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## microRNA-155 Regulates the Generation of Immunoglobulin Class-Switched Plasma Cells

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### Summary

MicroRNA-155 (miR-155) is expressed by cells of the immune system following activation and has been shown to be required for antibody production following vaccination with attenuated *Salmonella*. Here we show the intrinsic requirement for miR-155 in B cell responses to thymus-dependent and independent antigens. B cells lacking miR-155 generated reduced extra-follicular and germinal center responses and failed to produce high affinity IgG1 antibodies. Gene expression profiling of activated B cells indicated that miR-155 regulates an array of genes with diverse function-many of which are predicted targets of miR-155. The transcription factor Pu.1 is validated as a direct target of miR-155 mediated inhibition. When Pu.1 is over-expressed in wild type B cells fewer IgG1 cells are produced, indicating that loss of Pu.1 regulation is a contributing factor to the miR-155 deficient phenotype. Our results implicate post-transcriptional regulation of gene expression for establishing the terminal differentiation program of B cells.

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## Introduction

MicroRNAs (miRs) have been shown to regulate gene expression by sequence-specific base pairing with target mRNAs initiating inhibition of translation or degradation of the mRNA. In animals, miRNAs are transcribed as primary miRNA (pri-miRNA). The pri-miRNA are processed in the nucleus to precursor miRNA (pre-miRNA) by the microprocessor complex prior to their transport into the cytoplasm. In the cytoplasm, they are further processed to mature miRNAs by Dicer and incorporated into the RNA-induced silencing complex. (reviewed by (Bartel, 2004)). T-cell specific deletion of Dicer has revealed a role for this enzyme in thymic development and the differentiation of T lymphocytes (Cobb et al., 2006; Cobb et al., 2005; Muljo et al., 2005; Neilson et al., 2007). Although these data imply miRNAs may be important in lymphocyte development, essential functions for individual miRNAs have not yet emerged.

MiR-155 is contained within the non-coding B cell integration cluster (*Bic*) gene (Lagos-Quintana et al., 2002). *Bic* was first identified as a frequent site of integration for the avian leukosis virus and co-expression of *bic* with *c-myc* has been found to synergize for lymphomagenesis (Tam et al., 1997). In humans, high expression of *BIC* and miR-155 has been shown in Hodgkin's lymphoma, primary mediastinal B-cell lymphoma and diffuse large B-cell lymphoma, while very low expression of *BIC* and miR-155 were reported in adult Burkitt lymphoma (Eis et al., 2005; Metzler et al., 2004; van den Berg et al., 2003). When over-expressed as a transgene in B cells miR-155 gives rise to pre-B cell lymphomas (Costinean et al., 2006). In untransformed cells of the immune system *Bic* transcripts and miR-155 expression appear to be induced by antigenic stimulation (Haasch et al., 2002; Rodriguez et al., 2007). *Bic* is also expressed by macrophages following Toll-like receptor and type-I interferon stimulation (O'Connell et al., 2007); and in B cells following treatment with antibodies to surface IgM (van den Berg et al., 2003). Furthermore, a subset of human CD20 positive cells within the germinal center has been shown to express *BIC* by RNA *in situ* hybridization (van den Berg et al., 2003).

Previous studies show that miR-155 mutant mice display defective B and T cell immunity and abnormal function of antigen presenting cells (Rodriguez et al., 2007; Thai et al., 2007). Moreover, a reduced number of germinal centre B cells was observed in miR-155 deficient mice, whereas its over-expression led to the opposite phenotype (Thai et al., 2007). Neither of these studies identified the cellular basis of the defects *in vivo*. Both studies used miR-155 germ line mice and the conditional expression of miR-155 was driven by Cre under the control of the CD21 promoter. Thus, expression of miR155 was targeted to both B cells and follicular dendritic cells (Victoratos et al., 2006). Here we show that defects in humoral immunity following primary and secondary immunization are intrinsic to B lymphocytes. Microarray analysis of B cells activated under conditions that promote class switching to IgG1 revealed miR-155 regulates expression of many genes, a substantial fraction of which are predicted to be direct targets of miR-155. One of these genes was *Sfp1* (encoding the transcription factor Pu.1) which has a highly conserved functional miR-155 binding site in its 3'UTR. Moreover, Pu.1 is highly expressed in miR-155 deficient B cells and Pu.1 over-expression in wild type B cells results in reduced numbers of IgG1 switched cells. Our results indicate that miR-155 plays a key role in antigen-driven B cell maturation

and the persistence and/or differentiation of Ig class switched cells and that deregulation of Pu.1 is likely to be a contributing factor to the phenotype observed in miR-155-deficient mice.

## Results

### Deficiency in miR-155 leads to impaired primary and secondary immune response

It has been previously shown that miR-155 is dispensable for lymphocyte development but necessary for the generation of T and B cell responses *in vivo* (Rodriguez et al., 2007; Thai et al., 2007). To further understand the role of miR-155 in regulating the function of B cells we studied the requirement of miR-155 for T-independent type-I (TI-1) responses by immunizing mice with the dinitrophenylated lypopolysaccharide (DNP-LPS). We observed defective switched antibody responses at day 7 after immunization (Figure 1A). By contrast, the production of antigen-specific IgM was normal.

Next we studied the response of miR-155-deficient mice to the well characterized T-dependent (TD) antigen 4-hydroxy-3-nitrophenylacetyl conjugated to keyhole limpet hemocyanin (NP-KLH). Wild type and miR-155-deficient mice produced similar amounts of NP-specific antibodies of the IgM class following primary immunization (Figure 1B, left panel). On the other hand, the titers of primary IgG1 antibodies in miR-155 deficient mice were reduced to 20% of WT controls when NP<sub>17</sub>-BSA was used as the capture antigen (Figure 1B, middle panel). Using more stringent binding conditions with NP<sub>3</sub>-BSA as the capture antigen significant amounts of bound IgG1 were only seen in WT mice (Figure 1B, right panel), indicating impaired affinity maturation in the miR-155-deficient mice (Herzenberg et al., 1980). This defect can be readily seen by calculating the ratio of titers detected with NP<sub>3</sub>-BSA to those of NP<sub>17</sub>-BSA (Figure 1C). We also observed that re-immunization with soluble NP-KLH 70 days after primary immunization yielded impaired memory responses, suggesting that miR-155 is required for the generation and (or) maintenance or reactivation of memory B cells.

### Deficiency in miR-155 leads to reduced IgG1 secretion and impaired affinity maturation in a B cell autonomous manner

We wished to determine whether the impaired antibody production was intrinsic to B cells. To this end, we created mixed chimeras by transferring 20% of either wild type, or miR-155-deficient, bone-marrow cells with 80% of  $\mu$ MT deficient bone-marrow cells into sub-lethally irradiated mice double deficient in *Rag2* and the common cytokine receptor gamma chain (*Rag2*<sup>-/-</sup> *Il2rg*<sup>-/-</sup> mice), as previously described (Huntington et al., 2006). The  $\mu$ MT mutation prevents the generation of B cells, so the B cells in the animals receiving miR-155-deficient marrow will be miR-155 deficient, while the recipients of wild type marrow will have wild type B cells. The 20/80 ratio favors reconstitution of all the other hemopoietic lineages from wild type precursors (Huntington et al., 2006). Both groups of chimeras had similar proportions and numbers of B, CD4<sup>+</sup> and CD8<sup>+</sup> T cells (data not shown). We first studied the primary response to NP-KLH and found that the miR-155-deficient,  $\mu$ MT-deficient chimeras again had reduced IgG1 antibody production compared to the WT,  $\mu$ MT-deficient chimeras (Figure 2A-D) and recapitulated the phenotype observed in

miR-155-deficient mice; specifically, reduced amounts of NP-specific IgG1 in serum and impaired affinity maturation. We also observed that the defect in Ig secretion was not restricted to IgG1, as production of IgG2b and IgG3 subclasses was also significantly reduced ( $P<0.001$ , Figure 2E). These results demonstrate a B-cell intrinsic requirement miR-155 for the production of switched and high affinity antibodies.

