Jackson Laboratory (JAX Mouse Diversity Genotyping Array Service, Bar Harbor, MN). SNP array data has been deposited in the NCBI GEO database (Accession number GSE43377).

Consomic mouse strains

In order of sequence we constructed consomic chromosome substitution strain (CSS), interval specific congenic (ISC) strain, and subcongenic strain mice, as outlined in Supplemental Fig. 1. The reciprocal consomic B10.BR-Chr16^{BALB.K} and BALB.K-Chr16^{B10.BR} CSS mice were constructed by a marker-assisted speed congenics strategy as described elsewhere (Nadeau et al. 2000; Wakeland et al. 1997). Hereafter, B10.BR-Chr16^{BALB.K} and BALB.K-Chr16^{B10.BR} are designated B10.BALB-16 and BALB.B10-16, respectively. In brief, F1 hybrids of BALB.K female and B10.BR male mice were sequentially backcrossed to B10.BR mice to generate the B10.BALB-16 CSS, and to BALB.K mice to generate the BALB.B10-16 CSS. Forty-three chromosome 16 SNP markers were used to select progeny that were non-recombinant across chromosome 16 at each backcross generation. The B10.BALB-16 and BALB.B10-16 CSS were selected to the N8 and N7 backcross generations, respectively, and then interbred to generate homozygosity at the consomic interval across chromosome 16.

Interval specific congenic (ISC) mouse strains

The B10.BALB-16 CSS was then used to generate a panel of eighteen ISC mouse strains with unique and contiguously overlapping homozygous segments of BALB.K chromosome 16 for fine mapping. The ISC mice are designated B10.BALB-16.1, B10.BALB-16.2, B10.BALB-16.3, etc. Ten of these ISC lines were phenotyped for allogeneic BM engraftment and are reported here. For ISC line creation, F1 hybrids of B10.BALB-16 CSS males and B10.BR females were backcrossed to B10.BR parents. This produced backcross litters that were genotyped for SNP markers across chromosome 16 to identify desired recombinants. Background genes outside chromosome 16 for the F1 mouse, and all subsequent progeny, remains homozygous for B10.BR alleles and do not require reselection. Backcross progeny mice, which are heterozygous at target chromosome 16 congenic intervals, were interbred to produce homozygosity across congenic segments.

Subcongenic mouse strains

Lastly, F1 hybrids from two B10.BALB-16 ISC lines, selected on the basis of penetrant BM engraftment phenotypes, were intercrossed to generate five subcongenic mouse strains for

further fine mapping. The subcongenic strains were designated B10.BALB-16.C1, B10.BALB16-C1A, B10.BALB-16.C1B, B10.BALB-16.C1C, and B10.BALB-16.C1D. Homozygous congenic intervals on chromosome 16 in these subcongenic strain mice were identified by SNP genotyping and generated from interbreeding of selected recombinants.

Whole exome sequencing

Whole exome sequencing of BALB.K and B10.BR mice was performed by the Huntsman Cancer Institute Microarray Core Facility (University of Utah, Salt Lake City, UT). A genomic DNA library prep was used for target enrichment using the Agilent SureSelect XT Mouse All Exon Kit (Agilent Technologies, Santa Clara, CA). Next generation sequencing of exome capture libraries was performed using a 101 cycle paired end DNA sequencing protocol on an Illumina HiSeq 2000 instrument (Illumina, San Diego, CA). For analysis paired-end reads were aligned to the mouse mm9 genome assembly using Novoalign (Novocraft Technologies, Selangor, Malaysia). Local realignment and SNP and insertion-deletion variant calling was performed using GATK (Broad Institute, Cambridge, MA) (McKenna et al. 2010). Gene-based annotation of called variants was performed using ANNOVAR (Wang et al. 2010). Exome sequencing data has been deposited in the NCBI SRA database (Accession number SRA064150).

