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先天性免疫異常的 ENU 突變鼠的研究及分析
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行政院國家科學委員會補助專題研究計畫 **V 成果報告**
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成果報告類型(依經費核定清單規定繳交)：□精簡報告　v 完整報告

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Abstract

Keywords: STAT2, ENU-mutagenized mice, DC, type I IFN

Type I and type II interferons (IFN) are critical for regulating innate and adaptive immunity. We have recently identified one mutant mouse that displayed impaired response to IFNα through screening ENU-mutagenized mice. The phenotype of hypo-responsiveness of IFNα was recessive. Through genetic mapping and DNA sequencing, we have located a T→A point mutation, which was in the splicing donor of intron 4-5 of STAT2, resulting in pre-maturation of STAT2 mRNA and dramatic reduction of STAT2 protein expression in the mutant mice. To study the role of STAT2 in DC development and maturation, bone marrow cells of STAT2 mutant mice and wild type littermates were treated with Flt3L in the presence or absence of added type I IFN. Interestingly, IFNα suppressed the development of cDC but enhanced pDC development in wild type cells, which was blocked in STAT2 mutant cells. The expression of CD86, a maturation marker, on cDC was, however, enhanced wild type cells in response to IFNα which was also dependent on STAT2. To investigate the role of STAT2 in IFNα-mediated regulation of DC development, wild type or STAT2 mutant mice were treated with poly IC, an IFNα inducer. After treatment of poly IC in vivo, DC development in the BM was monitored by flow cytometry. These results suggested that there were, at least, two effects of STAT2 on IFNα-mediated modulation of DC development and maturation. It was reported that type I IFN could negatively regulate the development of dendritic cells in a STAT2-dependent but STAT1-independent pathway. This is in contrast to what has been known that the functions of type I IFN that are both STAT1 and STAT2-dependent. Therefore, we are currently investigating this unique phenomenon using in vitro and in vivo approaches. In addition, we are also generating mutant mice lacking both STAT1 and STAT2 and doing gene profiling using STAT1KO, STAT2 mutant and double mutant mice to understand specific and redundant roles of STAT1 and STAT2 in vivo.

中文摘要

關鍵詞：STAT2,ENU 突變鼠,樹突細胞,第一型干擾素

第一型和第二型干擾素(IFN)對先天和後天性免疫的調控極為重要。我們從 ENU 突變鼠中找到一隻對 IFNα 的反應有失常的老鼠。這隻老鼠對 IFNα 過低的反應是隱性遺傳。在遺傳定位及基因定序的實驗中，我們找到這隻異常鼠的一個 T→A 的點突變，它的位置是在 STAT2 的 intron 4-5 的剪接供應者。這個點突變導致 STAT2 mRNA 提早成熟及 STAT2 蛋白質表現的消失。為了解樹突細胞的發育與成熟，我們把 STAT2 異常鼠及正常鼠的骨髓細胞用 Flt3L 及第一型干擾素去刺激。有趣的是，IFNα 會抑制正常骨髓細胞生成 cDC 但促進 pDC 的產生。另外，一個成熟的標誌 CD86 在 cDC 的表現則是受到 IFNα 的促進，這現象也是需要 STAT2。為了解體內 STAT2 在 IFNα 調控 DC 的角色，我們使用一個 IFNα 的引發劑-poly IC 去做刺激。結果，我們發現 STAT2 至少有兩種的作用分別是影響 DC 的發育與成熟。最近有報導指出，第一型干擾素對樹突細胞的發育有負調節作用，而且這個現象是和 STAT2 有關但和 STAT1 無關。這結果和我們以往的認知-第一型干擾素的功能需同時仰賴 STAT1 和 STAT2 的作用的想法是相違背的。因此，我們目前正利用體內及體外的方法去進一步研究這個發現。另外，我們也要生產缺乏 STAT1 及 STAT2 的老鼠，其目的是要利用老鼠分別或同時缺乏 STAT1, STAT2 的老鼠做基因特徵的研究並瞭解 STAT1 和 STAT2 在體內中的特殊及共同的功能。