### **Impaired extrafollicular and germinal centre responses in the absence of miR-155 lead to reduced numbers of antigen specific IgG1 AFC**

The defects observed in the production of antigen-specific switched antibodies in response to NP-KLH prompted us to study the primary response using quantitative immunohistology. Immunization with T-dependent (TD) or T-independent (TI) antigens elicits an extrafollicular response which rapidly produces large amounts of IgM and switched antibodies providing a first line of defense. TD antigens, in addition, promote B cell growth in follicles where they form germinal centers resulting in the production of high affinity antibodies by plasma cells and generation of memory B cells. Examination of naïve miR-155-  $\mu$ MT double deficient chimeric mice reveals no overt abnormality in the structure of the white pulp (data not shown). However, after immunization with NP-KLH the extrafollicular response in miR-155-  $\mu$ MT double deficient chimeric mice was severely impaired. This was reflected by a significant reduction in the number of NP-specific plasmacytoid cells (plasmablasts and or plasma cells) seven days after immunization ( $P<0.05$ , Figure 3A-B). By day 14, the number of NP-specific plasmacytoid cells had fallen, as expected, in the wild type chimeric mice to 10% of their number at day 7. In the miR-155-  $\mu$ MT double deficient chimeric mice the numbers of NP-specific plasma cells had fallen to numbers that were not significantly different from those in non-immunized chimeras ( $P>0.05$ , Figure 3B). Kinetic analysis of the frequency of NP-specific IgG1 antibody forming cells (AFC) in the spleen, measured by enzyme-linked immunosorbent spot (ELISPOT), mirrored the emergence and disappearance of NP-specific plasmacytoid cells (Figure 3C). Moreover, the frequency of NP-specific IgG1 AFCs was significantly reduced in the absence of miR-155 ( $P<0.05$ , Figure 3C). The average spot size was similar to wild type cells ( $145\pm 24$  vs  $141\pm 17$ ,  $n=4$ , mean  $\pm$  SE), suggesting that miR-155 does not regulate secretion from plasmacytoid cells. Taken together, these results suggest that miR-155 regulates the output of antibodies produced in extrafollicular responses.

Analysis of the GC response showed that NP-specific GC were formed at day 7 in the miR-155-deficient,  $\mu$ MT-deficient chimeric mice (Figure 3A). However, the areas occupied by miR-155-deficient GCs were significantly smaller than those of wild type mice ( $P<0.001$ , Figure 3A-B). Similar results were observed at day 14 (Figure 3B), in agreement with a recent report (Thai et al., 2007). Antigen-specific plasma cells originating from the GC reaction preferentially migrate to the bone marrow where they reside for extended periods. We found that the frequency of NP-specific IgG1 AFC in the bone marrow 14 days after immunization was significantly reduced in the chimeric mice with miR-155- deficient B cells compared to those with WT B cells ( $P<0.05$ , Figure 3C). Reduced switched antibody production by miR-155 deficient B cells is therefore a consequence of lowered numbers of switched plasma cells arising from the extrafollicular and the GC responses.

### Impaired memory response in the absence of miR-155 is B cell autonomous

Secondary immunization of miR-155 deficient germ-line mice yielded reduced production of antigen-specific antibodies of low affinity (Figure 1B-E). To assess intrinsic defects of miR-155-deficient memory B cells we used the adoptive transfer method, as B cells influence T helper cell function and T cell memory (Linton et al., 2003; Linton et al., 2000; van Essen et al., 2000) and T cells, in turn, influence B cell memory function. B220<sup>+</sup> cells were purified from the spleens of  $\mu$ MT-deficient or miR-155- $\mu$ MT double deficient chimeras previously immunized with NP-KLH in alum and mixed with KLH primed T cells from C57BL/6 mice. The mixed cells were then transferred into *Rag2*<sup>-/-</sup> *Il2rg*<sup>-/-</sup> double deficient mice and boosted with soluble NP-KLH. The titers of anti-NP IgG1 antibodies were below detection when the mice were not immunized, suggesting no contamination by long-lived AFC in transferred B cells (Figure 4). Also, NP-IgG1 titers from mice that only received T cells were below the limit of detection, suggesting no contamination with wild type antigen-specific B cells (Figure 4). As observed in the primary response, we found reduced amounts of NP-specific IgG1 antibodies (Figure 4A-B) and impaired affinity maturation (Figure 4D). Thus, memory B cell function is regulated by miR-155 in a B cell autonomous manner. To assess whether miR-155 is required for the generation and/or maintenance of memory B cells we identified by FACS antigen-specific switched memory B cells 42 days after immunization with NP-KLH. At this time point, a stable population of IgG1<sup>+</sup>, NP<sup>+</sup>, B220<sup>+</sup> is found in the spleen (Blink et al., 2005). We observed a reduced proportion of switched memory cells in the absence of miR-155 (Figure 4E), suggesting that miR-155 may regulate the generation or maintenance of memory B cells.

### Normal rate of somatic hypermutation and class switch recombination in miR-155 deficient B cells

Impaired production of high affinity antibodies and reduced amounts of switched antibodies can result from dysfunction of the machinery for somatic hypermutation (SHM) and class switch recombination (CSR). To assess whether miR-155 was required for somatic hypermutation we compared the frequency and pattern of mutations between wild type and miR-155<sup>-/-</sup> mice in a system that is not biased by selection. This was achieved by sequencing the intronic V<sub>H</sub>J558-J<sub>H</sub> rearrangement-flanking regions from germinal center B cells obtained from Peyer's patches (Jolly et al., 1997). Our analysis showed no significant difference in the frequency of mutations between wild type and miR-155 deficient B cells (*P*=0.4, Figure 5A). Moreover, no difference in the pattern of nucleotide substitutions was observed between wild type and miR-155-deficient B cells (Figure 5A). We conclude that miR-155 does not regulate the machinery for SHM.

To examine CSR to IgG1 we used the well-characterized *in vitro* system of culturing B cells with LPS and IL-4 (Hodgkin et al., 1996). After culture with LPS and IL-4 miR-155 deficient B cells produced reduced IgG1 (Rodriguez et al., 2007). Defective secretion of IgG1 was accompanied by a reduction in the proportion of cells expressing IgG1 on the cell surface (Supplementary Fig. 1). Combinations of CD40 with IL-4 or IL-5 yielded similar results (Supplementary Fig. 1B). By contrast, the number of CD138 positive cells was only marginally impaired consistent with normal IgM secretion (Supplementary Fig. 1B). This inhibition was not reflected in a failure of B cells to proliferate, as judged by incorporation

of radioactive thymidine using a variety of stimuli (Rodriguez et al., 2007) (also Supplementary Fig. 1A). We next quantified the expression of activation-induced cytidine deaminase (*Aicda*) mRNA, as this enzyme regulates CSR, but found this was not reduced in the absence of miR-155 (Figure 5B). We also found similar expression of  $\mu$  and  $\gamma 1$  encoding sterile transcripts as well as  $\gamma 1$  encoding post-switch circle transcripts in miR-155-deficient and wild type B cells (Figure 5C). Thus, the failure of miR-155 deficient B cells to generate high affinity switched antibodies appears not to be due to a defect in SHM or CSR but more likely to a defect in the differentiation or survival of plasmablasts.

We examined whether the reduction in the number of IgG1<sup>+</sup> cells following *in vitro* differentiation of miR-155-deficient B cells with LPS and IL-4 reflected defective survival. As *bcl-2* promotes survival of lymphocytes both *in vivo* and *in vitro* (Vaux, 1993), we tested if the number of IgG1<sup>+</sup> cells could be rescued by exogenous expression of *bcl-2*. To this end miR-155 deficient B cells were transduced with a retrovirus expressing *bcl-2* with an IRES-GFP reporter to enable the identification of infected cells. The results indicate that *bcl-2* was unable to rescue the defect (Supplementary Fig. 2).

### Genome-wide analysis of gene expression shows enrichment of miR-155 binding sites in mRNA upregulated in miR-155<sup>-/-</sup> B cells

Our results thus far indicate that miR-155 is required for the maintenance and/or differentiation of IgG switched cells. To analyze how the absence of miR-155 affects the global gene-expression program underlying B cell differentiation we compared gene expression in wild type and miR-155-deficient B cells treated with LPS and IL-4. Kinetic analysis indicates that *Bic* and miR-155 expression peaks at 24 hrs after stimulation with LPS and IL-4 (Supplementary Fig. 3). This time point was chosen as it allows the examination of gene expression before the emergence of IgG1 cells. Since direct targets are predicted to have higher expression in absence of miR-155, we expected them to be enriched amongst the genes up-regulated in miR-155-deficient B cells.