Gene expression microarray

Gene expression profiling was performed using total RNA isolated from spleens of BALB.K and B10.BR mice at rest and 24 hours after TBI as previously described (X.C. and T.M.C., manuscript submitted). In brief, single-color Cy3 RNA labeling and hybridization to Agilent SurePrint G3 8x60K Mouse Gene Expression Arrays were performed by the Huntsman Microarray Core. Microarray data was analyzed using log-transformed and normalized signal intensities with Agilent GeneSpring GX 11 software. Statistical significance in gene expression was determined using *P*-values calculated using the unpaired Student's *t*-test with a Benjamini-Hochberg False Discovery Rate correction for multiple testing. All microarray data have been deposited in the NCBI GEO database (Accession number GSE43310).

Real-time PCR

Total RNA was isolated from spleens of mice 24 hours after TBI for reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA.). Real-time PCR using the LightCycler 480 SYBR Green I Master reagent on a LightCycler 480 instrument. PCR cycling parameters were as follows: initial incubation at 95°C for 10 min, 45 cycles of 95°C for 10 s, 65°C for 20 s, and 72°C for 30 s. Primer sequences are provided in Supplementary Table 2. Relative quantitation was performed by the $\Delta\Delta$ -CT method using β -Actin as internal controls.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. Differences in the proportions of engrafting mice between mouse strains were determined using the Fisher's exact test. Differences in the means of real-time PCR relative expression values was calculated using the Student's *T*-test. Statistical significance was conferred at the *P* < .05 level using two-tailed *P*-values.

RESULTS

Confirmation of Bmrg5 QTL in consomic mouse strains

Consomic mice, made by replacing a single chromosome in a host strain with the corresponding chromosome from a donor strain, have proven effective for localizing and

validating susceptibility trait loci (Nadeau et al. 2012; Nadeau et al. 2000). We used the strategy of constructing the reciprocal chromosome 16 consomic B10.BALB-16 and BALB.B10-16 CSS mice to confirm linkage of the *Bmgrp5* QTL. During line construction both CSS lines had been backcrossed to complete homozygosity for background alleles at 203 autosomal markers. Significant residual heterozygosity can be common, however, even in mouse reference populations when subjected to verification by high-density genotyping platforms (Simecek et al. 2011; Simmons et al. 2012). We therefore evaluated our CSS lines by genotyping using a 620K SNP array. As shown in Supplemental Table 3, both B10.BALB-16 and BALB.B10-16 CSS strains genotyped for donor alleles at almost all SNP loci across the chromosome 16 consomic interval. Elsewhere, almost all SNP markers genotyped for appropriate background alleles or, on chromosome 17 where the mouse MHC locus is located, alleles corresponding to the MHC donor strain. These results confirm that adequate elimination of residual background heterozygosity had been achieved in CSS line construction.

The B10.BALB-16 and BALB.B10-16 CSS mice were then evaluated for the BM engraftment phenotype. Recipient mice were conditioned with a non-myeloablative dose of TBI and transplanted with allogeneic BM from MHC-matched AKR/J donors. Engraftment was defined as the presence of donor T-cells measured at both 6 and 8 weeks post-transplant by flow cytometry of peripheral blood. As shown in Fig. 1, and consistent with prior observations (Cao et al. 2009b), parental BALB.K mice uniformly engrafted with AKR/J BM (23/23, 100%) when conditioned with TBI 400 cGy but not with TBI 300 cGy. TBI 400 cGy was not permissive for AKR/J BM engraftment in B10.BR mice (0/14, 0%), however, which required TBI 500 cGy for reliable engraftment. Testing for engraftment with TBI 400 cGy thus defines the discriminating trait characteristic that differentiates susceptibility versus resistance for allogeneic BM engraftment in our model. B10.BALB-16 and BALB.B10-16 CSS mice were therefore conditioned with TBI 400 cGy and infused with AKR/J BM. Among thirty-four B10.BALB-16 CSS mice that we transplanted, the proportion that engrafted (29/31, 91%) did not significantly differ from that observed with the susceptible parental BALB.K strain ($P = .27$). In contrast, none of eighteen BALB.B10-16 CSS mice conditioned with TBI 400 cGy achieved engraftment (0/18, 0%). All engrafting mice demonstrated comparable levels of partial T-cell donor chimerism. Multi-lineage donor engraftment in B-cell and myeloid cell compartments was documented in representative engrafted mice by real-time PCR (Supplemental Fig. 2). These results confirm localization of a highly penetrant BM engraftment QTL with large phenotypic effect to chromosome 16.