Background and significance

Genetic approaches either using transgenic or homologous recombination techniques have proved to be useful and powerful ways to understand the functions of specific genes in vivo. However, such methods do not scale well in the context of the whole genome. Neither do they provide detailed information of different functional domains of a gene in vivo. ENU-mediated genome-wide mutagenesis in mice can complement these drawbacks. Through the screening of ENU-mutagenized mice, we can acquire mice with abnormal phenotypes and identify genes that account for the alterations in the development or functions of the immune system. This phenotype-driven or forward genetic approach provides us with a unique opportunity to assign function to genes in an unbiased global manner. Furthermore, this approach is also useful to identify the cause of immune diseases that are mutligenic in nature such as autoimmune diseases. In addition to forward-genetics, we can also apply reverse-genetics to screen for mutations in a family of genes that are known critical for immune system in the ENU-mutagenized mice. With this we can easily study the effect of functionally closely related genes in immune response. This is completely different from traditional gene targeting approach in which only a mutant mouse can be generated at a time. This approach has recently been successfully employed to identify Zebrafish *rag1* mutations in a mutant library generated by ENU (1). Therefore, both forward and reverse-genetic approaches can be used simultaneously for screening to increase the chance of identifying mutations that affect the immune response.

IFNs come in various forms that are capable of interfering viral infection. Recent studies have demonstrated that IFNs not only participate in anti-viral response, they also display different functions, including modulation of immune system, promoting or impeding the differentiation of certain cell types, and inhibition of cell proliferation (2). Due to these effects, IFNs are now widely used in the treatment for chronic infection of hepatitis B and C, hairy-cell leukemia, kaposi's sarcoma, multiple sclerosis, and chronic granulomatous disease (3). IFNs can be classified into two types based on their structure and other properties. Type I IFN comprises mainly two serologically distinct groups; IFNα and IFNβ, that are produced by many cell types and induced by different substances. Viruses and double-stranded RNAs are amongst the best inducers. Type II IFN consists of only one kind of IFN, IFNγ that is mainly produced by T lymphocytes and NK cells when stimulated.

Signal transduction of IFNs is mainly mediated by JAK-STAT pathway. Upon ligand binding to IFNγ receptor, JAK1 and JAK2 are activated followed by activation of STAT1 by tyrosine phosphorylation. Activated STAT1 forms dimers and translocates into nucleus where they bind to gamma-activated site (GAS) in the promoter region of IFNγ-inducible genes and lead to the transcription of these genes. Likewise, binding of IFNα/β to their receptors triggers the activation of JAK1 and TYK2 followed by activation of STAT1, STAT2 and STAT3. STAT1, STAT2 and IRF9, a member of the IFN regulatory factor family, form hetero-trimer and bind to the IFN stimulated responsible element (ISRE) element in the promoter region of IFNα/β inducible genes. STAT1 and/or STAT3 can also form homo- and hetero-dimer, which bind to the GAS element (4, 5).

While the activation of JAK-STAT pathway in IFN response is more or less well characterized,

other pathways activated by IFNs are less clear. Several lines of evidence suggest the existence of such pathways. For example, the phenotypes of mice deficient in IFNα/β and/or IFNγ receptors are not completely equivalent to those of mice lacking STAT1, a shared signal mediator for both IFNs (6, 7). Besides, biochemical experiments also implied that in addition to JAK-STAT pathway, PI3 kinase and NFkB were also activated (8-10). Given the fact that phosphorylation of both tyrosine and serine residues is required for a full activity of STAT protein and that JAK kinases can only phosphorylate tyrosine residues suggests that another pathway is involved to phosphorylate the serine residue. Although p38 MAPK has been shown to enhance the transcription ability of STAT1, it, however, is independent of ser727 phosphorylation (11, 12). In any case, genetic approach using ENU-mutagenized mice allows us to identify pathways other than JAK-STAT in the IFN responses.

Results and discussions

We have identified a mouse with reduced IFNα responsiveness from ENU-mutagenized mice generated in the MMPCF at Sinica Academia. The pedigree 117 of the mutant mouse is shown in the Fig. 1. Clearly, the impaired IFNα response in the mutant mice of the pedigree 117 was recessive.

