This is a postprint of an article published in
Fleige, A., Alberti, S., Gröbe, L., Frischmann, U., Geffers, R., Müller, W., Nordheim, A., Schippers, A.
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(2007) Journal of Biological Chemistry, 282 (33), pp. 24320-24328.

SERUM RESPONSE FACTOR CONTRIBUTES SELECTIVELY TO LYMPHOCYTE DEVELOPMENT *

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Running Title: SRF is Required for Lymphocyte Development

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Serum response factor (SRF), is a crucial transcription factor for murine embryonic development and for the function of muscle cells and neurons. Gene expression data show that SRF and its transcriptional co-factors are also expressed in lymphocyte precursors and mature lymphocytes. However, the role of SRF in lymphocyte development has not been addressed *in vivo* so far, owing in part to early embryonic lethality of conventional *Srf*-null mice. To determine the *in vivo* role of SRF in developing lymphocytes we specifically inactivated the murine *Srf* gene during T or B cell development using lymphocyte-specific Cre transgenic mouse lines. T cell-specific *Srf* deletion led to a severe block in thymocyte development at the transition from CD4/CD8 double to single positive stage. The few residual T cells detectable in the periphery retained at least one functional *Srf* allele, thereby demonstrating the importance of SRF in T cell development. In contrast, deletion of *Srf* in developing B cells did not interfere with the growth and survival of B cells in general, yet led to a complete loss of marginal zone B cells and a marked reduction of the CD5[+] B cell subset. Our study also revealed a contribution of SRF to the expression of the surface molecules IgM, CD19, and the chemokine receptor 4 in B lymphocytes. We conclude that SRF fulfills essential and distinct functions in the differentiation of different types of lymphocytes.

Serum response factor (SRF) (1) is a widely expressed transcription factor belonging to an ancient family of DNA binding proteins. Its activity is regulated by the interaction with transcriptional co-factors, some of which are expressed in a cell type-selective fashion (2). SRF interacts directly with at least two classes of signal-regulated co-factors, the ternary complex factor (TCF) subfamily of Ets domain proteins (SAP-1, Elk-1, and Net) which respond to mitogen-activated protein kinase (MAPK) signalling (3) and members of the Myocardin-Related Transcription Factor (MRTF) family (4-6) which may be regulated through Rho GTPase/actin signalling. SRF activity can be triggered by a multitude of means such as serum, ionizing radiation, growth factors, and intracellular calcium-regulating agents. A steadily increasing set of SRF target genes is being identified, which includes immediate early genes (IEGs), cytoskeletal protein-encoding genes, and muscle differentiation genes (7,8) Regarding cellular function, recent *in vivo* and *in vitro* studies revealed essential contributions of SRF to murine embryogenesis (9), neuronal development (10-12), heart development (13-15), skeletal muscle function (16,17), programmed cell death (18,19), and processes of cell morphogenesis, adhesion and migration (20).

Cell culture analyses point to an additional requisite role for SRF in lymphocyte function and development. SRF is widely expressed in haematopoietic cell lines (21,22), activated in response to various cytokines (23), and responds to signalling via the receptors of T cells (24-26) and B cells (27,28). Furthermore, SRF is involved in the regulation of lymphocyte specific genes (e.g. Il2, IL2Rα, IFNγ) (24-26). MAP-kinase and Rho GTPase signalling, which have been shown to trigger SRF responses (2), are vitally important for

the selection, proliferation and maturation of thymocytes (24,29-32) and B cells (27,28,33).
Stimulation of lymphocyte antigen receptors (B cell receptor (BCR) and T cell receptor (TCR)) rapidly induces expression of *c-fos* and *early growth response factor 1* (*egr-1*) (28,33,34), which are immediate early target genes of SRF. Several studies support the importance of these genes for lymphocyte development: Overexpression of *c-fos* in mice augments the differentiation and accumulation of peritoneal B1b cells (35), marginal zone B (MZB) cells (36), and terminally differentiated antigen-specific B cells (37). Activator protein 1 is composed of members of the Fos and Jun family of DNA binding proteins. Induction of Activator protein-1 is required for activation of the germline ε promoter. This represents an essential step preceding immunoglobulin (Ig) isotype switching to IgE (38) and plays a central role in the regulation of promoter activity for Interleukin-2 (IL-2) and Interferon-gamma in T cells (25). The immediate early gene *egr-1* has been shown to play a role in limiting the number of T cell precursors and in the efficient differentiation and survival of thymocytes during the process of positive selection (39).
SRF has the potential to regulate cell growth, survival or apoptosis by altering the expression of specific genes involved in theses processes. Experiments in a human B cell line demonstrated that for apoptosis to proceed, the transcriptional events promoting cell survival and proliferation in which SRF is involved must first be inactivated by a caspase-mediated cleavage of the SRF protein (18). One mechanism by which SRF inhibits apoptosis is the transcriptional regulation of members of the B cell leukemia/lymphoma (*Bcl*) family of anti-apoptotic genes (*Bcl-2*, *Bcl-xl*, and *Myeloid cell leukemia sequence 1* (*Mcl-1*)). Neither *Bcl-xl* nor *Bcl-2* is absolutely required for T cell development to maturity (40-42). Conditional *Mcl-1* mutants, however, display a profound reduction in B and T lymphocytes (43).
TCFs are thought to function primarily through serum response elements via formation of ternary complexes with SRF. Recently, members of the TCF family of SRF co-factors have been characterized by knockout studies. Elk-1 deficient mice display normal immune responses and mildly impaired neuronal gene inactivation (44) and Net mutants show defects in cell migration (45), vasculature development (46) and impaired angiogenesis during wound healing (47). The strongest phenotype with regard to immune functions is observed in SAP-1 null mice where thymocyte development is severely impaired and a decrease in the amount of $CD4^+$ and $CD8^+$ single positive (SP) cells is seen resulting from defective thymocyte positive selection (48). The relatively mild phenotypes observed upon inactivation of single TCFs suggest functional redundancy between the different TCFs.

