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Leukocyte $\beta_7$ Integrin Targeted by Krüppel-like Factors

Melanie Alles,* Gleb Turchinovich,‡,§ Pumin Zhang,¶ Wolfgang Schuh,¶ Fabien Agenès,‖‡,#,¶ and Jörg Kirberg*

Constitutive expression of Krüppel-like factor 3 (KLF3, BKLF) increases marginal zone (MZ) B cell numbers, a phenotype shared with mice lacking KLF2. Ablation of KLF3, known to interact with serum response factor (SRF), or SRF itself, results in fewer MZ B cells. It is unknown how these functional equivalences result. In this study, it is shown that KLF3 acts as transcriptional repressor for the leukocyte-specific integrin $\beta_7$ (1gB7, Ly69) by binding to the $\beta_7$ promoter, as revealed by chromatin immunoprecipitation. KLF2 overexpression antagonizes this repression and also binds the $\beta_7$ promoter, indicating that these factors may compete for target sequence(s). Whereas $\beta_7$ is identified as direct KLF target, its repression by KLF3 is not connected to the MZ B cell increase because $\beta_7$-deficient mice have a normal complement of these and the KLF3-driven increase still occurs when $\beta_7$ is deleted. Despite this, KLF3 overexpression abolishes lymphocyte homing to Peyer’s patches, much like $\beta_7$ deficiency does. Furthermore, KLF3 expression alone overcomes the MZ B cell deficiency when SRF is absent. SRF is also dispensable for the KLF3-mediated repression of $\beta_7$. Thus, despite the shared phenotype of KLF3 and SRF-deficient mice, cooperation of these factors appears neither relevant for the formation of MZ B cells nor for the regulation of $\beta_7$. Finally, a potent negative regulatory feedback loop limiting KLF3 expression is shown in this study, mediated by KLF3 directly repressing its own gene promoter. In summary, KLFs use regulatory circuits to steer lymphocyte maturation and homing and directly control leukocyte integrin expression. The Journal of Immunology, 2014, 193: 1737–1746.

Krüppel-like factors (KLFs) are zinc finger family transcription factors with 17 members described for mammalian cells (1). KLFs have a highly conserved DNA binding domain comprised of three C2H2 zinc fingers near or at the C terminus and share similarity to the transcription factors of the specificity protein family (2–5). The KLF zinc fingers are thought to mediate binding to CACCC elements and GC-rich DNA sequences in target genes.

In Drosophila Schneider cells, KLF3 (BKLF) had been shown to act as a weak transcriptional activator (6). However, more recent data in murine cells implicate KLF3 as a repressor of transcription (7–9). Indeed, KLF3 has been shown to interact with the common transcriptional corepressors C-terminal binding protein 2 (CtBP2) and four and a half LIM domain protein 3 (FHL3) (8, 10, 11). A further KLF3-interacting transcription factor is serum response factor (SRF), as identified in muscle cells (12). Thus, KLF3 may act exclusively in concert with other transcriptional regulators.

In lymphocytes, KLFs play important roles in their development and function (7, 9, 13–20). When constitutively expressed under the B cell–specific CD19 promoter, KLF3 increases maturation toward marginal zone (MZ) B cells, resulting in a 5- to 10-fold increase of this subset, whereas, accordingly, MZ B cells were reduced in KLF3-deficient mice (7, 9). Interestingly, absence of KLF2 in B cells results in an increase of MZ B cells, mimicking the effect of constitutive KLF3 expression (15, 16, 19). Although the actual target genes mediating this effect are unknown, it has been demonstrated that the lymphocyte phenotypes of KLF2-deficient mice result from effects on the positioning, recirculation, and/or tissue-homing ability of these cells (13, 14, 19, 21–24). However, not all outcomes of KLF2 deficiency resulted from cell-autonomous processes (18).

Integrins perform crucial functions for the positioning of leukocytes by acting both as signaling and signaling-responsive adhesion molecules (25). The functions of the leukocyte-specific $\beta_2$ (CD18) and $\beta_7$ (Ly69) integrin subunits have been elucidated initially by blocking studies and subsequently by the respective gene-deficient mice. This led to a mouse model of leukocyte adhesion deficiency (LFA-A immunodeficiency, OMIM 116920) and specific defects in the migration of leukocytes to tissues such as skin and gut, respectively (26–31). Indeed, whereas leukocyte migration via the afferent lymph and within lymph nodes is not affected in the absence of all integrins, their extravasation is crucially dependent on them (32). With respect to $\beta_7$ integrin, this subunit can either pair with $\alpha_4$ (CD49d) to form lymphocyte Peyer’s patch (PP) adhesion molecule–1 (CD103), mediating binding to VCAM-1, mucosal addressin cell adhesion molecule 1 (MAdCAM-1, addressin), and fibroconnectin, or pair with $\alpha_6$ (CD103), a heterodimer found particularly among intestinal intraepithelial lymphocytes mediating binding to E-cadherin. Thus, $\beta_7$ expression is considered a hallmark of mucosal lymphocytes because $\alpha_4/\beta_7$ allows for binding to the high endothelial venules of gut
PP and mesenteric lymph nodes (MLn), and endothelial cells of the lamina propria, all specifically expressing MAdCAM-1 (33), and αβ/β₁ mediating the retention within the tissue by binding to E-cadherin, expressed in the gut epithelial layer. Whereas α₄ (CD103) only pairs with β₇, α₄ (CD49d) can pair with either β₂ or β₁ (CD29), the latter termed VLA-4 (α₄β₁, CD49d/CD29), mediating binding to VCAM-1, MAdCAM-1, and fibronectin. Whereas binding specificities thus overlap, the ratio of αβ/β₁ and αβ/β₁ determines lymphocyte-homing specificity because αβ/β₁ preferentially binds to MAdCAM-1 and αβ/β₁ has higher affinity for VCAM-1 (34–36). The relative expression of these integrins is set transcriptionally but also depends on intracellular pairing preferences. Apparently, under nonstimulated conditions, the expression of αβ and, particularly, β₁ is limited, such that the preferentially associating αβ/β₁ heterodimer does not fully outcompete the formation of αβ/β₁.