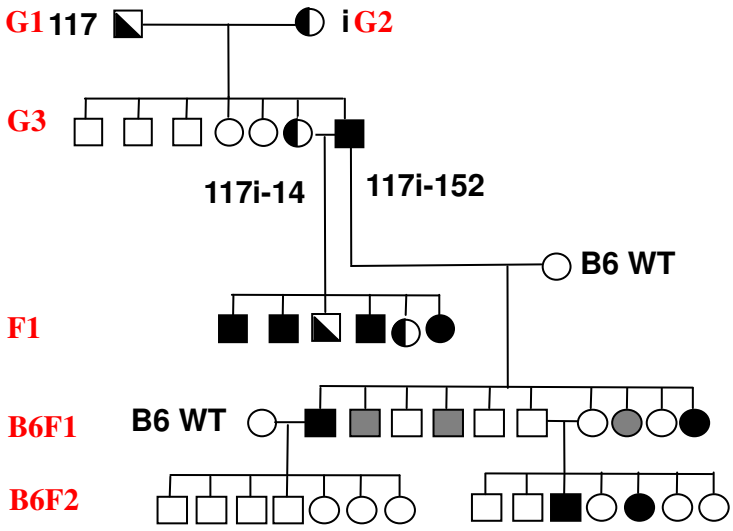


Fig. 1 Hypo-responsiveness to IFNα of the 117 pedigree mutant mice was recessive. Inheritance test of the hypo-responsive phenotype of the mutant mouse 117. 117i-152 G3 mouse was first generated from G1 (117) crossed with G2 (i). Intercross between 117i-152 with its sister 117i-149 generated many mice displaying the same phenotypes of 117i-152, suggesting that the phenotypes were inheritable. 117i-152 crossed with wild type C57BL/6 mice resulting in graded phenotypes in their offspring B6F1. B6F1 mice that showed no phenotype were intercrossed and around 25% of their offspring (B6F2) displayed phenotypes. A B6F1 was crossed with a wild type C57BL/6 and their offspring did not show any mutant phenotype.

To locate the mutation site of the mouse, the pedigree 117 was outcrossed to C3H mice, genetic background completely unrelated to B6 mice. After intercross of F1 (B6xC3H), the mice with or without mutant phenotype were subjected to rough mapping and the linkage of the mutant phenotype was mapped to chromosome 10 close to marker D10MIT233 (Fig. 2).

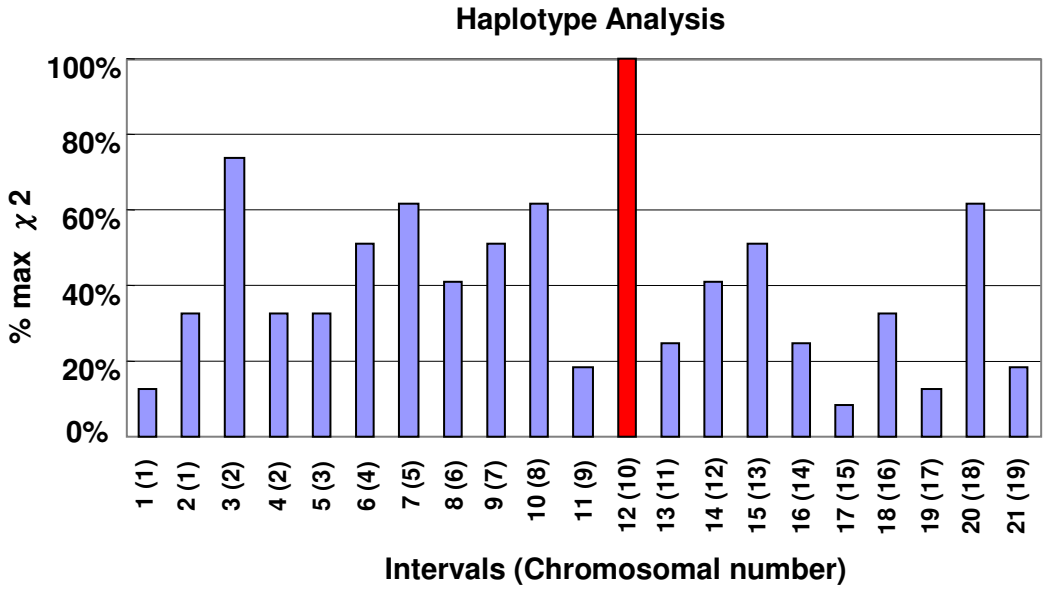
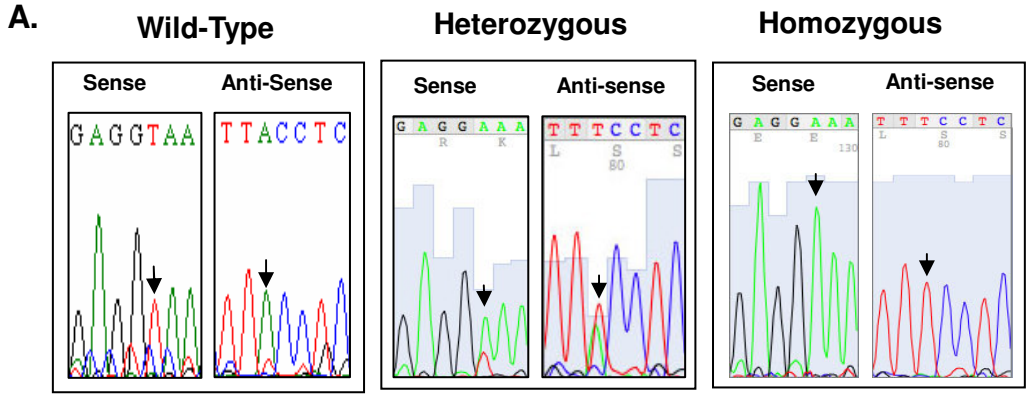