Fine mapping of *Bmgrp5* QTL in ISC mouse strains

To fine map the *Bmgrp5* QTL, the B10.BALB-16 CSS was used as the parental line to construct a panel of chromosome 16 interval specific congenic (ISC) strain mice (Darvasi 1997). These ISC mice were phenotyped for the BM engraftment phenotype and the results, along with a map of their congenic intervals, are shown in Fig. 2. Recipient ISC mice were conditioned with TBI 400 cGy and engraftment and donor chimerism was determined as before. Of eleven B10.BALB-16.17 ISC mice transplanted with AKR/J BM, none achieved engraftment (0/11, 0%). Conversely, fifteen of sixteen (94%) B10.BALB-16.18 ISC mice engrafted with donor chimerism. The engrafting phenotype for this ISC line was nearly fully penetrant, compared with susceptible BALB.K parents, and no other ISC line achieved significantly greater incidences for engraftment. Congenic boundaries for the B10.BALB-16.17 and B10.BALB-16.18 lines therefore demarcate, respectively, the proximal and distal boundaries of the *Bmgrp5* QTL. Collectively, the segregating engraftment phenotype in this ISC mouse panel confirmed the highly penetrant allele effect expressed by

the *Bmgr5* QTL and physically maps the support interval to a 3.4 Mb segment (Chr16:14.4–17.7 Mb).

Fractionation of *Bmgr5* QTL in subcongenic mouse strains

Previous studies using congenic mice for QTL mapping have shown that two or even three subloci can be found to underlie a single trait locus (Baguet et al. 2004; Lin et al. 2006; McAllister et al. 2003). To test for this possibility and to further fine map the *Bmgr5* QTL, a panel of subcongenic mice was constructed by crossing the B10.BALB-16.2 and B10.BALB-16.9 ISC mouse strains. These two ISC lines segregated with the penetrant BM engraftment phenotype and nest the *Bmgr5* support interval between non-overlapping congenic segments expressing BALB.K alleles. The resulting subcongenic lines thus represent phenotype-selected strains which, in the setting of other complex traits such as autoimmune and infectious diseases, have proven effective for enhancing traditional approaches for QTL isolation based on congenic mapping (Dai et al. 2009).

The genotypes on proximal chromosome 16 and engraftment phenotypes for the five subcongenic mouse strains are shown in Fig. 3. The subcongenic mice were tested for donor engraftment after TBI 400 cGy and infusion of AKR/J BM as before. Thirteen B10.BALB-16.C1C subcongenic mice failed to engraft (0/13, 0%), confirming the distal boundary of the *Bmgr5* QTL at 17.7 Mb. A similar lack of engraftment in the B10.BALB-16.C1B subcongenic strain (0/22, 0%) refined the proximal boundary slightly, by SNP marker rs51138904, to 14.6 Mb. Thus far, BALB.K alleles within congenic intervals of individual lines conferred either no (0% engraftment) or near complete (94–100% engraftment) permissiveness for the engraftment phenotype. Deviating from this pattern, the B10.BALB-16.C1D subcongenic line expressed a partial engraftment phenotype. The proportion of engrafting B10.BALB-16.C1D mice (11/25, 44%) differed significantly from both subcongenic lines that achieved no engraftment, B10.BALB-16.C1B and C1C, as well as from the two subcongenic lines that nearly fully engrafted, B10.BALB-16.C1 (31/33, 94%) and C1A (13/14, 93%). These results suggest that *Bmgr5* fractionates into two separate subloci, one between Chr16:15.8–17.7 Mb with partial phenotypic effect, and another between Chr16:14.6–15.9 Mb which contributes additional allele effects through genetic mechanism that cannot be determined.