Given the discrete binding preferences of the various integrin heterodimers, their relative expression has pronounced effects on the tissue specificity of extravasation. Consequently, there has been interest in the characterization of the promoter elements of various integrins and the molecules regulating expression. Factors of the specificity protein family of transcription factors have been directly defined as factors that control expression of this lymphocyte-specific integrin (43, 44).

In this study, it is shown that KLF3 and KLF2 both directly target the promoter of β₂ integrin, thereby contributing to the regulation of this gene in lymphocytes. Their relative expression levels determine by Ficoll density gradient centrifugation.

Materials and Methods

Mice

CD19-KLF3 transgenic mice [C57BL/6-Tg(Chloramphenicol acetyltransferase-cre)K1239/J (K1239, GCE, derived within the GUDMAP project [http://www.gudmap.org/]) (45, 46), B6.129-Tgfβr1tm1Ddg/J (47), B6.129Cd19tm1Cgn/J (48, 49), B6.SJL-PtprcaPepcb/BoyJ, C57BL/6-Itgb7tm1Cgn/J (30), and C57BL6/J (B6) mouse strains were obtained from The Jackson Laboratory (Bar Harbor, ME). B6.129P2(C)-Cd19tm1(cre)Cgn/J (45, 46), and B6.SJL-PtprcaPepcb/BoyJ, C57BL/6-Itgb7tm1Cgn/J (30), and C57BL6/J (B6) mouse strains were obtained from the GUDMAP project [http://www.gudmap.org/]. The B6;129P-Klf3tm1(cre/ERT2)Pzg/J (Klf3-GCE, as this phenotype is shown to be considerably, notably, the promoter for β₁ has been identified, but there has been a paucity of defined factors that control expression of this lymphocyte-specific integrin (43, 44).

In this study, it is shown that KLF3 and KLF2 both directly target the promoter of β₂ integrin, thereby contributing to the regulation of this gene in lymphocytes. Their relative expression levels determine by Ficoll density gradient centrifugation.

Cloning

Tagged versions of KLF3 were generated by PCR using vector pMT2/BLKLF (6) or derivatives as template (see Supplemental Fig. 1A). In the KLF3-ΔZF constructs, the zinc finger domain of KLF3 is deleted, such that a TAA stop codon follows amino acid Thr²⁷¹ of KLF3 (AA93256.1). In the KLF3-ΔDL construct, the PVDF motif in KLF3 was inactivated by Asp²⁷³→Ala and Leu²⁷⁵→Ser, as described (8). All expression constructs were verified by sequencing. They were shuttled into pMYc-IRES-GFP (50) or a derived pMYc-IRES-tTomato retroviral vector backbone for expression in target cells by retroviral transduction, pBMN-L2RS-IRES-GFP (51) served as vector backbone for the KLF2 construct.

Tissue culture

Cell culture was in IMDM (Biochrom, purchased as powder) supplemented with MEM nonessential amino acids, 100 IU/ml penicillin, 100 mg/ml streptomycin (Life Technologies), β-ME (50 μM), Gln (4 mM), 0.03% (w/v) Primatone RL (Quest, 5X59051, 10-kDa ultrafiltrate) (52), 5 μg/ml insulin (Sigma-Aldrich 15500), and 2% FCS (unless indicated differently) at 5–7.5% CO₂, 37°C.

Retroviral transduction

Retroviral vector particles were obtained by transient transfection of retroviral vector plasmid DNA into Plat-E producer cells (53). Briefly, the day prior to transfection, 5 × 10⁶ Plat-E cells were plated in a T75 flask in 8–10 ml medium (10% FCS). The next afternoon, chloroquine (25 μM final) (54) was added, and 1 h later cells were transfected using calcium-phosphate [to 1 ml 20 μg plasmid DNA in 0.295 M CaCl₂, 1 ml 50 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 mM NaCl, 1.5 mM Na₂HPO₄, (pH 7.16) was added, gassed with air for 20 s, and immediately the mixture was added to the cells] (55). The next morning, medium was exchanged. Virus particle-containing supernatants were taken 42, 56, 66, and 72 h after transfection. They were made cell free (10 min, 3300 rpm, 4°C), snap frozen in liquid nitrogen, and stored at −80°C. For transduction of some cells, retroviral particles were pseudotyped with the vesicular stomatitis virus glycoprotein G by adding plasmid pClVax-VSV-G (Stratagene) to the transfection.