Fig. 2 **Linkage of the mutant phenotype to Chromosome 10 closing to marker D10MIT233/ 113.7 Mb**. Tail DNA of the pedigree 117xC3H F2 mice that displayed abnormal phenotype were subjected to rough mapping, a service provided by the MMP Core Facility. Base on Chi square (χ 2) value and the lowest score of non-recombinant from unaffected group, the chance probability at the D10MIT233 marker is less than 0.001. As a result, the mutation is most likely linking to Chromosome 10.

Since the impaired response of the mutant mice was only restricted to IFNα but not to IFNγ (data not shown), we figured that the mutation that results in the mutant phenotype must have been signal mediators specifically operated in type I IFNs. We first aimed to look for mutation in STAT2 gene because first this molecule is only activated by IFNα but not IFNγ. Secondly, STAT2 gene is located in chromosome 10 close to the genetic marker D10MIT233 as revealed by the rough mapping shown in Fig. 2. After sequencing, we have identified a point mutation T→A located in the splicing donor of intron 4 to 5 (Fig. 3).



B. Exon 4…GAG g(t→a) aa…Intron 4-5

Fig. 3 T→A mutation at splicing donor of intron 4-5 of mouse STAT2 in the mutant mice. (A) Tail DNA prepared from wild-type, heterozygous and homozygous mutant mice were subjected to PCR-mediated sequencing. The corresponding site of mutation in sense or anti-sense strain was indicated by arrows. (B) The mutation was located in the splicing donor (ga) of intron 4-5 of STAT2.

To further confirm the finding of the mutation in STAT2, Western blotting was performed to measure the expression of STAT2 protein in the mutant mice. Interestingly, the expression of STAT2 protein in the spleen of homozygous mutant mice ($STAT2^{m/m}$) was greatly reduced as opposed to that of wild type mice although the level of STAT2 was still induced in response to IFNα treatment (Fig. 4A). Consequently, the levels of IFNα downstream genes such as OAS and

PKR were significantly reduced in response to IFNα when compared to that of wild type or heterozygous littermates (Fig. 4B and 4C).