A total of 101 protein coding genes were significantly increased in miR-155 deficient B cells whereas 84 were decreased in expression ( $P < 0.05$ ). Amongst the deregulated genes are transcription factors, signaling molecules, adhesion molecules and regulators of chemotaxis (Supplementary Tables 1 and 2). Several groups have computationally predicted miRNA targets based on perfect Watson-Crick complementarity of 6-8 nucleotides between the 5' end of a miRNA and the 3'UTR of mRNAs (Brennecke et al., 2005; Doench and Sharp, 2004; Lai, 2002), a region of the miRNA referred to as the "seed". To test whether up-regulated mRNAs in miR-155-deficient B cells may be direct miR-155 targets, we searched their 3'UTR for the presence of miR-155 seed matches. We found 97 up-regulated transcripts with annotated 3'UTRs, 60 of which contain at least one seed match for miR-155 (Figure 6A and Supplementary Table 2). As a control, we searched the up-regulated gene set for seed matches for all mouse miRNAs present in miRBase 9.1 (Giraldez et al., 2006; Griffiths-Jones et al., 2006). Amongst all possible 6nt, 7nt and 8nt seed sequences, only the enrichment of miR-155 matches was found to be statistically significant ( $P = 4.3 \times 10^{-5}$ , Figure 6B and Methods). When performing a similar analysis with all possible hexamers, the miR-155 6nt seed was found to be the top-ranked hexamer (data not shown). These

results demonstrate a highly significant enrichment of direct miR-155 targets amongst the mRNAs that were increased in abundance in miR-155 deficient B cells.

### **Pu.1 is a direct target of miR-155 and its over-expression in wild type B cells impairs the emergence of IgG1 switched cells**

Computationally predicted targets for miR-155 include transcription factors and regulators of cell differentiation and metabolism. Expression of *Aicda* was 1.6 fold increased in miR-155-deficient B cells (Supplementary Table 2), a trend we have also observed after 72 hours of *in vitro* culture with LPS and IL-4 (Figure 5B). We further validated the expression of five other genes up-regulated in miR155 deficient B cells which contain miR-155 binding sites in their 3'UTRs, by quantitative PCR (Figure 7A). We focused our attention on *Sfp1*, the gene encoding Pu.1, a member of the Ets domain-transcription factor family which plays a central role in many aspects of haematopoiesis. In B cells Pu.1 is required at early developmental stages (McKercher et al., 1996; Scott et al., 1994) and has overlapping roles, in the regulation of B cell function, with another Ets family member SpiB (Garrett-Sinha et al., 1999). Pu.1 is expressed at low amounts in lymphocytes and was not readily detected by immunoblot of lysates from wild type B cells. However, we could easily detect Pu.1 expressed in miR-155 deficient B cells (Figure 7B) consistent with Pu.1 being a direct target of miR-155. The 3'UTR of Pu.1 includes a phylogenetically conserved stretch of 8 nucleotides perfectly complementary to the miR-155 seed region (Figure 7C, left panel). When the Pu.1 3'UTR was coupled to a luciferase reporter and co-transfected with miR155 mimics into 293T cells we observed significant repression of the reporter ( $P < 0.0001$ , Figure 7C right panel). The repression was alleviated when 3 nucleotides from the predicted binding site were mutated (Figure 7C, right panel), indicating this binding site is functional. Taken together, these results indicate that Pu.1 is a direct target of miR155. We next tested whether over-expression of Pu.1 recapitulated aspects of the phenotype caused by deletion of miR-155. To this end, we transduced wild type B cells stimulated with LPS+IL-4 with a retroviral vector allowing bicistronic expression of Pu.1 and GFP. After 3 days, surface expression of IgG1 was determined in the fraction of B cells expressing Pu.1 which were identified on the basis of being GFP<sup>+</sup>. We observed a reduction in the proportion of IgG1 expressing cells (Figure 7D) which resembled the *in vitro* phenotype of miR-155 deficient B cells (Supplementary Figure 2). Similar results were observed when we measured intracellular expression of IgG1 (Figure 7E, left). The proportion of IgM positive cells was accordingly increased when Pu.1 was over-expressed (Figure 7E, right). As with miR-155 deficient cells, the inability to switch was not due to altered cell division. Staining with the dye PKH26, which allowed tracking of cell divisions by GFP expressing cells, showed a similar extent of division between wild type cells transduced with GFP only expressing virus or GFP and Pu.1 (Figure 7F). The switching was specific to Pu.1, as overexpression of another predicted target of miR-155, c-myb, resulted in impaired cell survival (data not shown). Taken together, our results suggest that miR-155 may affect the generation of IgG1<sup>+</sup> cells *in vitro* by regulating the expression of Pu.1.

## Discussion

Here we demonstrate that miR-155 is required for TI and TD responses, particularly the production of switched antibodies. Chimeric mice recapitulated all of the phenotypic alterations observed in the germ line mice, therefore B cells require miR-155 function in a cell-autonomous manner for the secretion of switched antibodies and affinity maturation in both primary and memory responses.

Our analysis of the primary TD response indicated the extrafollicular response was suppressed in miR-155 deficient B cells. This reduction was not due to defective proliferation, nor to reduced frequency of NP binding B cell precursors (data not shown). In addition, we found reduced humoral responses after immunization with the TI antigens DNP-LPS and DNP-Ficoll (data not shown), two antigens that promote a robust extrafollicular response. It is thus possible that NP-specific cells are being recruited into the response, but miR-155-deficiency prevents most of these cells maturing into plasmablasts. Alternatively, there may be a high death rate amongst miR-155 deficient plasmablasts. In contrast to our current study, where immunisation with haptenated proteins leads to normal IgM responses, infection of miR155 deficient mice with attenuated Salmonella resulted in reduced secretion of antigen specific IgM (Rodriguez et al., 2007). This difference may be due to the different responses these antigens elicit. Unlike NP-KLH, infection with Salmonella generates a massive T-dependent extrafollicular response which persists for up to 5 weeks (Cunningham et al., 2007). Furthermore, it is possible that reduction of IgM in the Salmonella infection is not B cell autonomous as the experiment has only been conducted in germ line mice.

The kinetics of formation of GCs was not affected by miR-155 deletion, despite a 50%-60% reduction in the size of the GCs. We found a 50% reduction in the proportion of GC B cells from Peyer's patches of miR-155 deficient mice (data not shown). The outcome of the GC response was also impaired as there were reduced numbers of antigen specific IgG1 plasma cells within the bone marrow and impaired production of high affinity antibody. The secondary IgM response of germ-line mice was defective suggesting that low affinity memory responses were impaired. Taken together, these data indicate that mir-155 may regulate the emergence or maintenance of memory cells.

SHM and CSR operate normally in the absence of miR-155 thus we suggest that miR-155-deficient B cells are likely able to class switch and hypermutate *in vivo*. The loss of affinity maturation in the presence of hypermutation suggests that there is a failure to select high affinity B cells. This might reflect impaired signaling through the BCR or other receptors during selection and may partly explain the reduction in size of the GCs. In support of that, *BIC* is expressed upon BCR crosslinking (van den Berg et al., 2003), by a subset of germinal centre B cells (van den Berg et al., 2003) and, in mice, upon CD40 stimulation (data not shown). Some limited selection must be occurring, as GC in the chimeras with miR-155 deficient B cells were small but persistent. Over-expression of *bcl-2* can prolong survival but cannot provide differentiation signals. Thus *in vivo* miR-155 deficient B cells may be receiving sufficient selection signals to survive, but still failing to receive, or respond to, signals that induce differentiation to plasma cells. Another contributing factor to the

defective germinal center response in miR-155 may be reduced production of cytokines by B cells as has been previously suggested (Thai et al., 2007). However, our histological analysis showed that the anatomy of the germinal centers (dark and light zones) was not affected by the absence of miR-155 in B cells (data not shown).

Our current model of plasma cell differentiation is based on the concerted action of several transcription factors (reviewed by (Shapiro-Shelef and Calame, 2005)). Our results show that miR-155 directly regulates the expression of Pu.1 and its over-expression impairs the emergence of IgG1 positive cells. It is currently unknown how Pu.1 may regulate terminal B cell differentiation as no phenotype was observed in mice with B cells deficient in Pu.1 (Polli et al., 2005). It is worth noting that Pu.1 is highly expressed in germinal centre B cells and is down-regulated in post-germinal centre cells (Cattoretti et al., 2006). Therefore, it is possible that miR-155 is required for the down-regulation of Pu.1, and, in the absence of miR-155, excessive Pu.1 affects the output of the germinal center reaction.