Gene targeting in mice has been particularly helpful in deciphering the genetic networks underlying lymphocyte development and function. Classical disruption of both Srf-alleles in mice leads to early embryonic lethality associated with a gastrulation defect (9), thereby precluding the analysis of SRF function in subsequent developmental processes. To investigate potential contributions of SRF to lymphocyte differentiation and function, we conditionally ablated the *Srf* gene in developing lymphocytes of the mouse. Potential functional redundancies of TCF proteins are thereby also addressed since SRF mutagenesis inactivates all TCF functions that are dependent on wildtype SRF. We show that mice with ablation of the *Srf* gene in T cells display a substantial reduction in mature SP thymocytes and nearly complete abrogation of peripheral T cells. B cell-specific depletion of SRF, on the other hand, resulted in the loss of MZB cells and decrease of B1 cells only, whereas the majority of the conventional B2 cells was not affected. Thus our study demonstrates for the first time *in vivo* that SRF is an essential transcription factor for murine lymphopoiesis.

Experimental Procedures
Lymphocyte Specific Deletion of Srf in Mice - Animals were bred at the Helmholtz Centre for Infection Research under specific pathogen-free conditions and all animal experiments were performed in accordance with institutional guidelines. *Srf* was deleted specifically in murine lymphocytes by using mice carrying the conditional *Srf* allele $Srf^{flex1neo}$(abbreviated *fl*), which is converted into the Srf^{lx} deletion allele (abbreviated *lx*) by Cre-mediated recombination (49). Conditional Srf^{fl} mice (49) were bred to CD4-Cre (50) or CD19-Cre (51) mice on a mixed 129SvEv / C57Bl/6 background. Detection of the Srf^{fl} allele was performed by polymerase chain

reaction (PCR) using two primer combinations (SRF-E/R and SRF-L/R) as previously described (49). The Cre transgene was detected by PCR using primer 1 (5'-ACGACCAAGTGACAGCA ATG-3') and 2 (5'-CTCGACCAGTTTAGTTAC CC-3'). *Srf* deletion was assessed by Southern blot analysis performed on *Bgl II* digested genomic DNA isolated from fluorescence activated cell sorting (FACS)-sorted lymphocytes. As described previously (49), hybridization with an external 3' probe allowed discrimination between wt (4.6 kb), *fl* (3.8 kb) and *lx* alleles *(*1.4 kb).

Histology - Formalin-fixed organs were embedded in paraffin and sectioned at 4 μm thickness. Immunohistochemistry for T lymphocytes was performed with a rat-anti-CD3 antibody (CD3-12, Serotec Ltd.). A biotinylated rabbit-anti-rat antibody was used for detection of the bound primary antibody. B lymphocytes were detected with a biotin-conjugated rat-anti-mouse-CD45R/B220 monoclonal antibody (RA3-6B2, BD Biosciences). The avidin-biotin-complex (ABC) method with diaminobenzidin as chromogen was used for detection of the biotinylated antibody.

For cryosections, spleens were embedded in OCT freezing medium on dry ice and 10 μm sections were prepared. Air dried sections were fixed and subsequently stained with a cocktail of fluorescent dye-coupled antibodies containing rat-anti-MOMA-FITC (MCA947F, Serotec, Oxford, UK), rat-anti-mouse-IgM-Alexa 594 (R33-24), and mouse-anti-mouse-IgD-Alexa 647 (clone 1.3) in Tris-Buffered Saline-Tween20 (TBST) with 2% rat serum. Pictures were processed with PhotoImpact software (version 10.0 SE, Ulead Systems).

Flow Cytometry - Single cell suspensions were prepared from thymus, spleen, lymph nodes, peritoneal cavity, and bone marrow. Peripheral blood was taken from the tail vein and heparinized using 375 I.E. heparin-sodium. Erythrocytes were depleted by lysis. After washing, cells were stained with various combinations of antibodies. To exclude dead cells, propidium iodide was used. The cell suspensions were measured on a FACS-Calibur flow cytometer (BD, San Jose, CA). Data was analyzed by FlowJo software (version 6.3.2 and 6.4.2). For cell sorting, cells were analyzed and collected on a MoFlo cell sorter (DakoCytomation). The following antibody conjugates were used: anti-CD4-PE-Cy5, anti-CD8-PE-Cy5, anti-Gr-1-PE, anti-Gr-1-FITC (*e*Bioscience, San Diego, CA), anti-CD19-APC, anti-CD49-FITC, anti-CXCR4-Bio, anti-CD21/35-FITC, anti-CD23-PE, anti-CD8-FITC, anti-CD5-Cy (BD Biosciences Pharmingen), anti-IgM-PE, anti-IgD-FITC, and anti-F4/80-PE (Serotec, Oxford, UK). The biotinylated anti-CXCR-4 antibody was detected by streptavidin-PE (BD Biosciences Pharmingen).