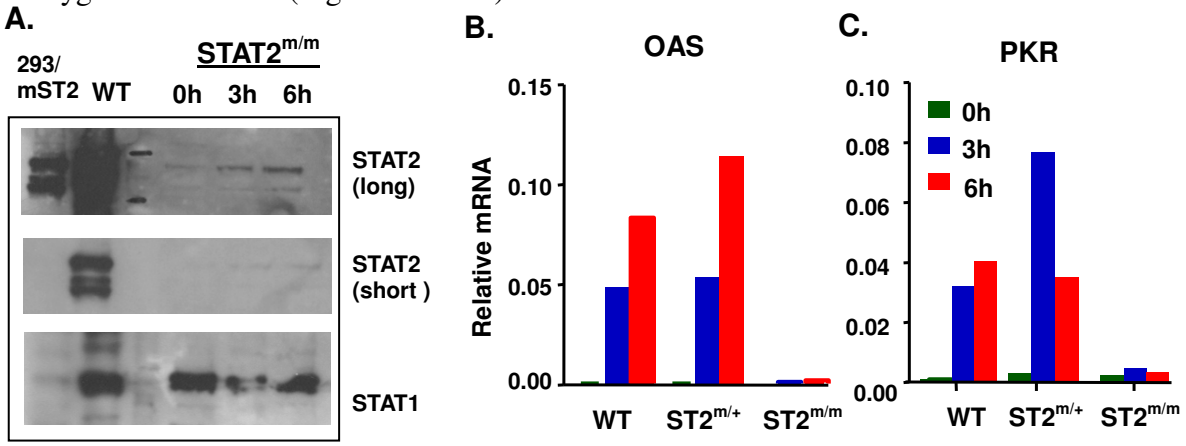
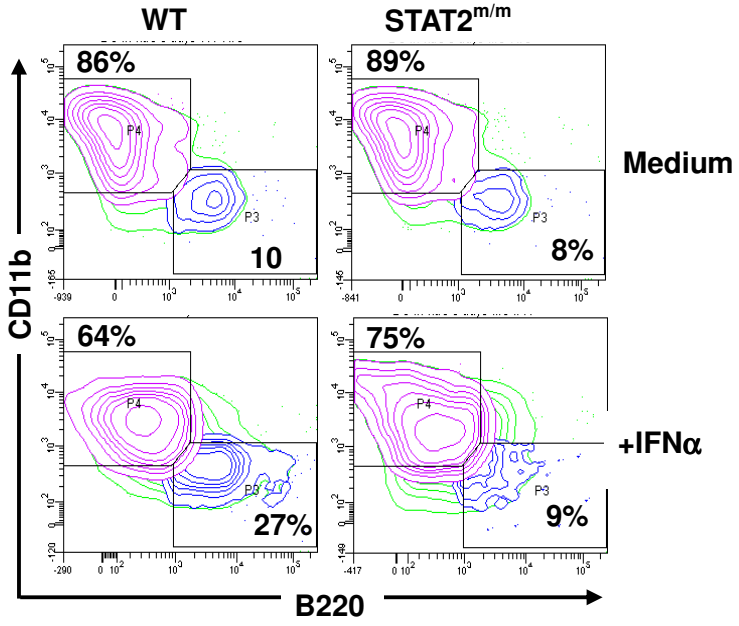


Fig. 4 Reduced expression of STAT2 and impaired response to IFNα in STAT2m/m mouse. Cell lysates prepared from mSTAT2 transfected 293T, or splenocytes of WT or STAT2m/m mouse that were treated with IFNα for 3h or 6h were subjected to Western blotting using antibodies to STAT1 or STAT2 (A). mRNA prepared from splenocytes of WT, STAT2$^{m/+}$ and STAT2$^{m/m}$ mice that were treated with IFNα for 3h and 6h were subjected to RT-QPCR using primers to OAS (B) or PKR (C). Relative mRNA was calculated by normalizing the values to that of β-actin.

It was previously shown that STAT2 may be involved in regulating the development of DC independent of STAT1 through IFNα (13). Therefore, we sought to determine the development of DC in the mutant mice. First of all, in vitro development of DC was performed by incubating BM cells of wild type or STAT2$^{m/m}$ mice with Flt3L in the absence or presence of IFNα and the percentage of cDC (CD11b+CD11c+B220-) or pDC (CD11b-CD11c+B220+) was measured. Interestingly, the development of cDC was suppressed and pDC was enhanced by the presence of IFNα. in wild type cells. This effect apparently was dependent on STAT2, as the abnormal phenotype seen in wild type cells was absent in STAT2$^{m/m}$ after treated with IFNα (Fig 5A). We also observed that though development of cDC was suppressed in wild type cells by IFNα, the maturation was, on the contrary, enhanced by the same treatment. As shown in Fig. 5B, CD86, a costimulatory molecule and a maturation marker was increased after IFNα treatment. This phenomenon was absent in STAT2$^{m/m}$ cells, suggesting that IFNα suppressed development but enhanced maturation of cDC and these effects are STAT2-dependent.