Microarray analysis showed that deletion of a single miRNA in B cells causes deregulation of a large number of genes. Amongst genes which showed increased expression more than half of them were predicted to be direct targets of miR-155. It is thus possible that the phenotypic alterations observed in miR-155 deficient mice are the result of deregulation other targets in addition to Pu.1. Such a view is consistent with recent reports of the role of mir-181a in setting the threshold for T-cell receptor signal transduction through the regulation of multiple phosphatases (Li et al., 2007) or in the regulation of cardiogenesis by miRNA-1-2 (Zhao et al., 2007). Elucidating how deregulation of Pu.1 contributes to the phenotype caused by deletion of miR-155 *in vivo* will be the next challenge.

## Experimental Procedures

### Generation of chimeric mice and immunisation

miR-155 deficient mice (*Bic<sup>m2</sup>* allele) were previously described (Rodriguez et al., 2007). For TI responses *Rag2<sup>-/-</sup> Il2rg<sup>-/-</sup>* mice were irradiated (5.0 Gy) and reconstituted with  $3 \times 10^6$  bone marrow cells. For the generation of mixed chimeras irradiated *Rag2<sup>-/-</sup> Il2rg<sup>-/-</sup>* mice received a mixture of 80% bone marrow cells of  $\mu$ MT origin and 20% wild type or miR-155 deficient. For TI responses, 25  $\mu$ g of DNP-LPS was administered intraperitoneally. For TD responses 100  $\mu$ g of alum precipitated NP-KLH (Biosearch Tech) was used.

### Adoptive transfer and immunization

Splenic B cells were enriched by positive selection using B220 beads (Miltenyi Biotech) from either  $\mu$ MT-deficient or miR-155- $\mu$ MT double deficient chimeras previously immunized with 100  $\mu$ g of NP-KLH precipitated in alum. T cells were enriched by negative selection using MACS system (Miltenyi Biotech) from the spleens of C57BL/6 immunized with NP-KLH in alum. The mixture of separated B cells ( $5 \times 10^6$ ) and T cells ( $2.0 \times 10^6$ ) were i.v. injected into *Rag2<sup>-/-</sup> Il2rg<sup>-/-</sup>* mice and subsequently immunized with 50  $\mu$ g of soluble NP-KLH.

## ELISA and ELISPOT assay

NP-specific AFC were detected using ELISPOT as previously described (Xiang et al., 2007). NP specific antibodies were detected by ELISA as previously described, except that antibody end point titers were used as a measure of relative concentration.

## Immunohistology

NP, IgG1 staining were detected as described previously (Cunningham et al., 2002; Luther et al., 1997). NP-binding cells were detected using NP conjugated to sheep IgG fraction. Biotinylated donkey anti-goat-Ig antibodies (Dako), which bind sheep Ig, were used as a conjugate to detect bound NP-sheep-Ig (Luther et al., 1997). The proportion of spleen sections occupied by germinal sections was determined using the point counting technique of Weible (Weibel, 1963). NP-specific plasmacytoid cells per mm<sup>2</sup> were counted at x 100 magnification using serial sweeps of each spleen section using a 1 cm<sup>2</sup> eyepiece graticule divided into 100 one mm<sup>2</sup> squares to define the section area being counted.

## B cell stimulation and quantitative PCR

B cells were purified by negative selection using the MACS system (Miltenyi Biotech) and stimulated with 10µg/ml LPS (Sigma) and 20 ng/ml IL-4 (Peprotech) for 3 days. Subsequently, RNA was extracted using TRIzol (Invitrogen) and converted to cDNA. Controls without reverse transcriptase were included for all samples. *Aicda*, *Sfp1l*, *Myb*, *Bat5*, *Rheb*, *Jarid2* mRNA and *Bic* mRNA were quantified using Taqman probes (Applied Biosystems) and normalized to the expression of  $\beta$ 2M (Applied Biosystems). Expression of  $\mu$  and  $\gamma$ 1 encoding sterile transcripts and post-switch  $\gamma$ 1 encoding circular transcripts were normalized using GADPH exactly as described (Reina-San-Martin et al., 2003).

## SHM

Germinal centre B cells were identified as and purified by FACS sorting. We analyzed somatic hypermutation by monitoring mutations in the intronic V<sub>H</sub>J558-J<sub>H</sub> rearrangement-flanking region in germinal centre B cells purified from Peyer's patches as described (Jolly et al., 1997). GC B cells were identified as B220<sup>+</sup>CD95<sup>+</sup> PNA<sup>high</sup> and purified by FACS sort from 4 month old mice (C57BL/6).

## Microarray analysis

B cells from five wild type and five miR-155 deficient mice were cultured with LPS and IL-4 for 24 hours as described above and RNA was extracted using TRIzol. Gene expression profiling was performed by hybridization to the mouse genome 430 2.0 Genechip arrays (Affymetrix) by Geneservice Ltd (Cambridge, UK). Genes with  $P < 0.05$  and a fold expression difference  $\geq 1.5$  were selected for further analysis. Microarray data for the data presented in this paper have been deposited in the ArrayExpress database with accession number E-MEXP-1325.

## Computational miRNA target search and calculation of binding site frequencies in mouse 3'UTRs

The method used to calculate miRNA binding site frequencies was essentially as described in (Giraldez et al., 2006). Mouse miRNAs in miRbase registry Release 9.1 (Griffiths-Jones, 2004) were included in our analysis. The set of up-regulated transcripts for which 3'UTR sequences could be obtained (test group) were examined for potential miRNA 'seed' matches. The average number of seed sequences found in the test group per 1 kb of sequence was used to calculate seed 'Observed Frequency' ( $F_{\text{obs}}$ ) values. The seed 'Genome Frequency' ( $F_{\text{genome}}$ ) values were calculated from all mouse 3'UTRs present on the microarray and represent the average occurrence for any given seed sequence pattern per 1 kb of sequence. The 'Fold Enrichment' (FE) value is calculated as:  $FE = \log(F_{\text{obs}} / F_{\text{genome}})$ . To correct for low-count biases those miRNAs whose 6nt(2) seed was present at less than 0.1 sites/kb in genomic 3'UTRs were removed from the analysis. To calculate the statistical significance of the FE for any seed, we randomly selected 10,000 sets with the same number of 3'UTR as the test group. From each one of these,  $FE_{\text{rand}}$  values were calculated and the mean and standard deviation (SD) were obtained. We can then describe any seed FE with a Z-score =  $(FE - \text{mean}) / SD$ . These Z-scores are essentially 'Fold Enrichment' scores that have been normalized taking into account the enrichment variability of that particular seed in the genome. A *P*-value was calculated for each Z-score as well as an E-value that takes into account the multiple numbers of seeds that we are testing.

### Luciferase assays

The *Sfp1* 3'UTR was amplified from genomic DNA, and inserted into the psiCheck-2 renilla luciferase reporter plasmid (Promega). This construct was used to derive a miR-155 'seed' mutant plasmid with the QuikChange Multi Site Mutagenesis Kit (Stratagene). The mutagenic primers used were: sense 5'-gaccccgccgcatagcaaataccgtcgcc-3' and antisense: 5'-ggcgacgggtatttctatggccgggggtc-3'. The correctness of all plasmids was confirmed by sequencing. Reporter assays were performed in 293T cells co-transfected in triplicates using Lipofectamine 2000 (Invitrogen) with test plasmid (3'UTR Pu.1 wild type or mutant) along with either murine miR-155 mimic or miR-124a control (Dharmacon) at a final concentration of 40 nM. Firefly luciferase was used as a normalization control. Reporter activity was detected 24 hrs post-transfection with the Dual-Glo Luciferase Assay System (Promega). Expression values were normalized against the average value for the corresponding plasmid as in (Rodriguez et al., 2007).

### Retroviral infection and FACS analysis

Stable cell lines with bicistronic expression of bcl-2 and GFP or GFP alone were kindly provided by Demin Wang (Wen et al., 2003). Retroviral control and GFP/Pu.1 expressing vectors were kindly provided by Rodney DeKoter (DeKoter and Singh, 2000). In this case, viral supernatants were collected 48hrs after transiently transfecting PlatE cells. Purified B cells were cultured with LPS and IL-4 as described above. After 16 hours, cell supernatant was removed and cells were resuspended in viral supernatant supplemented with 6µg/ml of polybrene, LPS and IL-4 and centrifuged for 30 minutes at 2500 rpm at room temperature. Cells were returned to 37C and the following day supernatant was removed and cells were

re-cultured in LPS and IL-4 until analyzed by FACS. Cultured cells were stained with the following antibodies: anti-Syndecan 1 (CD138): 281.2 (BD), anti-IgG1: A85-1 and anti-IgM: goat polyclonal (Jackson Laboratories). For intracellular staining cells were fixed for 20 minutes using Cytofixcytoperm (BD) and stained in Perm-wash solution (BD). Data were collected using FACs caliber or LSRII (BD) and analyzed using Flowjo (treestar).