Electrophoretic Mobility Shift Assay (EMSA) - EMSA studies were performed as described previously (52). A [α^{32}P]ATP-labeled DNA fragment containing the *c-fos* serum response element (SRE) was used as a DNA-binding probe either with 4 μg T cell lysates from mice with a T cell specific SRF deletion or 8 μg B or T cell lysates from mice with a B cell specific SRF deletion. To detect specific binding of SRF to the probe, SRF antiserum (G20, Santa Cruz) was included for supershift analysis. Control shifts were performed with the nuclear factor of activated T cells (NFAT) tandem site of the NFATc1-P1-promoter (53).

DNA Microarray Hybridization and Analysis - Sample preparation from FACS-sorted populations of splenocytes (B cells: $CD19^+IgM^+IgD^+$), hybridization, washing, staining, and scanning of Affymetrix GeneChips was performed as previously described (54). Data analysis was performed using the Affymetrix Microarray Suite 5.0, Affymetrix MicroDB 3.0, and Affymetrix Data Mining Tool 3.0. All array experiments were scaled to a target intensity of 150, otherwise using the default values of the Microarray Suite. Genes were considered as regulated when their fold change was greater than or equal to an increase or decrease of 1.5, the statistical parameter for a significant change was less than 0.001 or greater than 0.999 and the signal difference of a certain gene was greater than 40.

RESULTS

T Cell Lineage-Specific Deletion of Srf - To delete *Srf* specifically in lymphocytes we used a conditional Srf^{fl} mouse strain where Cre mediated recombination results in formation of the Srf^{lx} allele deleting the complete coding region of exon 1 (49). To the best of our knowledge, there is not a protein product being derived from this allele. However, the presence of such a truncated protein cannot be ruled out with 100% certainty (13). If such a hypothetical product were derived from the

Srf ^{lx} locus, it would be predicted to have lost all the important functions of nuclear localization, dimerization, and DNA binding. To restrict deletion of the *Srf* ^{fl} allele to developing T cells, the lymphocyte-specific CD4-Cre transgene (50) was introduced by breeding. The resulting Cre expression is directed by the murine CD4 enhancer/promoter/silencer, which leads to efficient Cre-mediated deletion in $CD4^+CD8^+$ double positive (DP) and $CD4^+$ SP thymocytes, in mature $CD4^+$ T cells, and, to a lesser extent, in $CD4^-CD8^-$ double negative (DN) thymocytes (55). The resulting mutant *CD4-CreSrf* $^{fl/fl}$ progeny were obtained at Mendelian frequency and exhibited no obvious phenotypic abnormalities.

T Cell-Specific Srf Deletion Leads to a Block in T Cell Development - We first looked whether *CD4-CreSrf* $^{fl/fl}$ mice still possessed T lymphocytes. Immunohistochemical staining of spleen sections with a T cell-specific anti-CD3 antibody showed a striking decrease of CD3 positive cells, as compared to control specimen (Fig. 1*A*). This was verified by FACS analysis, which showed decreased numbers of peripheral T cells in spleen, blood, and lymph nodes of *CD4-CreSrf* $^{fl/fl}$ mice when compared with littermate control mice (i.e. *Srf* $^{fl/fl}$, *Srf* $^{fl/wt}$ and *CD4-Cre-Srf* $^{fl/wt}$) (data not shown). The reduction of peripheral T cells suggested an impaired T cell development in the thymus. The overall number of thymocytes was similar in wildtype and SRF deficient mice and no major differences in DN and DP thymocyte populations were detected by FACS analysis. We observed, however, a dramatic reduction of SP $CD4^+$ (~ 80%) and $CD8^+$ (~ 50%) T cells among the thymocytes from SRF deficient *CD4-CreSrf* $^{fl/fl}$ mice (Fig. 1*B*).