For transduction, 1 × 10⁶ target cells/well were seeded in 12-well plates. Virus-containing supernatants were thawed on ice and used as such or aliquoted (1.5 ml), centrifuged (60 min, 14,000 rpm, 4°C), most supernatant aspirated, and the remaining liquid was resuspended and pooled to ~1 ml vol. Then polybrene was added to 5 μg/ml. Medium was removed from target cells, and the virus-containing solution was added, performing a spin infection (60 min, 1,800 rpm, 37°C). Thereafter, cells were incubated normally, changing medium 1 h later. Cells were split as needed, and on day 3 after transduction they were analyzed by FACS.

In vivo lymphocyte-homing assays

Donor lymphocytes were obtained from spleen (erythrocytes lysed using Tris-buffered ammonium chloride) and peripheral (all non-i.p.) lymph nodes of the indicated strains by passing through nylon meshes into PBS with 0.5% BSA (EQBAC62; Europa Bioproducts). Mixtures (1:1) based on cell counts were prepared and washed into PBS, and 10⁶–3 × 10⁷ cells were adoptively transferred into B6.Ly-5⁻ recipient mice i.v. Recipients were analyzed 15 h later, collecting PP from equivalent regions of the intestine, peripheral lymph nodes, MLN, and spleen. Blood lymphocytes were purified by Ficol density gradient centrifugation.

FACS analyses

Cells were isolated from the indicated organs, as described above. For samples from spleen or bone marrow, erythrocytes were lysed (Tris-buffered ammonium chloride). Adherent cells from tissue culture were detached with trypsin/EDTA. Staining was performed in PBS with 0.5% BSA using reagents at predetermined optimal dilution, maximizing signal to noise, and adding 2.402 (CD16/32) hybridoma supernatant or normal mouse Ig to block nonspecific staining. Dead cells were excluded by staining with Sytox-blue (Invitrogen). Abs were from BD Biosciences and eBioscience, or prepared by protein G affinity chromatography, followed by derivatization using standard techniques. Flow cytometric analyses were performed on a LSRII-SORP (BD Biosciences) instrument with Diva 4.1.6.1 software. Data were analyzed using FlowJo 7.6.3-7.6.5 software. Unless specified differently, numbers indicate the proportion of cells within the given gate or region. The p values (Student’s t test) from pairwise comparisons are indicated within figures or their legends.

Chromatin immunoprecipitation

Cells (2 × 10⁷) were washed into PBS and fixed with formaldehyde (0.75%, 15 min). Fixation was stopped with glycine (125 mM final). Fixed cells were washed into PBS, then lysed (1 h, 4°C, in 1.2 ml lysis buffer containing 50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.1% Na-deoxycholate, 1% SDS, protease inhibitors [Complete ULTRA; Roche]). After sonication to an average fragment size of 500–1000 bp (Bandelin Sonopuls HD2200, Sonotrode 3300 rpm, 80°C), the lysates were cleared (7,640 g, 2 min), aliquoted, and snap frozen.

For chromatin immunoprecipitation (ChIP), protein G magnetic beads (NEB) were blocked in radioimmunoprecipitation assay buffer (RIPA) containing 0.1 mg/ml BSA and one-tenth volume Roti-Block (Carl Roth). Lysates representing the equivalent of 1.33 × 10⁶ cells were diluted 1/5 with RIPA buffer, and then either goat anti-KLF3 (Acrris) or rabbit anti-KLF2 (56) was added (~10 μg per 400 μl diluted lysate). Then, 15-μl beads were added, agitating overnight at 4°C. Thereafter, beads were
Itgb7 site3_a, 5

 FIGURE 2A; for an overview of the various constructs, see Supplemental are as follows: 

 KLF3 1a promoter), and one general control primer pair amplifying an site1_s, 5

 case for many, but clearly not all, follicular B cells (Fig. 1A, T-3

 (Furin)_s, 5

 (58), known to express

 B cells of CD19:KLF3 transgenic mice. Notably though, this is the

 expression, we resorted to a murine mastocytoma cell line, P815

 downregulated in KLF3 transgenic cells while being somewhat

 (Supplemental Fig. 1B). Because KLF3 had been demonstrated to

 gene use were gene use. Primer sequences are as follows: 380,_-10 kb KLF3-a, 5-GGC TCA TCT CCT CGC TAT CCT CC-3; 381-_10 kb KLF3-a, 5-GGC TCA TCT CCT CGC TAT CCT CC-3; 382_Itgb7 site3-a, 5-CAG GGT GGT TTT AGC TTA TC-3; 383_Itgb7 site3-a, 5-CAG GGT GGT TTT AGC TTA TC-3; 384_Itgb7 site1-s, 5-GCA GCC GCT TGC TAC CTG TGT G-3; 385_Itgb7 site1-s, 5-GCA GCC GCT TGC TAC CTG TGT G-3; 386_Itgb7 site2-s, 5-CAG GAT GAT GAT GAT CAA GAG AGT GGT CAA GAG CC-3; 387_Itgb7 site2-s, 5-CAG GAT GAT GAT GAT CAA GAG AGT GGT CAA GAG CC-3; 388_Itgb7 site2-s, 5-CAG GAT GAT GAT GAT CAA GAG AGT GGT CAA GAG CC-3; 389_Itgb7 site2-s, 5-CAG GAT GAT GAT GAT CAA GAG AGT GGT CAA GAG CC-3;

 The suitability of primer pairs for QRT-PCR was tested by amplification

 of titrated amounts of genomic mouse DNA. All primer pairs had com-

 the MESA Blue qPCR MasterMix Plus for SYBR Assay no ROX (Euro-

 7. Interestingly, expression of the CtBP interaction domain mu-

 9

 -TGG ATG TGA TTG AGG CTG GG-3

 2 cycle thresholds) and gave a con-

 D

 1200 RPM, 15 min), then adding a 2X mastermix for the qPCR, which

 levels (Fig. 3, 10 kb to the

 7 expression data were first normalized on basis of

 2

 2

 10 kb_KLF3_s, 5

 7 expression (data not shown).