### Proliferation

The following B cell stimuli were used at the indicated concentrations: anti-IgM F(ab)<sub>2</sub> fragment, 5µg/ml; anti-CD40 (3/23 clone), 10µg/ml; mIL-4 (Peprotech), 20ng/ml; mIL-5 (Sigma), 25ng/ml. B cell proliferation was measured after addition of [<sup>3</sup>H]-thymidine for the final 16 hours of culture.

### *Bic* and miR-155 expression

Expression of *Bic* was measured by quantitative PCR as previously described (Rodriguez et al., 2007). miR-155 expression was determined by Northern blot as previously described (Rodriguez et al., 2007).

### Statistical analysis

Statistical analysis was performed using two-tailed Student-t test, one-way or two-ways ANOVA, as indicated in the figures legends, using GraphPad Prism 4 or Graphpad InStat 3 software.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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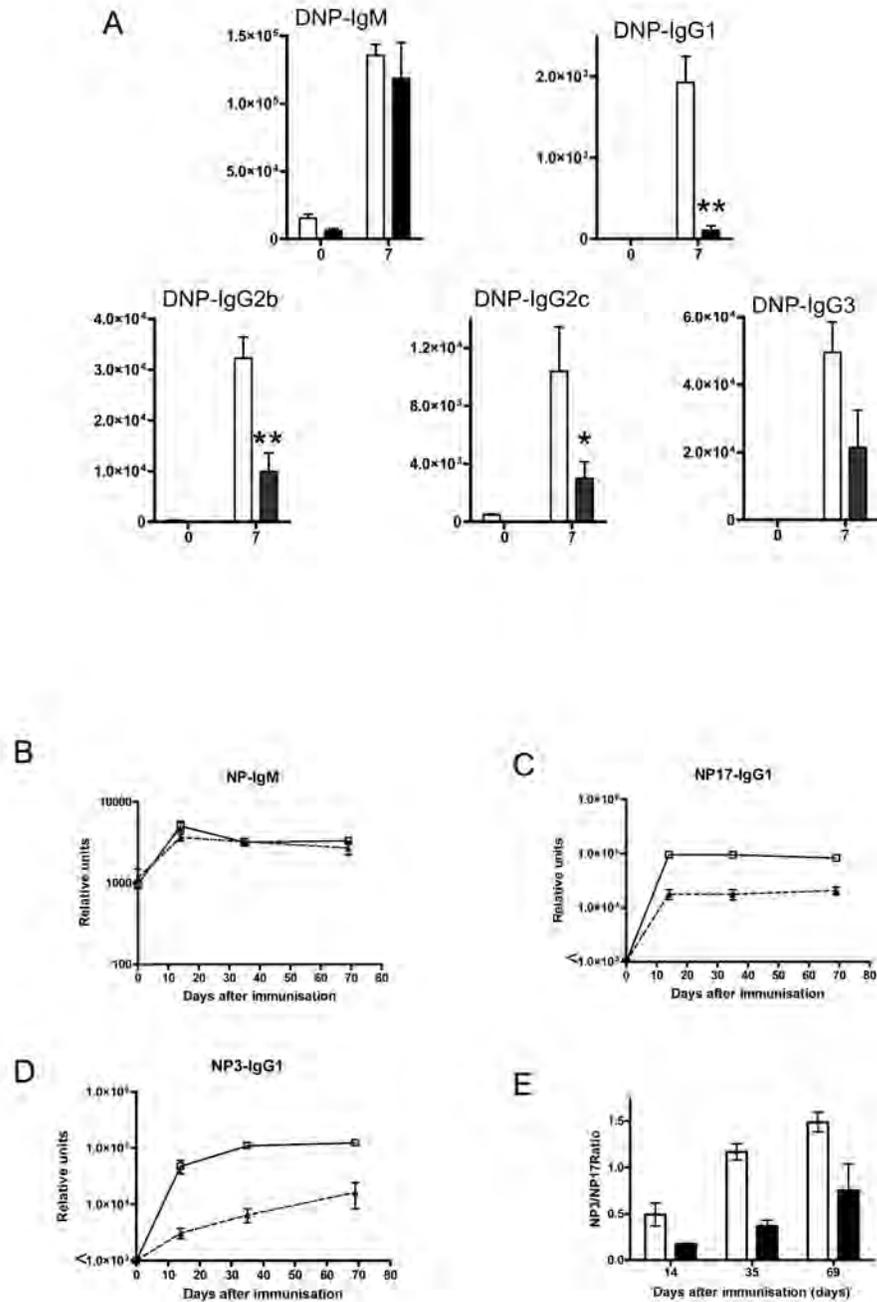
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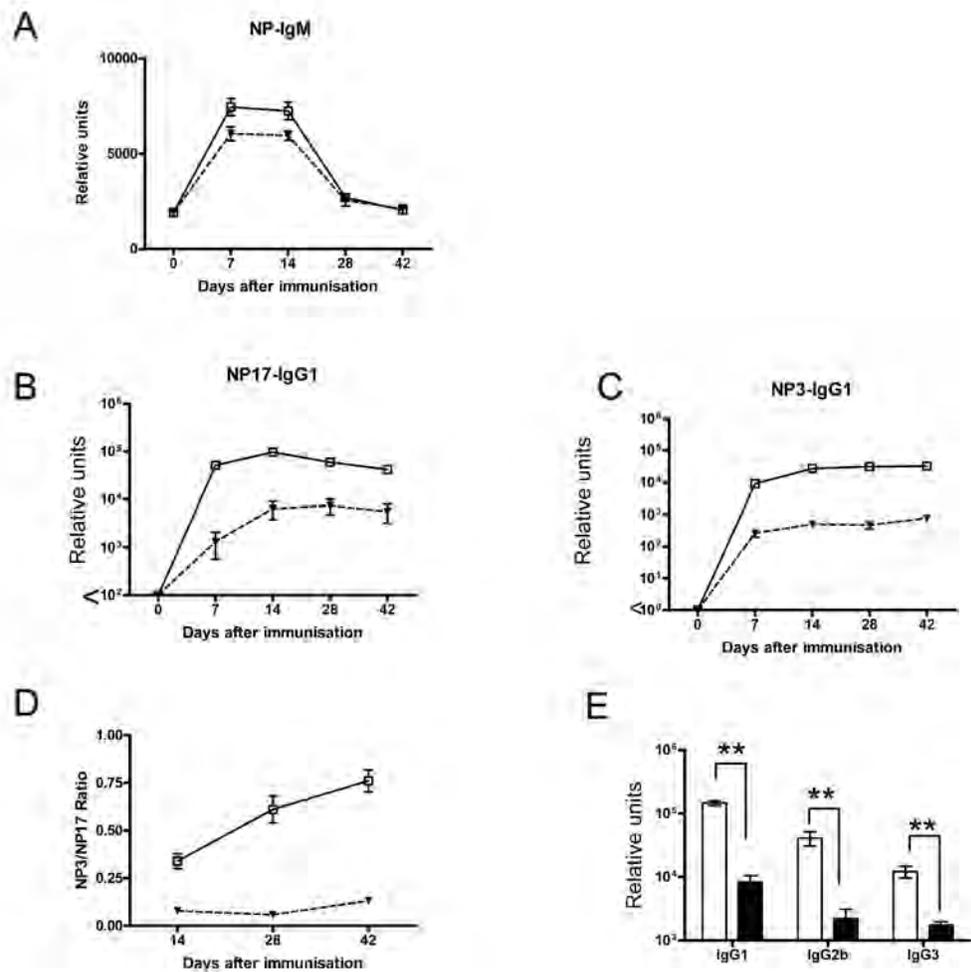
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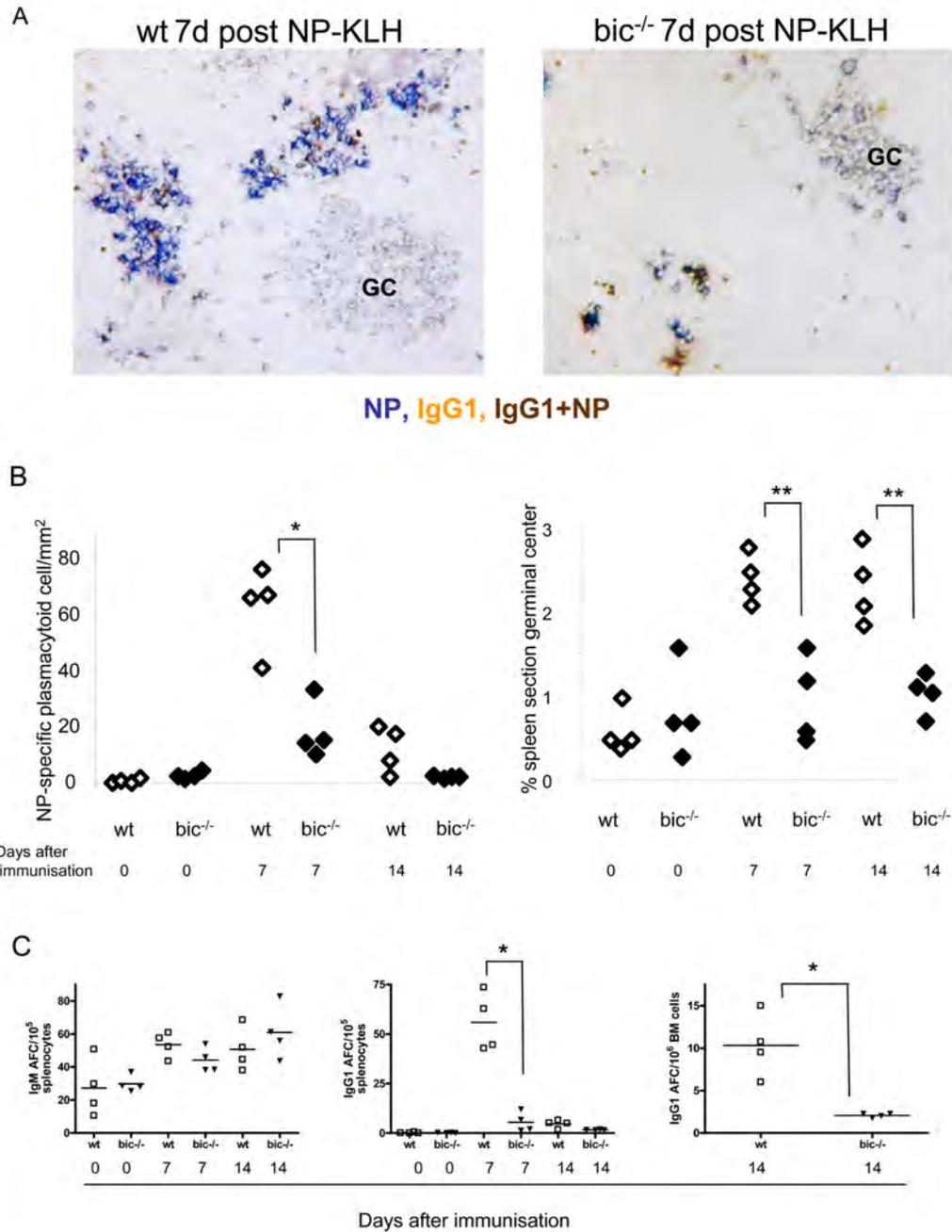
**Figure 1. miR-155 deficient mice produce reduced amounts of low affinity IgG1 antibodies**  
 (A) Wild type (open squares) or miR-155 deficient chimeric mice (filled triangles) were immunized with DNP-LPS and anti-DNP specific antibody titers were measured 7 days later. “\*” corresponds to  $P < 0.05$  and “\*\*\*” to  $P < 0.005$  using Student t-test. (B-C) Wild type (open squares) or miR-155 deficient mice (filled triangles) were immunized with NP-KLH in alum at day 0 and day 70, indicated by the arrowheads. Production of NP specific antibody titers were measured at the indicated times. (B) Titers of IgM anti-NP were measured using NP<sub>17</sub>-BSA (left). Titers of IgG1 anti-NP measured using NP<sub>17</sub>-BSA