Whole exome sequencing of BALB.K and B10.BR mice

The proximal congenic boundary for B10.BALB-16.C1D mice, as also shown in Fig. 3, included a 0.1 Mb interval, between SNP markers rs4164958 and rs4164967, where derivation from either BALB.K or B10.BR mice was unknown. It was thus possible that heterozygous causative alleles, within this interval, not fixed to homozygosity and randomly distributed among B10.BALB-16.C1D littermates segregated in engrafted individuals to cause the partial phenotype. In considering this possibility, we first performed whole exome DNA sequencing of the parental B10.BR and BALB.K strains using DNA extracted from mice maintained in our colony. Significant de novo mutation due to genetic drift can be frequent even between closely related sublines of inbred mouse strains (Bryant 2011), thus variants private to our mice could not be excluded. As shown in Supplemental Table 4, paired end sequencing achieved > 20X depth coverage in 97% of exon capture windows. No exon and splice site sequences had fewer than 10X coverage within the *Bmgr5* support interval between Chr16:14.6–17.7 Mb (data not shown).

Exome sequencing results, shown in Table 1, revealed a total of 40 novel DNA variants polymorphic between our BALB.K and B10.BR mice within the entire *Bmgr5* interval. The only coding nonsynonymous variants identified from exome sequencing, however, were two previously characterized SNP in the *Prkdc* gene that cause R2140C and M3844V amino acid

substitutions. This gene encodes the catalytic subunit of the DNA-dependent protein kinase. R2140C is encoded by rs4164958, which had already been genotyped as fixed to a homozygous state in all congenic strains. The other variant, M3844V, resided within the congenic boundary for B10.BALB-16.C1D mice and thus its genotype for this subline was unknown. Thirteen B10.BALB-16.C1D littermates, along with ten randomly selected BALB.K and B10.BR mice, were therefore genotyped for the M3844V variant. As shown in Table 2, all B10.BALB-16.C1D mice are homozygous for the resistant B10.BR allele at this locus. The most parsimonious interpretation of these results, collectively, is that the *Bmgr5* QTL sub-fractionates into 2 separate subloci which we termed *Bmgr5a* and *Bmgr5b*. Confirmation of sublocus effects will require further validation in independent congenic lines.

Hematopoietic expression of *Bmgr5* genes mediate engraftment

To functionally characterize *Bmgr5* allele effects for candidate gene prioritization, we determined whether expression of *Bmgr5* susceptibility alleles in hematopoietic as opposed to solid tissues conferred permissiveness for engraftment. We generated radiation chimeras by lethally irradiating B10.BR mice and transplanting BM from B10.BR, BALB.K, or B10.BALB-16 CSS mice. After twelve weeks to allow for hematopoietic reconstitution, the radiation chimeras were evaluated for engraftment of AKR/J BM after TBI 400 cGy as before. As shown in Table 3, none of ten B10.BR radiation chimeras reconstituted with control syngeneic BM achieved engraftment when transplanted with allogeneic AKR/J BM. In contrast, all (BALB.K → B10.BR) and nine of ten (B10.BALB-16 CSS → B10.BR) radiation chimeras engrafted with AKR/J BM. These results show that expression of *Bmgr5* susceptibility alleles solely in hematopoietic tissues is sufficient to confer permissiveness for the engraftment phenotype. Furthermore, these results suggest that evaluating gene expression in hematopoietic cells would be most relevant for prioritizing candidate *Bmgr5* genes.

Gene expression analysis of spleen response to TBI

The spleen was selected as the hematopoietic tissue for RNA isolation and microarray gene expression analysis. As summarized in Supplemental Table 5, we compared the expression changes in resting spleens of BALB.K and B10.BR mice. Separately we evaluated BALB.K and B10.BR mice 24 hours after TBI for comparison with un-irradiated controls. TBI was given at doses permissive for engraftment of AKR/J BM, which was 400 cGy for BALB.K and 500 cGy for B10.BR. A genelist of microarray probe features that were significantly differentially expressed, at $P < .05$ levels, and at least 2.0 fold up- or down-regulated, for resting BALB.K versus B10.BR is provided as Supplemental File 1; for BALB.K mice given TBI 400 cGy versus no TBI as Supplemental File 2; and for B10.BR mice given TBI 500 cGy versus no TBI as Supplemental File 3. Genes in these genelists co-localizing to the QTL support intervals can be prioritized as *Bmgr5* candidate genes.