 KLF3 can act despite its PVDLT motif being inactivated, lacking the potential to recruit CGBP2 (8).

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 washed twice each at low (0.05% SDS, 1% Triton X-100, 2 mM EDTA

 (pH 8.0), 500 mM NaCl, 20 mM Tris (pH 8.0)), DNA was eluted from beads by adding 120 µl 1% SDS, 100 mM NaHCO3 (15 min, 30°C). The eluate was diluted with RIPA buffer (to 400 µl), adding RNase, then proteinase K, to liberate the DNA and to reverse the formaldehyde fixation (65°C, 4–5 h). Finally, the DNA was purified by phenol/chloroform extraction. Glycogen was added as carrier for ethanol precipitation. Recovered DNA was resuspended in water and stored at 4°C until analysis. To this end, quantitative real-time PCR (QRT-PCR) was performed on a Light Cycler 480 instrument (Roche) and the MESa Blue qPCR MasterMix Plus for SYBR Assay no ROX (Eurogentec), using the absolute quantification method, to determine the relative DNA abundance for the primer-defined DNA region in the given ChIP sample. Three primer pairs for the β7 integrin promoter, one pair for the KLF3 1b promoter region (57) and a corresponding control (10 kb to the KLF3 1a promoter), and one general control primer pair amplifying an irrelative mouse genomic region (Furin) were used.

 Comparing follicular and MZ B cells of normal and CD19:KLF3

 7 integrin expression. Shown are B cells as gated on basis of being CD19:CD93+ . Differentiation into follicular and MZ B

 KLF3 represses and KLF2 upregulates β7 integrin expression in P815 mastocytoma cells

 To further characterize the ability of KLF3 to regulate β7 expression, we resorted to a murine mastocytoma cell line, P815 (58), known to express β7 integrin. Consistent with the decrease in β7 expression in B cells of CD19:KLF3 transgenic mice, expression of KLF3 in P815 cells strongly decreases β7 expression (Fig. 2A; for an overview of the various constructs, see Supplemental Fig. 1A). In contrast, expressing a truncated version of KLF3 lacking its zinc fingers, KLF3-DZF, does not change β7 expression. This demonstrates that KLF3 requires its zinc finger domain for repression, apparently utilizing this domain for binding to promoter elements in cellular DNA. Additional data confirmed that KLF3-DZF localized to the nucleus as does the full-length protein (Supplemental Fig. 1B). Because KLF3 had been demonstrated to interact with CtBP2 via a defined motif (8, 11), it was possible to test whether this interaction is also relevant for the repression of β7. Interestingly, expression of the CtBP interaction domain mutant of KLF3, KLF3-DΔL, still leads to significant downregulation of β7 expression in P815 cells (Fig. 2A). Thus, in this setting,

 KLF3 represses β7 integrin expression in vivo

 Comparing follicular and MZ B cells of normal and CD19:KLF3 transgenic mice by mRNA array analysis, β7 integrin was strongly downregulated in KLF3 transgenic cells while being somewhat higher expressed in the absence of KLF3 (7, 9). Thus, it was tested whether KLF3 represses β7 as determined by cell surface staining. Indeed, downregulation of β7 is observed among splenic follicular B cells of CD19:KLF3 transgenic mice. Notably though, this is the case for many, but clearly not all, follicular B cells (Fig. 1A, left histogram panels). With respect to MZ B cells, expressing less β7 normally, all cells appear to be affected (Fig. 1A, right histogram panels).

 Results

 KLF3 represses β7 integrin expression in vivo

 KLF3 represses and KLF2 upregulates β7 integrin expression in P815 mastocytoma cells

 KLF3 can act despite its PVDLT motif being inactivated, lacking the potential to recruit CGBP2 (8).

 KLF2 partially appears to possess functions that are opposing to those of KLF3, such as in the generation of MZ B cells or lower β7 expression in KLF2-deficient B cells (15, 16, 19). Indeed, upon KLF2 overexpression in P815 cells, β7 expression is increased (Fig. 2B). The opposing functions of KLF3 and KLF2 are thus reproducible in this cell line.

 KLF2 and KLF3 work antagonistically on β7 integrin expression

 To determine how KLF3 and KLF2 interact, they were simultaneously transduced into P815 cells, followed by monitoring β7 integrin expression. As above, KLF3-transduced cells exhibit reduced, whereas KLF2-transduced cells exhibit elevated β7 integrin levels (Fig. 3, top center histogram panels). When using a three-dimensional plot, it appears that the relative level of the two factors, as determined by the respective marker gene expression GFP and tdTomato, determines the level of β7 expression (data not shown). Because β7 integrin levels of KLF2 and KLF3 double-expressing cells, as gated in Fig. 3 (top right panel), approached
KLFs affect β7 integrin expression in P815 mastocytoma cells. (A) P815 cells expressing KLF3 or variously altered versions of it, as schematized above each dataset, were obtained by retroviral transduction. They were analyzed for β7 integrin cell surface expression. Transduced and nontransduced cells are those being marker gene positive (GFP+) or being GFP−, respectively, and stem from the same culture. Transduction by empty vector served as additional control. (B) Similar to (A), however, cells were transduced with a retrovirus encoding KLF2. (A and B) The cell surface expression of α4 integrin was monitored at the same time, but no alterations could be detected (data not shown). Each experimental condition was reproduced independently at least two times, giving comparable results.