(middle) or NP<sub>3</sub>-BSA (right). (C) Ratio of the IgG1 titers detected using NP<sub>3</sub>-BSA to those with NP<sub>17</sub>-BSA. Each symbol corresponds to one mouse. Statistical analysis: two-way ANOVA was performed for panels (B)-(C) and  $P < 0.0001$  for the genotype and time effect for NP-specific IgG1 antibodies of low and high affinity. Similar experiments were done twice.



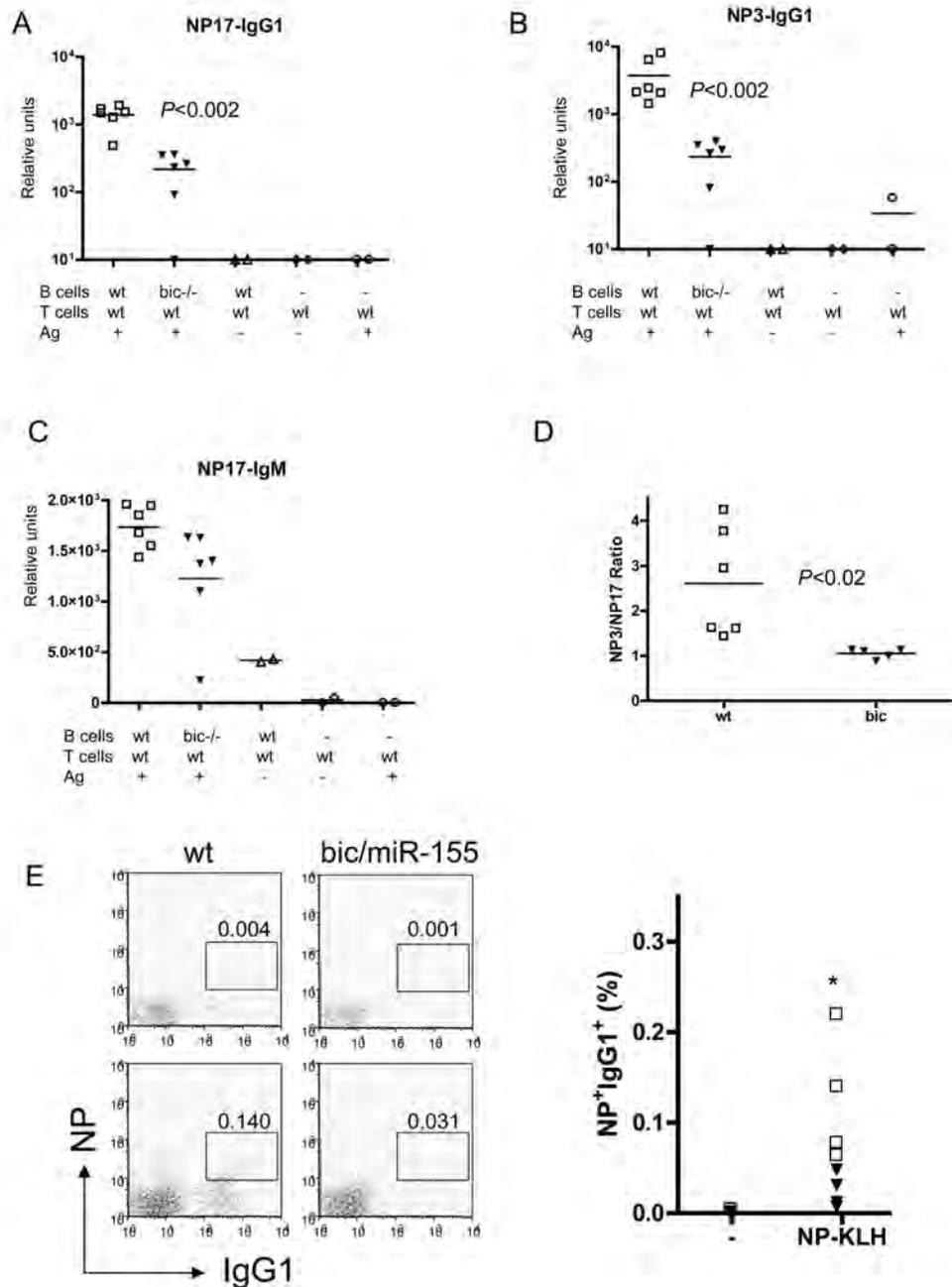
**Figure 2. The deficiency in switched antibody production in miR-155 deficient mice is B cell intrinsic**

Wild type (open squares) or miR-155 deficient (filled triangles) chimeras were immunized with NP-KLH in alum at day 0. Titers of IgM anti-NP (A) or IgG1 (B) were measured using NP<sub>17</sub>-BSA. (C) Titers of IgG1 anti-NP measured using NP<sub>3</sub>-BSA. (D) Ratio of the IgG1 titers detected using NP<sub>3</sub>-BSA to those with NP<sub>17</sub>-BSA. (E) Titers of IgG1, IgG2b and IgG3 anti-NP at day 14 after immunization. Each symbol corresponds to one mouse. Statistical analysis:  $P < 0.0001$  for the genotype and time effect of a two-way ANOVA analysis for panels (B)-(D). For panel (E) the Student-t test was used: “\*\*” indicates  $P < 0.001$ . Similar experiments were done twice.