Bmgr5a and *Bmgr5b* candidate genes

The genomic interval extending from 16.7 Mb to the distal boundary of the *Bmgr5* interval, at 17.7 Mb, is identical by descent (IBD) between BALB/c and C57BL/10 strains (Yang et al. 2011). Consistent with the assignment of local IBD in this region, no DNA variant polymorphic between our BALB.K and B10.BR mice was ever detected across several platforms including genotyping by the JAX SNP array, whole exome sequencing, and high resolution melt curve analysis of individual SNP markers curated from public databases (Supplemental Table 6). We therefore excluded this IBD region in prioritizing candidate *Bmgr5* genes. Protein coding genes within the QTL intervals, based on the mm9 assembly, are listed in Table 4. The only gene in this segment expressing nonsynonymous coding variants, confirmed by exome sequencing, is *Prkdc*. Significant gene expression changes

include up-regulation of *Pkp2*, or plakophilin 2, in un-irradiated BALB.K compared with B10.BR mice. *Mcm4* (minichromosome maintenance deficient 4 homolog) is down-regulated in BALB.K mice given TBI 400 cGy, while *Fgd4* (FYVE, RhoGEF and PH domain containing 4) is up-regulated in B10.BR mice given TBI 500 cGy. The Snail homolog 2 gene, or *Snai2*, is up-regulated in both BALB.K and B10.BR mice after TBI. Significant microarray gene expression changes were verified by real-time PCR (Supplemental Fig. 3).

DISCUSSION

Allogeneic engraftment barriers are mediated by multiple mechanisms, both immune and non-immune, working in concert through many cell types, including T-cells and natural killer cells, utilizing more than one molecular pathway including those that enable cell cytotoxicity (Shizuru et al. 2010). Because of this complexity, precise delineation of host cell types and novel mechanisms through which donor BM cells are resisted remains elusive. Recent work has highlighted innate immune mechanisms such as the phagocytosis of donor hematopoietic stem cells by host macrophages, an activity that involves CD47 (Jaiswal et al. 2009). A crucial early trigger leading to donor cell clearance via this mechanism may be activation of host macrophages by the TLR4/TRIF signaling pathway (Xu et al. 2012). An additional link between innate and adaptive immunity in BM graft rejection may be alternative pathway complement components. Specifically, the C3a and C5a complement factors were recently shown to be important regulators of CD4 T-cell help in a CD8 T-cell mediated organ allograft rejection model (Vieyra et al. 2011). In allogeneic hematopoietic cell transplants between MHC-matched donor-recipient pairs, host T-cells are generally considered the largest barriers to BM engraftment.

We previously identified the *Bmgr5* QTL by genetic linkage in a clinically relevant MHC-matched mouse model of T-cell mediated allogeneic BM rejection (Cao et al. 2009b). We now report validation of QTL localization to chromosome 16 using consomic mice. Using congenic mice for fine mapping we resolved the trait locus to two subloci, termed *Bmgr5a* and *Bmgr5b*. By itself the *Bmgr5b* QTL segregated with a partial engraftment phenotype, whereas co-expression of *Bmgr5a* and *Bmgr5b* produced near fully penetrant allele effects. Whether this results from additive interactions between the two QTL, as opposed to independent phenotypic effects exerted by each, is unknown and this was because the effect size that the *Bmgr5a* QTL would confer by itself cannot be determined with the current mapping panel. It is possible that expression of *Bmgr5a* susceptibility alleles will also become associated with a partial BM engraftment phenotype. If so, then there is the additional possibility of epistatic effects between the two QTL, where the combined allele effect is greater than the sum of the two alone. Confirming and defining the genetic effects conferred by *Bmgr5a* and *Bmgr5b*, in independent congenic lines, will be crucial for understanding the molecular mechanisms mediated by their underlying causative genes.

From original QTL identification, the salient feature of our genetic model has been the large phenotypic effect expressed by *Bmgr5* susceptibility alleles. This was demonstrated by our ability to essentially reproduce the complete allogeneic BM engraftment phenotype in the B10.BALB-16 CSS and to mitigate all permissiveness for engraftment in the reciprocal BALB.B10-16 CSS. Consomic strains introgress susceptibility alleles across an entire chromosome, however, and can be associated with spuriously large phenotypic effects possibly due to complete removal of background genetic variance (Shao et al. 2008). We did not, however, observe any significant degradation in the engraftment phenotype in segregating congenic lines until the QTL sub-fractionated with the B10.BALB-16.C1D subcongenic strain. While the availability of congenic lines allowed us to breed mice in

adequate numbers to assign statistical significance to segregating alleles and thus their phenotypic effects, the detection of distinct subloci was not obscured by small effect size.