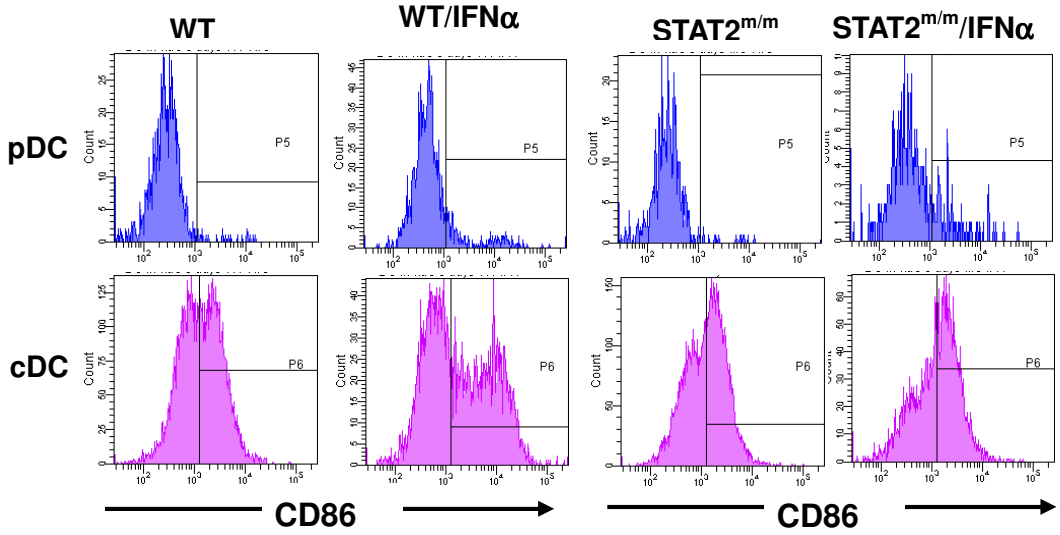
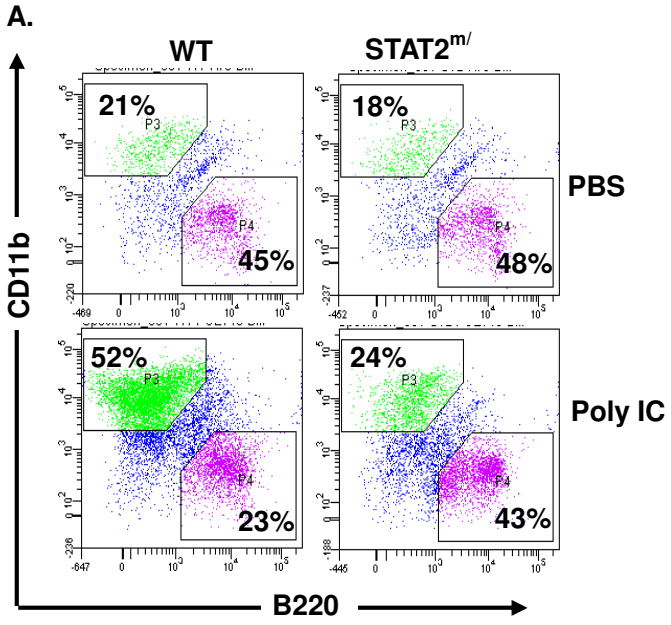


Fig. 5 Promotion of pDC development and cDC differentiation *in vitro* by IFNα is STAT2-dependent. cDC or pDC were derived from BM cells of WT or STAT2$^{m/m}$ mice that were cultured with Flt3L in the presence of absence of IFNα were four-color stained with anti-CD11b, anti-CD11c, anti-B220 and anti-CD86 followed by FACS analysis by gating on CD11c positive cells. The percentages of CD11c^{+}CD11b^{+}B220^{-} (cDC) and CD11c^{+}CD11b^{-}B220^{+} (pDC) cells were shown (A). The expression of CD86, a maturation marker, on pDC (upper panels) and cDC (lower panels) in WT (B) or STAT2$^{m/m}$ (C) in the absence or presence of IFNα was shown.