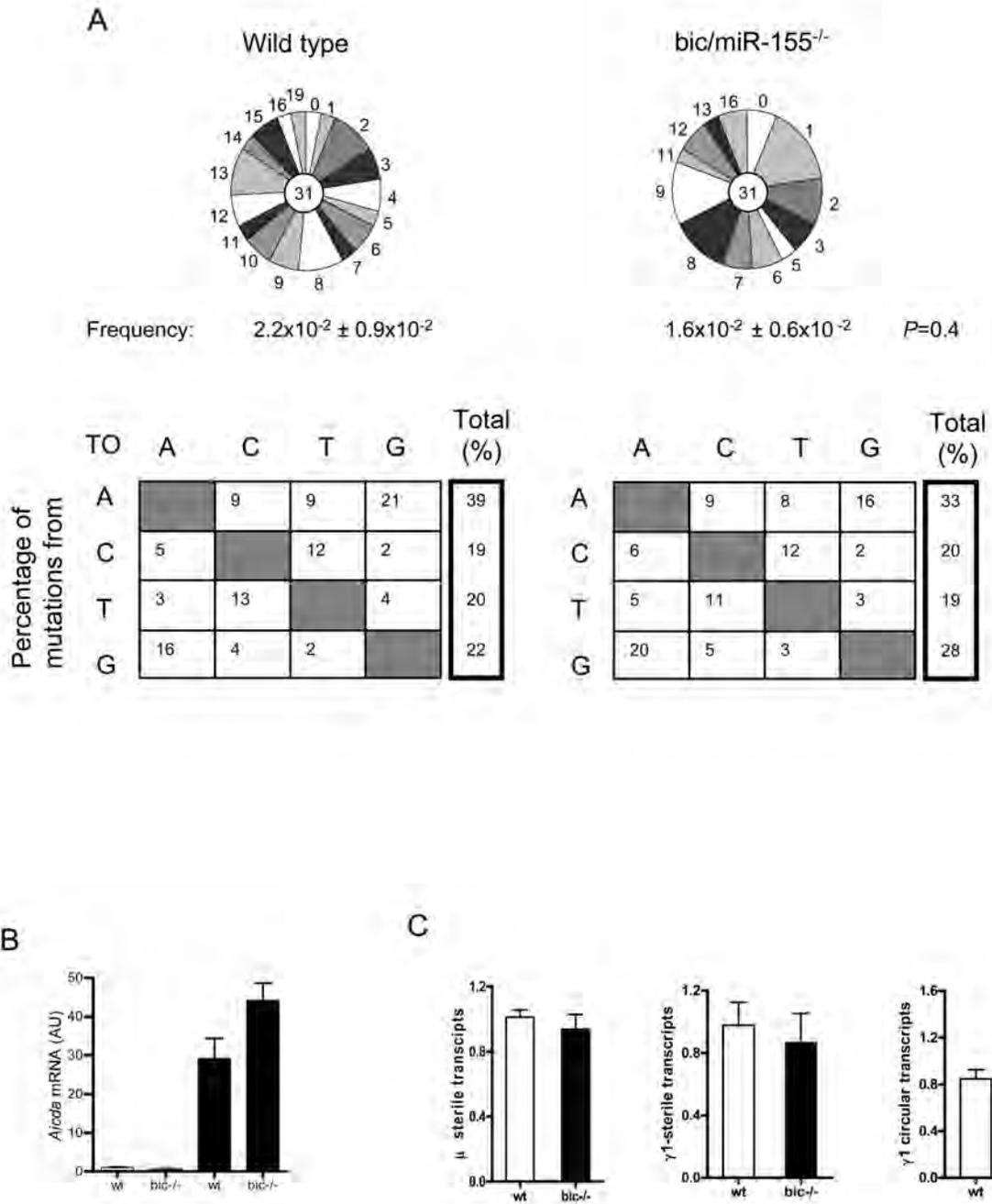
**Figure 3. Impaired extrafollicular and germinal centre response in the absence of miR-155**  
 (A) Immunohistochemical analysis of splenic sections from wild type (WT) or miR-155 chimeric mice showing NP-binding cells (blue) and IgG1 positive cells (orange) on day 7 after immunization with NP-KLH. (B) Wild type (open squares) or miR-155 deficient (filled triangles) chimeras were immunized with NP-KLH. The graph on the left shows the number of NP-specific extrafollicular plasmacytoid cells per mm<sup>2</sup> of spleen section. The graph on the right shows the percentage of spleen section occupied by germinal centers. (C) ELISPOT analysis of splenic NP-specific IgG1 AFC (left panel) or bone marrow NP-

specific IgG1 AFC (right panel). (B-C). Each symbol represents one mouse and the horizontal bar corresponds to the media. Statistical analysis was performed using one-way ANOVA. “\*” indicates  $P < 0.05$ , “\*\*\*”  $P < 0.001$ . In panel (B) no significance difference ( $P > 0.05$ ) was observed when comparing miR-155 deficient NP-specific extrafollicular plasmacytoid cells per  $\text{mm}^2$  at day 0 with day 7 or day 14. Similar experiments were repeated 2-3 times.



**Figure 4. The defective memory response in miR-155 deficient mice is B cell intrinsic** NP-primed B cells from wild type (WT) or miR-155 deficient (Mir-155) chimeras were mixed with wild type carrier-primed T cells and transferred into *Rag2*<sup>-/-</sup> *Il2rg*<sup>-/-</sup> deficient mice. Subsequently, recipient mice were immunized with soluble NP-KLH and one week later antigen-specific IgM and IgG1 measured. Control groups are indicated. Titers of IgG1 anti-NP measured using NP<sub>17</sub>-BSA (A) or NP<sub>3</sub>-BSA (B). (C) Titers of IgM anti-NP using NP<sub>17</sub>-BSA. (D) Ratio of the IgG1 titers detected using NP<sub>3</sub>-BSA to those with NP<sub>17</sub>-BSA. Each symbol represents an individual mouse and horizontal bars indicate the average. The