Genomic resources enabling genetic studies in mouse models continue to expand at a rapid pace (Bucan et al. 2012). The steady addition of mouse SNP markers first through array-based resequencing (Frazer et al. 2007), then high-density microarray SNP genotyping (Yang et al. 2009), and finally whole genome shotgun sequencing of classical inbred strains (Keane et al. 2011) created an inventory of polymorphic markers that was a critical tool during our congenic line construction effort. Along with this wealth of strain variation data came increasingly reliable and detailed assignment of local IBD in common laboratory mice (Cervino et al. 2006; Yang et al. 2011). Importantly, the distal portion of the *Bmgr5b* QTL is a genomic region IBD between our parental mouse strains and thus is not prioritized for consideration of candidate genes. Our current list of *Bmgr5* causal candidates will need to be expanded, however, to recognize more broadly noncoding and intergenic variants. Results from the Encyclopedia of DNA Elements (ENCODE) project show that relatively little of the non-protein coding genome is functionally inactive (Bernstein et al. 2012). The remainder is represented by discrete regions that are transcribed to an assortment of noncoding RNA, are interaction sites with DNA binding proteins, or bear reproducible biochemical signatures such as distinct histone marks. These epigenetic features are associated with gene regulation, and there are compelling evidence implicating them as causative risk variants underlying complex disease traits (Harismendy et al. 2011; Schaub et al. 2012; Sur et al. 2012). Further, it is possible that congenic intervals can identify regions with regulatory elements but not their distally located target genes (Sanyal et al. 2012). It has been shown, for example, that trans-acting expression QTLs commonly correlate SNP alleles with expression features located on entirely different chromosomes (Fehrmann et al. 2011). Data from the Mouse ENCODE effort (Stamatoyannopoulos et al. 2012), a parallel project applying the same experimental pipeline used by ENCODE to the mouse genome, will be informative for evaluating causative *Bmgr5* gene variants.

Our candidate genes currently include *Prkdc*, which resides in the *Bmgr5a* QTL and encodes the catalytic subunit of the DNA-dependent protein kinase. *Prkdc* is the only protein coding gene with non-synonymous SNPs throughout the QTL interval. It is pivotal for non-homologous end joining and repair of DNA double-strand breaks, such as those that occur from exposure to TBI, and the amino-acid changing SNP variants expressed by the BALB.K strain are known to compromise catalytic function and cause radiosensitivity (Fabre et al. 2011). The genetic mechanism associated with BALB.K alleles and *Prkdc*-mediated radiosensitivity, which is recessive (Okayasu et al. 2000), is not consistent with *Bmgr5*-mediated permissiveness for engraftment, however, where BALB.K alleles are dominant (Cao et al. 2009b). *Snai2*, also known as Slug, is a zinc finger transcription factor and is another attractive candidate gene based on functional criteria. *Snai2* is transcriptionally induced by p53 upon irradiation and protects against apoptosis by repressing p53-mediated transcription (Wu et al. 2005). Thus, *Snai2* protects hematopoietic progenitor cells from TBI and overexpression in recipients could represent a barrier to engraftment. Based on microarray and real-time PCR data, however, induction of *Snai2* transcription after non-myeloablative TBI do not differ between BALB.K and B10.BR mice. *Mcm4*, the minichromosome maintenance deficient 4 homolog, differs from this in that it is significantly down-regulated in BALB.K but not B10.BR mice. *Mcm4* is a gene important in DNA replication and has been implicated in cancer susceptibility (Bagley et al. 2012), but a causal role in an allogeneic BM engraftment phenotype is not clear. *Pkp2* and *Fgd4* are two other genes in the QTL interval differentially expressed between BALB.K and B10.BR mice. However, *Pkp2* is expressed at very low transcript abundance in hematopoietic tissues (data not shown), and *Fgd4* has mainly been associated with neurologic phenotypes (Stendel et al. 2007).