KLF3 represses β7 integrin expression in TK1 T cell lymphoma cells

The data above show that KLF3 and KLF2 work antagonistically in the regulation of β7 integrin expression in P815 mastocytoma cells. To analyze whether these functions are also relevant for lymphoid cells, the murine T lymphoma cell line TK1 was used, known to express high levels of β7 and lacking β1 integrin expression (59–62). Downregulation of β7 integrin expression is evident upon KLF3 expression in these cells, whereas the zinc finger domain-deficient variant KLF3-ΔZF has no effect (Fig. 4, upper center panels). An attempt was also made to increase β7 expression by introduction of KLF2; however, TK1 cells express relatively high amounts of KLF2 endogenously, and no increase in β7 expression is observed when introducing the KLF2-encoding retrovirus (Fig. 4, right panel). As TK1 cells lack expression of β1, β7 repression is accompanied by a decrease in α4 integrin surface expression (Fig. 4, lower panels).

KLF2 and KLF3 directly bind to the β7 integrin promoter

As demonstrated above, KLF3 and KLF2 act antagonistically in the regulation of β7 integrin expression. In the case of TK1, lack of β7 expression on the cell surface could have also been caused by the repression of α4 expression. The results in P815, remaining α4 positive following introduction of KLF3 while β7 expression vanishes, would argue against this. In any case, to determine whether KLF3 was directly involved in the regulation of β7 integrin expression, ChIP experiments were performed. As TK1 cells express only low levels of endogenous KLF3 (data not shown), they were also transduced to express higher levels. Using a KLF3-specific antisera in ChIP, the precipitated material is enriched for DNA corresponding to two nonoverlapping regions of the β7 promoter region (~−270 to −130 nt and +920 to +1050 nt relative to the transcriptional start site of β7) (44) (Fig. 5A). This binding of KLF3 to the β7 promoter is detectable both in normal TK1 and, yielding increased signals, in TK1 cells overexpressing KLF3. Because KLF3 and KLF2 act antagonistically, as demonstrated above, the ChIP was repeated using a KLF2-specific antisera. As TK1 cells express KLF2 endogenously, there was no need for overexpression. Indeed, KLF2 also binds to the β7 promoter regions (Fig. 5B). Together, these results demonstrate that KLF3 and KLF2 both directly bind to the β7 integrin promoter, giving a molecular explanation for their antagonistic function in the regulation of β7 expression.

KLF3 expression impacts lymphocyte homing

As KLF3 directly represses β7 expression, we tested whether this would affect lymphocyte homing. To this end, medium-term (15-h)
Migration assays were performed because our focus was on B cells (29). Equivalent numbers of lymphocytes obtained from peripheral lymph nodes and spleens of B6.Ly-5a/b control and various Ly-5b experimental mice, either B6, CD19:KLF3, or Itgb7−/−, were transferred into B6.Ly-5a recipients. As expected for cells having reduced or lacking β7 expression (29–31), homing to PP and MLn was severely abolished (Fig. 6, Supplemental Fig. 2). For cells from CD19:KLF3 transgenic mice, this only affected B cells, consistent with the specificity of transgene expression. Interestingly, the remaining fraction of CD19:KLF3 transgenic cells detected in PP and MLn showed β7 expression, whereas in the other organs β7 expression was effectively repressed (Supplemental Fig. 2). This demonstrates that β7 indeed is the essential driving force for the homing to PP and MLn in these experiments. Interestingly, although β7-deficient B cells could effectively reach peripheral lymph nodes, this was less pronounced for CD19:KLF3 transgenic B cells (Fig. 6A).

In β7-deficient mice, KLF3 still drives MZ B cell maturation

Because KLF3 both repressed β7 and led to the accumulation of MZ B cells in CD19:KLF3 transgenic mice, β7-deficient mice were analyzed with respect to this B cell subset. As to this, β7 deficiency has no impact on the proportion of MZ B cells as defined by staining for various marker combinations (CD1d versus CD23, CD21/35 versus IgM, or CD21/35 versus CD23; Fig. 7A, 7C, and data not shown). This could also be confirmed by immunohistological analysis (Supplemental Fig. 3). Moreover, when the CD19:KLF3 transgene is bred onto the β7-deficient background, the presence of the transgene still leads to an enrichment for MZ B cells, demonstrating that the effects of KLF3 can be clearly separated with respect to the regulation of β7 versus the induction of MZ B cell maturation (Fig. 7B, 7C).