Peripheral T Cells Lacking SRF are Non-Viable - The remaining T cells may have survived without functional SRF or may represent a minority population that failed to delete the *Srf* gene. To address this question we sorted T and B cells from thymus and spleen and performed Southern blot analysis to distinguish the active *Srf* ^{fl} allele from the inactive *Srf* ^{lx} allele (Fig. 2*A*). Consistent with the absence of CD4 promoter activity in B cells, only the *Srf* ^{fl}, but not the deleted *Srf* ^{lx} allele could be detected in B cells from spleens of *CD4-CreSrf* $^{fl/fl}$ mice (Fig. 2*A*, right panel). In contrast, the *Srf* ^{fl} allele was completely recombined to *Srf* ^{lx} in $CD4^+CD8^+$ DP cells from thymi of *CD4-CreSrf* $^{fl/fl}$ mice (Fig. 2*A*, left panel). Thus, CD4-Cre mediated excision of *Srf* ^{fl} resulted in selective perturbations of T cell populations. The *Srf* ^{lx} allele was also detectable in peripheral T cells isolated from the spleens of mutant animals (Fig. 2*A*, center). However, in these splenic T cells we detected an additional band, which was specific for the intact *Srf* ^{fl} allele. We then decided to investigate SRF protein content of lymphocyte cells directly by electrophoretic mobility shift assays (EMSA). Protein extracts prepared from $CD4^-CD8^-$ DN cells (in which Cre is not yet expressed) from thymi of mutant mice contain SRF as shown by the protein-DNA complex in Fig. 2*B* (left panel). In contrast, no such SRF-DNA complex was visible when we used $CD4^+CD8^+$ DP cell extracts from *CD4-CreSrf* $^{fl/fl}$ mice. The presence of SRF protein in these shifted complexes was verified by supershift experiments with SRF antiserum. To prove the general capability of our protein extracts for specific DNA binding, we performed EMSA control experiments employing a tandem DNA binding site for the NFAT transcription factor (data not shown). Furthermore, when lymphocyte extracts from spleen were used for gel shift assays we detected SRF protein not only in B cells of *CD4-CreSrf* $^{fl/fl}$ mice, but also in the remaining peripheral T cells (Fig. 2*B*, right panel). The latter result is in agreement with the Southern blot analysis, indicating the presence of the non-recombined *Srf* ^{fl} allele in some splenic T cells. Taken together, our data suggest that peripheral T cells lacking both copies of *Srf* are non-viable and that the residual T cells found in *CD4-CreSrf* $^{fl/fl}$ mice are derived from cells that have escaped Cre-mediated deletion.

B Lineage-Specific Srf Deletion - The CD19-Cre mouse strain used here for B cell-specific recombination of the *Srf* ^{fl} allele has been generated by a knockin of the Cre recombinase into the CD19 locus. CD19-Cre hemizygous mice are phenotypically normal and can be used for B lineage-specific deletion of a floxed target gene (56). To disrupt *Srf* specifically in B cells we crossed CD19-Cre mice with *Srf* $^{fl/fl}$ animals. Mutant CD19-Cre*Srf* $^{fl/fl}$ mice were born with Mendelian distribution and appeared phenotypically normal. Southern blot analysis showed a complete B cell-specific deletion of the *Srf* ^{fl} allele in FACS-sorted splenocytes of CD19-Cre-*Srf* $^{fl/fl}$ mice (Fig. 3*A*). Presence of SRF in the

protein-DNA complexes was monitored by EMSA, including supershift assays with SRF antiserum (Fig. 3*B*). Again, NFAT DNA-binding was used in control EMSAs to check for the integrity of cell extracts (data not shown). The remaining SRF protein in splenic B cells from CD19-Cre*Srf* $^{fl/fl}$ mice is in contrast to the complete recombination of *Srf* seen in the Southern blot analysis and can probably be attributed to the long half-life of the SRF protein (15,57). This has also been noticed in embryonic myocytes (15) and in fibroblasts where a half-life of at least 12 h was estimated for SRF (57).

B Cell-Specific Srf Deletion Leads to a Decrease in B Cell Numbers, CD19 and IgM Expression - We next analyzed the overall distribution of B cells in spleen, bone marrow, peripheral blood, and lymph nodes and found that the respective cell populations of pre-B cells (IgM^-IgD^-), newly generated B cells (IgM^+IgD^-), and mature recirculating B cells (IgM^+IgD^+) exhibited comparable percentile representations in both SRF deficient (CD19-Cre*Srf* $^{fl/fl}$) and control animals (*Srf* $^{fl/fl}$ and CD19-Cre*Srf* $^{fl/wt}$) (data not shown). Quantification of the number of lymphocytes and $CD19^+$ cells from bone marrow, however, showed a decrease in the overall number of $CD19^+$ lymphocytes (Fig. 4*A*, left panel), which could not be attributed to the reduction of an individual B cell population (Fig. 4*A*, right panel). To our surprise, SRF deficient mature B cells (CD19-Cre*Srf* $^{fl/fl}$) from spleen, lymph nodes, bone marrow, and blood displayed a lower expression of the surface molecule IgM in comparison to controls (Fig. 4*B*, left; shown for blood only), whereas the expression of IgD, which is cotranscribed, remained constant (Fig. 4*B*, right). We also observed down-regulation in the expression of CD19 in SRF deficient B cells from peripheral blood. This is only partly caused by the hemizygosity of the CD19 allele, as shown by FACS analysis comparing B cells from CD19-Cre*Srf* $^{fl/fl}$ mice with B cells from control animals expressing Cre recombinase (CD19-Cre*Srf* $^{wt/wt}$ and CD19-Cre*Srf* $^{fl/wt}$) (Fig. 4*C*). Next, we tested the ability of SRF deficient splenic B cells to elicit a calcium response to the F(ab')2 fragment of anti-IgM antibodies. No differences in $[Ca^{2+}]_i$ between wildtype and mutant cells were observed when the splenic B cells were stimulated by suboptimal or optimal concentrations of anti-IgM antibodies (data not shown). This analysis confirms that the BCR complex on SRF deficient B cells is capable of receiving and delivering signals. In addition, there was no difference in the basal serum amounts of IgM, IgG1, IgG2, IgG3 and IgA of CD19-Cre*Srf* $^{fl/fl}$ mice in comparison to controls (data not shown).