If these candidate genes encode causal variants for engraftment susceptibility conferred by the *Bmgr5* QTL, they may do so by exerting subtle allele effects not detectable by examining cellular (Cao et al. 2009b) and gene expression responses to TBI conditioning in bulk splenocyte populations. At the same time, selectivity with regards to cell types and underlying molecular pathways, mediated by susceptibility alleles with large phenotypic effect, hold promise that positionally cloned *Bmgr5* genes can be exploited as attractive therapeutic targets to overcome allogeneic engraftment barriers. Importantly, we showed that hematopoietic expression of *Bmgr5* susceptibility alleles mediate permissiveness for engraftment. Positional cloning strategies that avoid transgenic mouse construction, such as those used to clone the *Latexin* gene as a regulator of hematopoietic stem cell compartment size (Liang et al. 2007), can thus be used to identify causative *Bmgr5* genes. Success in this endeavor may lead to new transplant preparative regimens that are less toxic for patients with established malignant and non-malignant indications for the procedure, as well as for patients who may benefit from donor-specific tolerance derived from the procedure were the toxicities not prohibitive.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Engraftment of allogeneic AKR/J BM in consomic mice

Recipient BALB.K, B10.BR, and B10.BALB-16 or BALB.B10-16 CSS mice were treated with TBI 300 cGy, 400 cGy or 500 cGy and injected with 1×10^7 BM from MHC-matched AKR/J donors. Shown are percent mice with donor engraftment and percent donor T-cell in peripheral blood at 8 weeks post-transplant. Results are pooled from a minimum of three experiments for each experimental group.

Fig. 2. Engraftment of allogeneic AKR/J BM in ISC mice
Recipient B10.BALB-16 ISC mice were treated with 400 cGy and injected with 1×10^7 AKR/J BM. Shown are percent mice with donor engraftment and percent donor T-cell in peripheral blood at 8 weeks post-transplant. Also shown for each ISC line is the congenic interval on a map of chromosome 16. The chromosome segments are highlighted in white if derived from BALB.K, black if derived from B10.BR, and gradient-filled if the derivation is unknown. The map is annotated with SNP markers at congenic boundaries that type as B10.BR alleles, marker physical map (Mb) and genetic map (cM) location, confidence intervals for the *Bmgrp5* and *Gvh1* QTLs from linkage analysis (dashed line), and physical support interval for the *Bmgrp5* QTL from fine mapping using the ISC panel (solid line). Results are pooled from a minimum of three experiments for each experimental group. Fisher exact test two-tailed *P*-values are for comparison with proportion of BALB.K mice engrafting (23/23, 100%) from the experiment in Figure 1.

Fig. 3. Engraftment of allogeneic AKR/J BM in phenotype-selected subcongenic mice
 Recipient B10.BALB-16 subcongenic mice were treated with 400 cGy and injected with 1×10^7 AKR/J BM. Shown are percent mice with donor engraftment and percent donor T-cell in peripheral blood at 8 weeks post-transplant. For each subcongenic line, shown is the congenic interval on a map of the proximal portion of chromosome 16. Illustrated for reference are congenic intervals for the B10.BALB-16.2 and -16.9 ISC lines, which were parental mice used for subcongenic line derivation. Also shown is a map of the B10.BALB-16.C1D subcongenic line spanning the congenic boundary overlaid with the UCSC Genes track, based on the mm10 genome assembly. The chromosome segments are highlighted in white if derived from BALB.K, black if derived from B10.BR, and gradient-filled if the derivation is unknown derivation. The map is annotated with selected SNP markers, marker physical map (Mb) location, and physical support intervals for the *Bmgr5a* and *Bmgr5b* QTLs from fine mapping using the subcongenic panel (solid line). Results are pooled from a minimum of three experiments for each experimental group. Fisher exact test two-tailed *P*-values are for comparison with proportion of BALB.K mice engrafting (23/23, 100%) from the experiment in Figure 1, or proportion of engrafting B10.BALB-16.C1D subcongenic mice.

Table 1

Whole exome sequencing results.