To further determine the effect of IFNα on DC development, we injected poly IC, a TLR3 ligand and an IFNα inducer, in vivo to confirm the phenotype seen in vitro. Wild type or STAT2m/m mice were injected with poly IC daily for three day to maintain a certain levels of IFNα in vivo. BM cells of treated mice were then subjected to FACS analysis for DCs. Interestingly, increased percentage of cDC but reduced percentage of pDC was observed in wild type mice after poly IC treatment. Again, this phenomenon was STAT2-dependent as no change was observed in STAT2$^{m/m}$ mice (Fig. 6A). The injection of poly IC also induced maturation of both cDC and pDC as indicated by the increased expression of CD86. Nonetheless, poly IC-induced pDC but not cDC maturation was only affected in STAT2$^{m/m}$ mice (Fig. 6B).



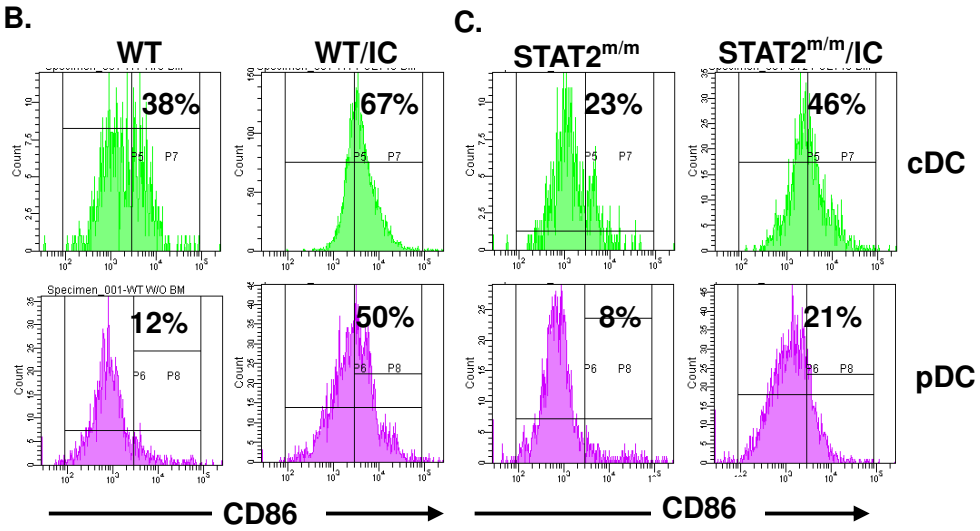


Fig. 6 TLR3 signaling-induced enhancement of cDC development and pDC maturation *in vivo* were STAT2-dependent . BM of WT or STAT2$^{m/m}$ that were i.p. injected without (upper panels) or with (lower panels) poly IC (100 mg) for 3 times in 3 days were subjected to four color staining with anti-CD11b, anti-CD11c, anti-B220 and CD86 followed by FACS analysis by gating on CD11c positive cells. The percentage of CD11c^{+}CD11b^{+}B220^{-} (cDC) and CD11c^{+}CD11b^{-}B220^{+} (pDC) was shown in (A). The expression of CD86 on cDC (upper panels) and pDC (lower panels) of WT (B) or STAT2$^{m/m}$ (C) with or without poly IC treatment was shown.

In sum, we have identified a mutant mouse from ENU-mutagenized mice. This mouse displayed a decreased response to type I but not type II IFNs. Inheritance test suggests that the phenotype is a recessive trait. Genetic mapping suggests that the mutation(s) accounts for the phenotype lies in chromosome 10 near to a genetic marker DM10MIT233. By genomic sequencing, we have located a T→A mutation, which is located in the intron 4 to 5 of STAT2 gene. The expression of STAT2 protein in the mutant mice was dramatically decreased although it can still be induced by the treatment of IFNα. Development of different cell types such as T, B, granulocytes, NK and macrophages are not affected in the mutant mice, suggesting that STAT2 does not participate in hematopoiesis. However, STAT2 is required for regulation of development and maturation of DC by type I IFNs. In mutant mice, IFNα-mediated suppression of cDC development and enhancement of cDC maturation in vitro are STAT2-dependent. Besides, the treatment of poly IC results in enhanced development of cDC but reduced development pDC. Maturation of both cDC and pDC are also enhanced by the treatment of poly IC. Nonetheless, only maturation of pDC is largely dependent on STAT2. Apparently, some discrepancies between in vitro and in vivo treatment are observed. Therefore, more detailed analysis is required to sort out the mechanisms of IFNα- and STAT2-depedent regulation of DC development and maturation.

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