B Cell-Specific Srf Deletion Leads to Absence of Marginal Zone B Cells and Decrease of CD5$^+$ B Cells - Analysis of the cell surface expression of CD21/35 and CD23 discriminates between immature B cells ($CD21/35^{neg}CD23^{neg}$), follicular B cells ($CD21/35^{int}CD23^{high}$) and MZB cells ($CD21/35^{high}CD23^{neg\text{-}low}$) (58). This analysis with SRF deficient splenocytes showed that MZB cells were specifically absent (Fig. 5*A*). MZB cell populations were also examined by microscopy of splenic cryosections stained with anti-IgM and anti-IgD (Fig. 5*B*). Normal micro-anatomic structures containing T cell areas, B cell follicular structures, and marginal zone and metallophillic macrophages were present and correctly localized. However, in agreement with the cytometric results, the characteristic ring of IgM^+IgD^- MZB cells (arrows in Fig. 5*B*) peripheral to follicles was missing in the absence of SRF. This result confirms the absence of MZB cells in CD19-Cre*Srf* $^{fl/fl}$ mice despite normal follicular architecture.

Next, we examined a possible influence of SRF on the self-renewing mature $CD5^+$ B1 B cells. Analysis of peritoneal cells from CD19-Cre*Srf* $^{fl/fl}$ mice revealed a substantial decrease of $CD19^+IgM^+CD5^+$ B cells in comparison to littermate controls (*Srf* $^{fl/fl}$ and CD19-Cre*Srf* $^{fl/wt}$) (Fig. 6).

Down-Regulation of SRF Target Genes in the B Cell-Specific Srf Mutant - To identify SRF target genes that could account for the observed phenotypic differences, we compared the gene expression profiles of FACS-sorted splenic B cells from mutant (CD19-Cre*Srf* $^{fl/fl}$) and control mice (*Srf* $^{fl/fl}$) using cDNA expression arrays (Affymetrix MOE430A GeneChip, 22690 transcripts) in two independent experiments. The microarray data were deposited in the NCBI gene expression and hybridization array data repository with assigned accession number (GEO Series Acc GSE7412). We filtered for genes whose expression was at least 1.5 fold differentially regulated. In addition to the expected down-regulation of known SRF target genes, such as the immediate early genes *egr-1*, *junB*, and *c-fos* and

the structural genes encoding *γ-actin*, *β-actin* and *vimentin*, a contribution of SRF was observed to the regulation of the accessory immunoglobulins *Igα* and *Igβ*, and *CD19*, which we already knew from our FACS analysis (see Fig. 4C), and the chemokine receptor *CXCR4*. Also, in the case of CXCR4 we were able to confirm its down-regulation on protein level by FACS analysis (Fig. 7).

DISCUSSION

Our data show substantial evidence that SRF is involved in controlling lymphocyte maturation. We deleted the conditional *Srf* ^{fl} allele (49) using mice expressing the Cre recombinase in developing T cells (CD4-Cre) (50) or developing B cells (CD19-Cre) (51). The phenotypes of these mutant mice reveal distinct requirements for SRF in the developmental control of different lymphocytes.

T cell-specific *Srf* knockout mice showed a substantial reduction in mature SP thymocytes and nearly complete absence of peripheral T cells, prohibiting a further functional analysis of this specific T cell subset. The few remaining T cells in the periphery still contained the non-recombined *Srf* ^{fl} allele and SRF DNA-binding activity, suggesting that the residual peripheral T cells had undergone population expansion from the few T cells that had escaped Cre-mediated deletion. This data provides evidence of the requirement of SRF for T cell maturation.

T cell development is controlled at multiple stages by signals from cell surface receptors. The reduced percentage of mature cells in the T cell specific *Srf* mutant mice suggests that SRF is required for positive selection, an advanced stage of T cell differentiation. Here, $CD4^+CD8^+$ DP T cells interact with peptides associated with major histocompatibility complex (MHC) proteins to either differentiate or undergo apoptosis. If positively selected, immature DP thymocytes down-regulate either CD4 or CD8 to become SP T cells (59). A contribution of SRF to the process of positive selection is consistant with data showing the necessity of the Ras/Erk (MAPK) pathway for this process. MAPK signalling is known to trigger SRF via its TCF co-factors. Dominant interfering forms of Ras, MEK, and ERK are able to block positive selection (31,60). This was confirmed by the phenotype of ERK deficient mice (60). The transcription factor egr-1 is targeted by activated ERK in thymocytes (61) and *egr-1* deficient mice exhibit impaired positive selection (39), whereas enforced *egr-1* expression promotes positive selection (62). The TCFs are known to regulate a MAPK-responsive subset of SRF target genes, including *egr-1*, *c-fos* and *Mcl-1*. Recent results from Costello and colleagues prove the TCF SAP-1 to be a direct link between ERK signalling and the transcriptional effectors of positive selection for T cells (48). SAP-1 is rapidly phosphorylated upon TCR activation via the ERK pathway, and SAP-1 deficiency caused impairment of *egr-1* and *Id-3* gene activation and decreased the amount of positively selected thymocytes. T lineage-specific deletion of *Srf* increased the severity of the SAP-1 phenotype leading to stronger reductions in the proportion of $CD4^+$ SP and $CD8^+$ SP thymocytes and peripheral T cells. This probably reflects the functional redundancy between the TCFs. Although SAP-1 was demonstrated to be the main constituent of ternary complexes in thymocytes, Elk-1 was also shown to be present (48). We demonstrated that SRF is required for T cell development following the DP stage, coinciding temporally with CD4-Cre expression and *Srf* deletion. However, it is likely that SRF has important functions also in the early, TCR-independent phase of T cell development, during e.g. T-lineage commitment, the transition from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP stage, or for the survival of mature peripheral T cells. These assumptions are supported by the finding that SRF binding activity is already abundant early in thymocyte development at the DN stage (26). Moreover, studies with *egr-1* knockout mice demonstrated defective positive selection and an increased amount of DN thymocyte precursor cells (39). Also, conditional deletion of the SRF target gene *Mcl-1* arrested T cell development at the DN stage, whereas deletion in peripheral T cell populations resulted in their rapid loss (43). The effects of a SRF deficiency on the very early stages of T cell development may be addressable using lck-Cre mice (63), permitting Cre-mediated deletion at the most immature $CD44^+$ DN stage. On the other hand, an influence of SRF on late stages of T cell development may be detectable using inducible CD4-Cre or inducible mx-Cre mouse strains (64).