	Number of DNA variants	
	BALB.K	B10.BR
Different from C57BL/6J reference	523,697	55,021
Different from C57BL/6J reference (Chr 16)	10,806	325
Different from C57BL/6J (Chr 16:14.6–17.7M)	139	15
Novel variants	25	15
Intergenic	12	12
Intronic	11	0
3' UTR	2	0
Known variants		
Coding synonymous SNP	1	0
Coding non-synonymous SNP	2	0
<i>Prkdc</i> ^a	2	0

Abbreviations: Chr16, chromosome 16; SNP, single nucleotide polymorphism; UTR, untranslated region.

^aThe *Prkdc* coding nonsynonymous variants are two SNPs that produce R2140C and M3844V residue substitutions.

Table 2Genotype of *Prkdc* M3844V SNP (rs48580935).

	<i>n</i>	Genotype, No. (%)		
		A/A	A/G	G/G
BALB.K	10	0 (0%)	0 (0%)	10 (100%)
B10.BR	10	10 (100%)	0 (0%)	0 (0%)
B10.BALB-16.C1D	13	13 (100%)	0 (0%)	0 (0%)

Table 3

Allogeneic BM engraftment in B10.BR radiation chimeras.

	Engrafted, No. (%)	% Donor T-cells	<i>P</i> -value ^a
AKR/J → (B10.BR → B10.BR)	0/15 (0%)	0 ± 0	-
AKR/J → (BALB.K → B10.BR)	15/15 (100%)	66.2 ± 5.9	< .0005
AKR/J → (B10.BALB-16 → B10.BR)	9/10 (90%)	62.4 ± 6.4	< .0005

Abbreviations: BM, bone marrow

^a*P*-value for number of mice engrafted versus (B10.BR → B10.BR) radiation chimeras.

Table 4

Candidate genes within the *Bmgr5a* and *Bmgr5b* genomic support intervals.

Gene Symbol	Chr	Mb	CnSNP	Gene expression microarray data (FC)			Gene name
				BALB.K vs. B10.BR	BALB.K TBI 400 cGy vs. TBI 0 cGy	B10.BR TBI 500 cGy vs. TBI 0 cGy	
<i>Bmgr5a</i> (14,618,906 – 15,829,660)							
<i>A630010A05Rik</i>	16	14.56	none	ns	ns	ns	RIKEN cDNA
<i>Snai2</i>	16	14.71	none	ns	+2.35	+2.59	Snail homolog2
<i>Efcab1</i>	16	14.91	none	ns	ns	ns	EF hand calcium binding domain 1
<i>F830005K03Rik</i>	16	15.09	none	ns	ns	ns	RIKEN cDNA
<i>Ube2v2</i>	16	15.55	none	ns	ns	ns	Ubiquitin-conjugating enzyme E2 variant 2
<i>Mcm4</i>	16	15.62	none	ns	-3.74	ns	Minichromosome maintenance deficient 4 homolog
<i>Prkdc</i>	16	15.64	R2140C M3844V	ns	ns	ns	Protein kinase, DNA activated, catalytic polypeptide
<i>Bmgr5b</i> (15,829,660 – 17,663,457 bp)^a							
<i>Mzi2</i>	16	15.85	none	ns	ns	ns	Mitotic spindle organizing protein 2
<i>Cebpd</i>	16	15.89	none	ns	ns	ns	CCAA T/enhancer-binding protein delta
<i>2310008H04Rik</i>	16	15.89	none	ns	ns	ns	RIKEN cDNA
<i>Pkp2</i>	16	16.21	none	+2.03	ns	ns	Plakophilin 2
<i>Yars2</i>	16	16.30	none	ns	ns	ns	Tyrosyl-tRNA synthetase 2
<i>Dnm1l</i>	16	16.31	none	ns	ns	ns	Dynamin 1-like
<i>Figd4</i>	16	16.42	none	ns	ns	+2.54	FYVE, RhoGEF and PH domain containing 4
<i>Olfir19</i>	16	16.67	none	ns	ns	ns	Olfactory receptor 19
<i>4933404G15Rik</i>	16	16.71	none	ns	ns	ns	RIKEN cDNA

Abbreviations: CnSNP, coding nonsynonymous SNP; FC, fold change; ns, not significant; TBI, total body irradiation

^aNot listed are genes in the QTL that reside within the interval between 16,714,199 – 17,663,457 bp that is identical by descent.