SRF is irrelevant both for KLF3 repressing β7 and driving MZ B cell maturation

The transcription factor SRF physically associates and synergizes with KLF3, thereby working as a transactivation complex, as shown for a muscle-specific target gene (12). Furthermore, mice in which SRF was deleted in B cells show a loss of MZ B cells (63), a phenotype shared with KLF3-deficient mice (7, 9). Thus, these factors may act together to drive the formation of MZ B cells. To investigate this possibility directly in vivo, the contribution of SRF for MZ B cell maturation and β7 repression driven by KLF3 was analyzed. As expected, in the absence of SRF, the MZ B cell subset is significantly reduced (Fig. 8A [upper two dot plots], 8B). MZ B cells, however, are rescued on the SRF-deficient

**FIGURE 4.** KLF3 expression affects β7 and α4 integrin expression in TK1 T lymphoma cells. TK1 cells expressing KLF3 or a variant of KLF3 lacking its zinc finger domain, as schematized above the dataset, or expressing KLF2, were obtained by retroviral transduction. They were analyzed for β7 (top row) and α4 integrin (bottom row) cell surface expression. Transduced and nontransduced cells are those being marker gene positive (GFP+) or being GFP−, respectively, and stem from the same cultures. Transduction by empty vector served as additional control. The experiment was performed twice, giving comparable results.

**FIGURE 5.** ChIP demonstrating KLF3 and KLF2 binding to the β7 integrin promoter. ChIP was performed on formaldehyde-fixed, sheared lysates of normal (white bars) and KLF3-transduced TK1 cells (gray bars, using the strep-tagII-myc-tag-KLF3 construct as in Fig. 4). For immunoprecipitation, either a goat polyclonal anti-KLF3 serum (A) or a rabbit polyclonal anti-KLF2 serum (B) was used. DNA recovered by immunoprecipitation was quantified with respect to the indicated target sequences by QRT-PCR. The percentage of input indicates the quantity of DNA corresponding to the given sample/PCR target sequence, as recovered following immunoprecipitation normalized relative to the input DNA quantity before immunoprecipitation. Results for two primer pairs amplifying nonoverlapping regions of the β7 integrin promoter are shown (Itgβ7 sites 1 and 2). Control samples included those in which the antiserum was omitted (beads only control) or where the QRT-PCR was performed with a primer pair specific for an irrelevant genomic region. Data derive from duplicate determinations (shown are mean values and range). Results are representative of two experiments using independently generated lysates.
background by the CD19:KLF3 transgene, demonstrating that the interaction of SRF with KLF3 is dispensable for MZ B cell maturation (Fig. 8A [lower left dot plot], 8B). Similarly, β7 repression by KLF3 is unaltered when SRF is absent (Fig. 8A [histogram plots], 8C). As in the normal situation, repression of β7 by KLF3 is more efficient among MZ B cells compared with their follicular counterpart. Thus, both KLF3-driven MZ B cell maturation as well as β7 repression is SRF independent.

Feedback regulation of KLF3 in vivo

In CD19:KLF3 transgenic mice, endogenous KLF3 expression is repressed as determined on mRNA and protein levels (7). To assay this effect more directly and to relate it to the repression of β7, the CD19:KLF3 transgene was combined with a newly available KLF3 knock-in allele (Klf3-GCE). In this allele, a GFP-Cre-ERT2 transgene was combined with a newly available KLF3 promoter. The outcome is either increased expression or repression in vivo. However, even in the presence of the CD19:KLF3 transgene, the penetrance of this repression is significant, but does not affect all cells. Thus, up to ~40% of the cells show repression of β7 and/or GFP, with the remaining unaffected proportion being β7^GFP^+. This may indicate that (a) further cofactor (s), only expressed in a fraction of the cells, is/are essential for KLF3 to function as repressor.

Because the above results indicate that KLF3 exerts a feedback loop, ChIP was performed to determine whether there is direct binding of KLF3 to the KLF3 promoter. Indeed, KLF3 binding to the KLF3 1b promoter, driving expression from the second noncoding exon [exon 2 in (57), exon 1b in (65)], is detected in this study, either in normal or KLF3-overexpressing TK1 cells (Fig. 10A). Unfortunately, no suitable primer pair could be derived to determine binding to the exon 1 promoter, possibly due to the region being GC-rich (partially >80% GC). In contrast to the β7 promoter, in which both KLF3 and KLF2 can bind, there is no evidence by ChIP for KLF2 binding to the KLF3 promoter (Fig. 10B).

Discussion

Target genes of the KLF transcription factor family have only partially been elucidated. In this study, we show that two factors of this family, known to be relevant in lymphocytes, act as regulators of the lymphocyte-specific β7 integrin by directly binding to its promoter. The outcome is either increased expression or repression, as mediated by KLF2 or KLF3, respectively. This antagonistic outcome is reminiscent of the experimental result in vivo, in which KLF3 is required for efficient B cell maturation toward the MZ B cell subset, whereas KLF2 limits this process (7, 9, 15, 16, 19). Notably, it has been demonstrated that integrins mediate the retention of MZ B cells within the MZ of the spleen by binding ICAM-1 and VCAM-1 and, evidently for this purpose, MZ B cells express increased levels of LFA-1 (α/L/β2) and α/β1 integrin,
while having less β7, as compared with follicular B cells (66). Accordingly, a possible hypothesis was that β7 repression by KLF3 allows for an augmented cell surface expression of α4/β1, having higher affinity toward VCAM-1. This would increase adhesion, and thus retention, within the MZ, thereby promoting MZ B cell survival and/or maturation. However, although β7 was clearly downregulated, no change in the cell surface expression of α4 or β1 resulted, neither on MZ B cells of CD19:KLF3 transgenic mice nor on KLF3-transduced P815 cells (data not shown), arguing against this simple scenario. Still, due to preferential pairing of β1 with α4 (37), small changes to the rather large pool of α4/β1 heterodimers may preclude the experimental detection of relevant expression differences, once β7 is repressed. To specifically address the importance of β7 repression, β7-deficient mice were thus analyzed. No difference was detected as to the relative proportion of follicular and MZ B cells, indicating that KLF3-mediated repression of β7 is not the mechanism mediating the increase in MZ B cells of CD19:KLF3 transgenic mice. Consequently, the CD19:KLF3 transgene remained effective in the promotion of MZ B cells when bred onto the β7-deficient background. Thus, the exchange of α4/β7 to α4/β1, as mediated by β7 repression, has no relevance for the final share of MZ B cells to the B2B cell pool.