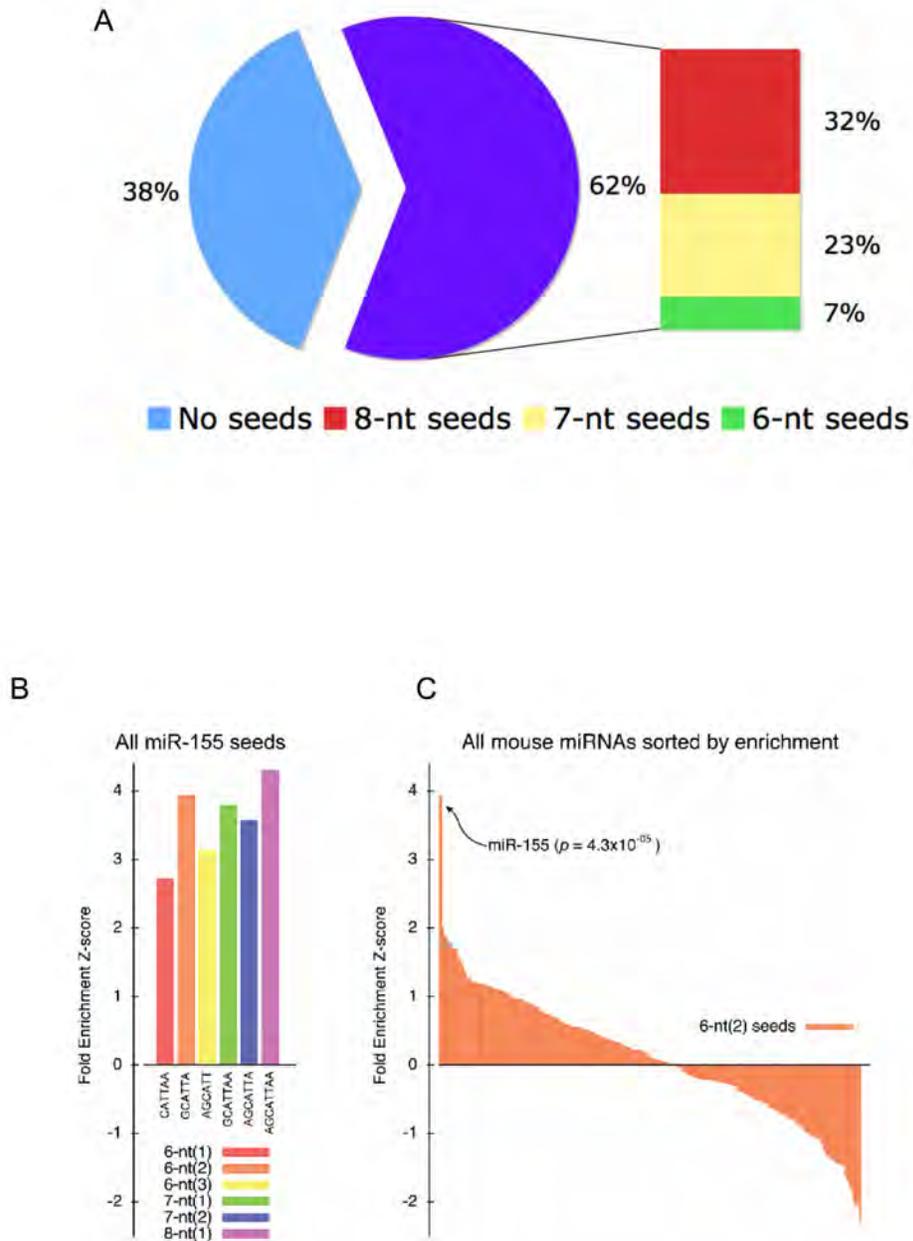
indicated “*P* values” were calculated using Student’s *t*-test. (E) Frequency of memory B cells after 42 days of immunization with NP-KLH. Red blood cell depleted splenic cells were gated as B220<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>-</sup>, IgM<sup>-</sup>, IgD<sup>-</sup>, Gr-1<sup>-</sup>, F4/80<sup>-</sup>, PI<sup>-</sup>, and analyzed for the expression of NP and IgG1. Dot plots correspond to a representative example of a mouse of each genotype. The top panel corresponds to non-immunized mice, the bottom panel to NP-KLH immunized mice. The boxes indicate the frequency of NP<sup>+</sup>, IgG1<sup>+</sup> cells. The graph summarizes the results of 4 mice each genotype, open squares correspond to wild type, filled triangles to miR-155 deficient mice. A significance reduction in the proportion of NP<sup>+</sup> IgG1<sup>+</sup> cells after immunization was observed in the absence of miR-155 (*P*=0.03, by Student-*t* test).



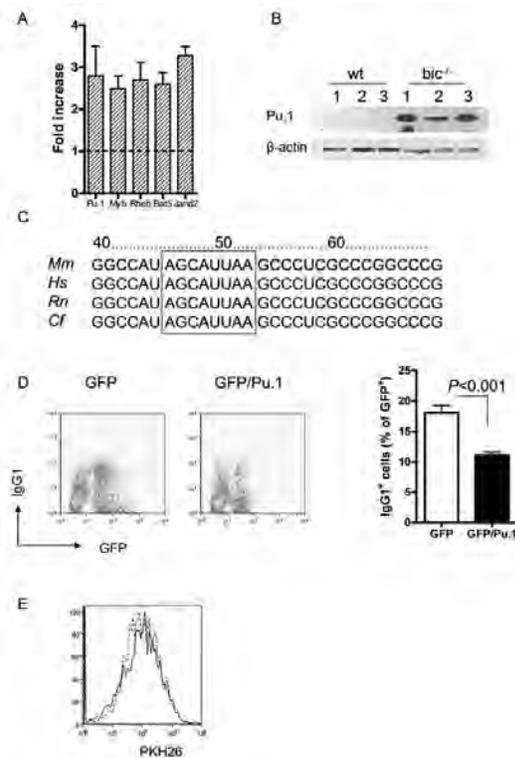
**Figure 5. Hypermutation in miR-155 deficient B cells in Peyer’s patch GC despite impaired IgG1 secretion**

(A) Analysis of mutations in the intronic  $V_H J558-J_H$  rearrangement-flanking region of wild type and miR-155 deficient germinal centre B cells purified from Peyer’s Patches. Segment sizes in the pie charts are proportional to the number of sequences carrying the number of mutations indicated in the periphery of the charts. The total number of sequences analyzed is indicated in the centre of the chart. The frequency of mutations is expressed as the mean  $\pm$  SD for 3 mice per group and the  $P$  value was calculated using Student t-test. The tables indicate the percentage nucleotide substitution. The total number of mutations was 275 for

the wild type and 196 for the miR-155 deficient mice. (B-C) Splenic B cells were cultured with LPS+IL-4 for 3 days. Each symbol corresponds to B cells isolated from an individual mouse and the horizontal bar indicates the average, open squares correspond to wild type B cells, filled triangles to miR-155 deficient. (B) Induction of *Aicda* (C) Expression of  $\mu$  encoding sterile transcripts (left panel),  $\gamma 1$  encoding sterile transcripts (middle panel) and post-switch encoding  $\gamma 1$  circular transcripts (right panel). This experiment was repeated 2-3 times.



**Figure 6. Enrichment for miR-155 “seeds” in genes upregulated in miR155 deficient B cells**  
 (A) Representation of miR-155 “seeds” in the group of upregulated genes in miR-155 deficient B cells. Genes were grouped according to their content of 6-nt seeds, 7 nt(1), 7 nt(2) or 8 nt- miR-155 seeds and represented in a pie chart. (B) Fold enrichment for 6-nt seeds, 7 nt(1), 7 nt(2) or 8 nt-miR-155 “seeds”. (C) Fold enrichment of mouse 5’miRNA “seed” sequences contained in the 3’UTRs of significantly upregulated genes. The *P*-value for miR-155 is indicated.



**Figure 7. Pu.1 is a direct target of miR155 and its over-expression in wild type B cells results in a reduction of IgG1 switched cells**

(A) Expression of *Sfp1l*, *Myb*, *Rheb*, *Bat5* and *Jarid2* was assessed by q-PCR. Data is represented as the fold increase in miR-155 relative to wild type amounts set at 1 (n=4). For all genes  $P < 0.05$ . (B) Protein expression of Pu.1 from B cell cultures from 3 wild-type and 3 miR-155 deficient mice. (C) Left panel, sequence alignment of part of the 3'UTR of PU.1 from different species. The 8nt-miR155 binding site is boxed. The AAU sequence indicated below the alignment shows the mutagen derivative created to assess miR155 dependent translational repression in the luciferase reporter assay. Wild type (WT) or mutant Pu.1 (mutant) plasmid were cotransfected with miR-155 mimic (closed bars) or control miR-124a (open bars). The right panel shows a significant miR-155 specific repression of Pu.1 reporter.  $*P < 0.0001$  in comparison with wild type plasmid treated control mimic by Student's *t* test. (D-F) Wild type or miR-155 deficient B cells were cultured in the presence of LPS and IL-4. After 16 hours cells were transduced with retrovirus expressing GFP or GFP and Pu.1 and cultured for 3 more days. (D) Cultures were then stained for surface IgG1 expression. The left panel shows a representative example of cultures from one mouse transduced with each virus. The graph summarizes the results observed for cultures arising from four mice. (E) Same as (D) expect cells were stained for intracellular IgG1 (icIgG1) expression (left) or surface IgM (sIgM, right). (F) Before culture, B cells were stained with PKH26 to follow proliferation. Representative histograms gated on GFP<sup>+</sup> cells for B cells transduced with GFP virus (black line) or GFP-Pu.1 (dashed line) are shown. This experiment was repeated 2-4 times.