B cell development in adult mice occurs in the bone marrow. Progenitor and precursor cells

differentiate into immature B cells after expressing a surface immunoglobulin receptor (IgM). Newly generated B cells leave the bone marrow and some of these cells, after further positive and negative selection steps, give rise to mature B cells. Based on phenotypic, topographic and functional characteristics, B cells are classified into at least three subsets. The heterogeneous recirculating B2 cells localize to the B-lymphoid follicles of lymph nodes and spleen, the self-renewing nonrecirculating B1 B cells are enriched in the pleural and peritoneal cavities, and the mostly nonrecirculating MZB cells are enriched in the marginal zone of the spleen (65).

Our findings imply a specialized role for SRF in $CD5^+$ B1 cells and MZB cells. No obvious B cell function was described for the SRF cofactor SAP-1 (48), possibly indicating functional redundancy of different TCFs in B1 cells. These populations are substantially decreased or even lost, respectively, in the B cell specific *Srf* mutant, which precludes a further functional analysis of these cell types. Surprisingly, we found SRF to be largely dispensable for the development of conventional B2 lymphocytes. These display no obvious change in BCR function and there is no difference in the basal serum immunoglobulin concentrations in mutant animals as compared to wildtypes.

FACS analysis of the remaining B2 cells revealed a down-regulation of the surface molecules CD19, IgM and CXCR4. Using gene arrays we detected decreased expression of *c-fos, egr-1, junB, Igα, Igβ* and several genes coding for structural proteins. As IgM and IgD are transcribed as a common pre-RNA, a transcriptional change should lead to altered expression of both genes, but the level of IgD in our mutants remained unchanged. We interpret this to be due to the reduced *Igα* message observed in the gene array. Igα is necessary for the display of IgM and IgD on the cell surface, but IgD in contrast to IgM, can be alternatively anchored via a lipid tail in the cell membrane (66).

An interesting yet difficult to address question concerns the mechanism by which SRF promotes the development of MZB and B1-type B cells. There is distinct uncertainty in the field regarding the identity of immediate precursors to these B cell subsets and signals leading to their development. The fact that numerous mutations that modulate BCR signalling (67,68) result in altered development of MZB or B1 cells led to the conclusion that increased BCR signalling is involved in the development of these B cell subtypes. If this were correct one would imagine an interplay between SRF signalling and BCR signalling. Hints for this were found in the observed reductions in the amount of the B cell coreceptor CD19, the surface molecule IgM, and the associated signal transducing elements *Igα* and *Igβ* in the remaining B cells from our mutants. In that respect, it is worth mentioning that CD19 deficient mice present multiple B cell defects, including a severe reduction in MZB and B1 cells (56,69). However, we did not detect any obvious change in BCR function as determined by measuring calcium flux activation.

Our array data proved *c-fos* to be an SRF target gene in B cells. As overexpression of *c-fos* in mice elicited an increase in peritoneal B 1 cells and MZB cells (35,36), up-regulation of *c-fos* by SRF could be one possible mechanism to influence the size of these specific B cell subsets. Defects in cell migration might similarly account for the observed phenotypes in our B cell specific SRF mutants. Indeed, ES cells lacking SRF display impaired cell-cell interactions, disorganization of the cytoskeleton, and down-regulation of surface proteins (20). Furthermore, an influence of the actin cytoskeleton and integrin-mediated processes in lymphocyte retention and localization to the marginal zone has been shown (70,71). Altered cytoskeletal activity in SRF deficient B cells may be indicated by the down-regulation of the *actin*, *vimentin* and *vinculin* genes, as detected by our array analysis. In addition, we noticed a slight decrease in the amount of beta1 and beta7 integrin (data not shown). Decreased expression of the chemokine receptor CXCR4 could likewise contribute to the phenotypic differences seen in our SRF mutant. B cell-specific inactivation of *CXCR4* affects B cell migration leading to reductions in the B1 and MZB cell compartments (72). However, a general migration defect in B cells from our mutants can be excluded, as they can be detected in the peripheral lymph nodes.