**FIGURE 7.** Follicular and MZ B cell subsets of β7-integrin-deficient, CD19:KLF3 transgenic mice. (A) Splenic B cells of Itgβ7-heterozygous and -homozygous mice were analyzed for CD1d, CD23, IgM, and CD21/35 to determine the presence and proportion of MZ B cells. Shown are CD19+CD93+ cells. In total, n = 9 Itgβ7+/+ and n = 8 Itgβ7−/− animals were analyzed; one representative experiment is shown. (B) Splenic B cells of Itgβ7−/− and Itgβ7−/− CD19:KLF3 mice were analyzed for CD1d, CD23, and CD21/35. Shown are CD19+CD93+ cells. In total, n = 8 Itgβ7−/− and n = 3 Itgβ7−/− CD19:KLF3 animals were analyzed; one representative experiment is shown. (C) Shown are the proportions of follicular and MZ B cells among CD19+CD93+ cells as analyzed in (A) and (B). Data from Itgβ7+/+ and Itgβ7−/− animals were pooled. The p values are indicated within the figure.

**FIGURE 8.** Neither KLF3-mediated expansion of MZ B cells nor Itgβ7 repression depends on the KLF3-interacting factor SRF. (A) Splenic B cells of SRFfl/fl mice, in which SRF was ablated in B cells by being CD19cre heterozygous, with or without the CD19:KLF3 transgene, were analyzed for CD21/35 and IgM to determine the proportion of MZ B cells. Shown are CD19+CD93+ cells. Differentiation into follicular and MZ B cells is based on the gates, as shown on the left. Histograms depict the expression of Itgβ7 among these subsets. Gray shades have been positioned into histograms at the same expression level to aid in the visualization of changes. A total of n = 4 SRFfl/fl CD19+/+, n = 5 SRFfl/fl CD19cre/+, and n = 5 SRFfl/fl CD19:KLF3 transgenic mice was analyzed; one representative experiment is shown. (B and C) Shown are the proportions (B) and the geometric means and SDs for β7 expression (C) of follicular and MZ B cells, as analyzed in (A). The p values of relevant pairwise comparisons are indicated within the figure.
β7 integrin has been prominently known as an adhesion molecule mediating the homing and retention of lymphocytes to mucosal sites (34). Indeed, β7-deficient mice were reported to have smaller but normal numbers of PP (30). Accordingly, in CD19:KLF3 transgenic mice, fewer B cells would be expected to home to this site, due to the lack of β7 expression on many of them. We compared T versus B cell proportions within PP of CD19:KLF3 transgenic and normal mice but found no difference (data not shown). It is quite possible, though, that the population of B cells showing unaffected β7 expression in CD19:KLF3 transgenic mice suffices to populate this tissue to normal numbers. To thus elucidate the relevance of β7 repression by KLF3 in vivo, we performed lymphocyte-homing assays, transferring mixtures of normal and CD19:KLF3 transgenic or β7-deficient cells. Indeed, the outcome was that CD19:KLF3 transgenic B cells could not efficiently reach PP and MLn, thus mimicking β7-deficient cells. Interestingly, the few CD19:KLF3 transgenic B cells that could still home to this region showed β7 expression. This affirms that β7 is the one relevant integrin controlled by KLF3 for homing to this side. Also, the occurrence of a small subset of cells in which CD19:KLF3 is unable to repress β7 explains why we could not detect a B cell paucity in these locations of CD19:KLF3 mice, unlike the situation in animals lacking β7 throughout (30). Interestingly, CD19:KLF3 transgenic B cells fail to home efficiently to peripheral lymph nodes, but also do not accumulate in blood or spleen, which is different from β7-deficient cells. This will require further analyses.

As stated, not all B cells show β7 repression in CD19:KLF3 transgenic mice. Cells that do not downregulate β7 were observed among bone marrow cells (within Hardy fractions E and F) and follicular B cells, whereas most MZ B cells appear to be responsive. Interestingly, the resistant cells are identical to those that also escaped the negative feedback loop when the CD19:KLF3 transgene was bred onto the KLF3GCE strain, appearing as β7+/β7−.
GFP+ cells. This may indicate that either counteracting factors, such as KLF2, may get even more upregulated at these stages, or that there are further cofactors involved in the KLF3-mediated repression of β2 and in the feedback regulation of KLF3. The aforementioned B cell subsets would differ in the expression of these cofactors. Identified cofactors with which KLF3 interacts are FHL3, CtBP2, and SRF (7, 9, 63). Interaction with SRF may also be important in lymphocytes, that is, SRF may act in conjunction with FHL3, CtBP2, and SRF (8, 11, 12). Some of these may indeed be relevant in lymphocytes, that is, SRF may act in conjunction with these cofactors. Identified cofactors with which KLF3 interacts are the aforementioned B cell subsets would differ in the expression of KLF3). 7. Turchinovich, G., T. T. Vu, F. Frommer, J. Kranich, S. Schmid, M. Alles, J.-B. Loubert, J.-P. Goulet, U. Zimber-Strobl, P. Schneider, et al. 2011. Programming of marginal zone B cell fate by basic Kruppel-like factor (BKLFL/KLF3). Blood 117: 3780–3792.


Suppl. Fig. 1: Tagged KLF3 constructs and determination of nuclear localization

(A) Tagged KLF3 variants containing N-terminal combinations of strep-tag II, tobacco etch virus protease cleavage site (TEV), and the myc-tag, with or without the zinc finger (ZF) domain of KLF3, were cloned. All constructs contain an optimal Kozak translation initiation consensus sequence GCC ACC preceding the start codon. Constructs with the N-terminal myc-tag are as described (1). The N-terminus of constructs with the strep-tag II, TEV and myc-tag is NH₂-M-Sť-WSPQFEK-GPG-ENLYFQG-EQKLISEEDL—... (aa written in italic letters added as linkers). In the ΔZF construct the zinc-finger domain is deleted, such that a TAA stop codon follows amino acid Thr(271) of KLF3 (AAA93256.1). In the KLF3-ΔDL mutant the PVDLT motive in KLF3 was inactivated by Asp(63)->Ala and Leu(64)->Ser, as described (2). All constructs were transferred into pMYc-IRES-GFP or pMYc-IRES-tdTomato retroviral vector backbones.

(B) NIH-3T3 (ATCC CRL-1658) cells were transduced with the indicated KLF3 construct. Following adhesion, then fixation (4% paraformaldehyde in PBS on ice), cells were permeabilized (1x Perm/Wash buffer (BD Biosciences)), unspecific binding was blocked (3% BSA in Dulbecco’s PBS), and, following another washing step (1x Perm/Wash buffer), cells were stained with the primary antibody to KLF3 (rat αKLF3 mAb 511.7D12, gift of Elisabeth Kremmer, Helmholtz Center, Munich, Germany). Staining was revealed by labeled mouse anti-rat-α (MAR18.5) using Bisbenzimide (Hoechst-33258, 2 µg/ml final) as counterstain for DNA. Cells were embedded in Mowiol (10,24% Mowiol 4-88, 4,26% PEG-400 in PBS) and analyzed on a LSM510 meta confocal microscope using Zeiss LSM software. For graphical representation, this and Photoshop and/or Illustrator CS5.1 (Adobe) software was used. Shown is the merged picture of KLF3 (red) and Hoechst (blue); areas stained by both appear in white.

Suppl. Fig. 2
(Alles et al.)

Donor lymphocytes from peripheral lymph nodes and spleen of Ly-5<sup>a</sup> and Ly-5<sup>b</sup> donors of the indicated genotype were mixed (1:1) and adoptively transferred into Ly-5<sup>a</sup> recipients as described in materials and methods. 15 h after transfer, cells from the indicated organs were isolated and analyzed with respect to their origin, based on staining for Ly-5 allele expression, and gating on mature B cells (CD19<sup>+</sup>, large dot plots). Numbers indicate the proportion of Ly-5<sup>a/b</sup> and Ly-5<sup>b</sup> donor B cells. Also shown is the expression of β<sup>7</sup> on Ly-5<sup>a/b</sup> or Ly-5<sup>b</sup> donor B cells (small dot plots; numbers report the geometrical mean of β<sup>7</sup> expression).

Data derive from one experiment in which each donor cell combination was analyzed in two recipient mice. Two additional experiments were performed that gave similar results.
Suppl. Fig. 3 (Alles et al.)

Suppl. Fig. 3: Spleen immunohistology of β7-deficient mice
For immunohistology, cryosections (5 µm) were cut, placed on Superfrost Plus slides (Thermo), fixed in methanol:acetone, blocked (PBS with 0.5 % BSA, 50 % 2.4G2 (CD16/32) hybridoma supernatant and/or 1-5 % normal mouse serum), and stained with the indicated antibodies. Sections were mounted in Fluoromount G (Southern Biotech Assoc) or a mixture of 1 part glycerol with 2 parts of 25 % (w/v) Moviol 4-88 in 0.2 M Tris pH 8.5. Pictures were acquired on a Zeiss Apotome (Axio ComMR camera) using Zeiss Axiovision software. For some micrographs, the Zeiss MosaIX Axiovision software module was used. For graphical representation, ImageJ (1.48g, http://imagej.nih.gov/ij), Photoshop and Illustrator software (CS5.1, Adobe) were used.
Suppl. Fig. 4 (Alles et al.)
Gating strategy for Hardy-fractions within bone-marrow cells

1°-gates for
(a) lymphoid cells,
(b) alive cells (DNA neg.),
(c) singlets (doublet-discrimination)

2°-gates on markers for indicated fractions A-F

Suppl. Fig. 4: Gating strategy for Hardy-fractions within bone-marrow cells
Shown is the gating strategy used to identify the various subsets compromising discrete stages of B cell development in the bone marrow (3).