In summary, we showed that SRF is required for the development of T cells from the DP stage onward and for the development of the B1 and MZB subsets of B cells. Surprisingly, SRF deficiency does not perturb the development or survival of conventional B cells, which suggests that SRF and its cofactors mediate distinct

physiological functions in different types of lymphocytes. Thus, SRF ablation does not have pleiotropic effects on the general transcription machinery in all lymphocytes, suggesting that alternative transcriptional programs exist to regulate the survival and maintenance of B2 cells. In addition, there has to be a redundancy in factors regulating known SRF-target genes like *Mcl-1*, which was shown to be required for the survival of all sets of B lymphocytes. However, as the CD19-Cre regulatory element used to direct Cre recombinase is not active from the beginning of B lymphocyte development one could imagine that deletion at an earlier time might result in a more severe phenotype. This hypothesis is supported by the general decrease in the number of conventional B cells seen in our mutants. Using a Cre transgenic mouse line deleting earlier in B cell development, i.e. MB1-Cre (73) should reveal additional roles of SRF in the early processes of B cell differentiation.

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FOOTNOTES

* We thank A. Samuels, M. Ebel, H. Petrat, M. Schwarzkopf, and S. Keilholz-Gast for assistance, E. Serfling (Würzburg) for NFAT probes, A. Gruber for technical help and B. Prochnow for critical reading the manuscript.
Alfred Nordheim acknowledges financial support by the EU (HPRN-CT-2002-00255), the BMBF (Hepatosys), and the DFG (NO120/12-2), Werner Müller was supported by NGFN and MUGEN.

The abbreviations used are: SRF, serum response factor; TCF, ternary complex factor; MAPK, mitogen-activated protein kinase; BCR, B cell receptor; TCR, T cell receptor; MZB, marginal zone B cells; Bcl, B cell leukaemia/lymphoma; Mcl-1, Myeloid cell leukaemia sequence 1; SP, single positive; PCR, polymerase chain reaction; TBST, Tris-Buffered Saline-Tween20; SRE, serum response element; NFAT, nuclear factor of activated T cells; DP, double positive; DN, double negative; BM, bone marrow.

FIGURE LEGENDS

Fig.1. T cell-specific *Srf* deletion leads to a block in T cell development. Comparative analysis of *SRF* $^{fl/fl}$ control mice and CD4-*CreSRF* $^{fl/fl}$ mutant mice (abbreviated CD4-Cre$^-$ and CD4-Cre$^+$, respectively). *A*, Representative micrographs of paraffin sections from spleens which were stained with anti-CD3 antibody to visualize T cells or anti-B220 antibody to visualize B cells. Original magnification is equal for all pictures. *Srf* deletion leads to a strong decrease in the amount of CD3 positive cells. *B*, CD4 and CD8 expression of thymocytes from mutant and control mice was analyzed by flow cytometry. Shown is one representative example. Percentages of CD4$^+$ (SP), CD4$^+$CD8$^+$ (DP), CD8$^+$ (SP) and CD4$^-$CD8$^-$ (DN) cells are indicated in the quadrants. *Srf* deletion leads to dramatic reduction in the amount of SP T cells.

Fig.2. Peripheral T cells lacking SRF are non-viable. Comparative analysis of *SRF* $^{fl/fl}$ control mice and CD4-*CreSRF* $^{fl/fl}$ mutant mice (abbreviated CD4-Cre$^-$ and CD4-Cre$^+$, respectively). *A*, Southern blot analysis of *Bgl II* digested genomic DNA isolated from sorted DP thymocytes, SP splenocytes (T cells) and CD19$^+$ splenocytes (B cells) from mutant and control mice. Bands specific for the intact *Srf* ^{fl} allele (3.8 kb) and the deleted *Srf* ^{lx} allele (1.4 kb) are indicated. No band from the intact Srf ^{fl} allele is remaining in the DP T cells from mutant mice, whereas there is no complete *Srf* deletion in the peripheral T cells of these mice in the spleen. *B*, EMSA of SRF DNA-binding activity in nuclear extracts isolated from sorted CD4$^+$CD8$^+$ (DP) thymocytes, CD4$^-$CD8$^-$ (DN) thymocytes, CD19$^+$ (B) splenocytes and CD4$^+$/CD8$^+$ single positive (T) splenocytes. As control in parallel reactions SRF antiserum (anti-SRF) was added to the binding reactions (indicated at top of figure), generating a supershifted band with slower mobility (ssSRF). SRF-DNA complexes are absent in extracts from DP T cells of mutant mice, whereas the protein is clearly seen in the extracts of peripheral splenic T cells from these mice.

FIG. 3. B lineage-specific Srf deletion. *A*, Comparative analysis of CD19-Cre positive *Srf* $^{fl/wt}$ mice (fl/wt CD19-Cre+), CD19-Cre negative *Srf* $^{fl/wt}$ mice (fl/wt CD19-Cre-) and CD19-Cre positive *Srf* $^{fl/fl}$ mice (fl/fl CD19-Cre+). Southern blot analysis was performed on *Bgl II* digested genomic DNA isolated from sorted CD19$^+$ splenic B cells and CD4$^+$/CD8$^+$ single positive splenic T cells. Bands specific for the Srf ^{wt} allele, the intact *Srf* ^{fl} allele (3.8 kb) and the deleted *Srf* ^{lx} allele (1.4 kb) are indicated. In splenic B cells of CD19-Cre positive Srf $^{fl/fl}$ mice a complete deletion of the Srf ^{fl} allele is observed. *B*, Comparative analysis of CD19-Cre positive *Srf* $^{fl/fl}$ mice (fl/fl CD19-Cre+) and CD19-Cre negative *Srf* $^{fl/fl}$ mice (fl/fl CD19-Cre-). EMSA to show SRF DNA-binding activity was performed on nuclear extracts of sorted CD19$^+$ B cells (B) and CD4$^+$/CD8$^+$ SP T cells (T) from spleen. As control in parallel reactions SRF antiserum (anti-SRF) was added to the binding reactions (indicated at top of figure), generating a supershifted band with slower mobility (ssSRF). SRF-DNA complexes in extracts from splenic B cells of mutant mice are strongly decreased.

Fig. 4. B cell-specific *Srf* deletion leads to a decrease in B cell numbers, IgM and CD19 expression. Data are representative for at least three separate experiments. The indicated percentages always refer to gated lymphocytes. *A*, Flow cytometric quantification of bone marrow (BM) B cells shown for five CD19-Cre negative *Srf* $^{fl/fl}$ mice (filled squares) and five CD19-Cre positive *Srf* $^{fl/fl}$ mice (open squares), respectively. The left panel shows the percentage of CD19$^+$ cells in the BM, the right panel shows the distribution of the same cells at different B cell developmental stages: pre-B cells (IgM$^-$IgD$^-$), newly generated B cells (IgM$^+$IgD$^-$), and mature recirculating B cells (IgM$^+$IgD$^+$). In mutant mice a decrease in the overall number of CD19$^+$ lymphocytes is observed. *B*, IgM and IgD expression of mature CD19$^+$ peripheral blood B cells from two CD19-Cre positive *Srf* $^{fl/fl}$ mutant mice (fl/fl CD19-Cre+), two CD19-Cre positive *Srf* $^{fl/wt}$ control mice (fl/wt CD19-Cre+), and two CD19-Cre negative *Srf* $^{fl/wt}$ control mice (fl/wt CD19-Cre-), respectively, was analyzed by flow cytometry. *Srf* deletion leads to a decrease in the expression of IgM, whereas the expression of IgD remains constant. *C*, CD19 expression of mature CD19$^+$ peripheral blood B cells from CD19-Cre negative wildtype mice (wt/wt CD19-Cre-), CD19-Cre positive "wildtype" mice (wt/wt CD19-Cre+), CD19-Cre negative *Srf* $^{fl/wt}$ mice (fl/wt CD19-Cre-), CD19-Cre positive *Srf* $^{fl/wt}$ mice (fl/wt CD19-Cre+), CD19-Cre negative *Srf* $^{fl/fl}$ mice (fl/fl CD19-Cre-), and CD19-Cre positive *Srf* $^{fl/fl}$ mice

(fl/fl CD19-Cre+) was analyzed by flow cytometry. *Srf* deletion leads to an additional decrease in the expression of CD19 as compared to the decrease which is brought about by the hemozygosity of the CD19 allele in CD19-Cre positive mice.

Fig. 5. B cell-specific Srf deletion leads to absence of MZB cells. *A*, CD21/35 and CD23 expression of $CD19^+$ splenic B cells from CD19-Cre positive $Srf^{fl/wt}$ control mice (fl/wt CD19-Cre+) and CD19-Cre positive $Srf^{fl/fl}$ mutant mice (fl/fl CD19-Cre+) was analyzed by flow cytometry. Shown is one representative example. Boxed areas represent the percentages of $CD21/35^{high}CD23^{neg\text{-}low}$ MZB cells, which are missing in the mutant. *B*, Representative micrographs of cryosections from spleens of CD19-Cre positive $Srf^{fl/wt}$ control mice (fl/wt CD19-Cre+) and CD19-Cre positive $Srf^{fl/fl}$ mutant mice (fl/fl CD19-Cre+) which were stained with anti-MOMA-1 antibody (green), anti-IgM antibody (red), and anti-IgD antibody (blue). The arrowhead indicates the position of IgM^+IgD^- MZB cells, which are missing in the mutant. Original magnification is equal for all pictures.

Fig. 6. B cell-specific Srf deletion leads to a decrease in the amount of $CD5^+$ B1 B cells. IgM and CD5 expression of $CD19^+$ peritoneal B cells from CD19-Cre positive $Srf^{fl/wt}$ control mice (fl/wt CD19-Cre+) and CD19-Cre positive $Srf^{fl/fl}$ mutant mice (fl/fl CD19-Cre+) was analyzed by flow cytometry. Shown is one representative example. Boxed areas represent the percentages of $CD19^+IgM^+CD5^+$ B cells which are under-represented in the mutant mice.

Fig. 7. CXCR4 expression of $CD19^+$ splenic B cells from CD19-Cre negative wildtype mice (wt/wt CD19-Cre-), CD19-Cre positive "wildtype" mice (wt/wt CD19-Cre+), CD19-Cre negative $Srf^{fl/wt}$ mice (fl/wt CD19-Cre-), CD19-Cre positive $Srf^{fl/wt}$ mice (fl/wt CD19-Cre+), CD19-Cre negative $Srf^{fl/fl}$ mice (fl/fl CD19-Cre-), and CD19-Cre positive $Srf^{fl/fl}$ mice (fl/fl CD19-Cre+) was analyzed by flow cytometry. Shown is one representative example. *Srf* deletion leads to a decrease in the expression of CXCR4.