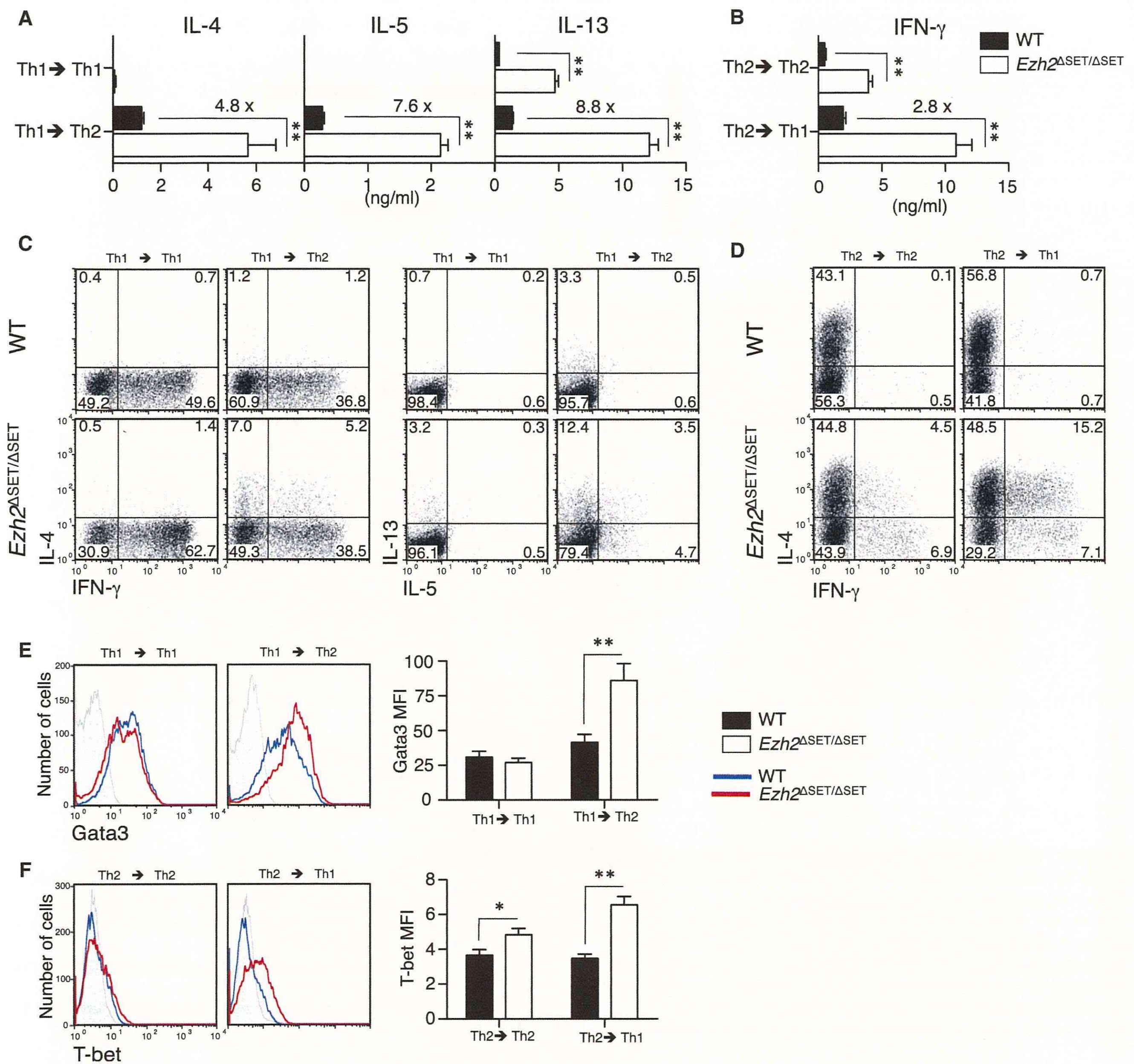

Figure 6. Loss of Ezh2 Results in Enhanced Plasticity of Th1 and Th2 Cells

Naive CD4+ T cells were polarized for 3 days under Th1 (A, C, and E) or Th2 (B, D, and F) cell-inducing conditions with 10 or 100 ng/ml of IL-12 or IL-4, respectively, and then recultured under the same condition or under the opposing condition for a further 2 days as indicated.

(A and B) Production of IL-4, IL-5, and IL-13 (A) and IFN-γ (B) measured by ELISA after 24 hr restimulation with anti-TCRβ, n = 3, mean ± SD.

(C–F) Cytoplasmic staining for IFN-γ with IL-4 (C and D) or IL-5 with IL-13 (C) and Gata3 alone (E), or T-bet alone (F) after 5 hr restimulation with anti-TCRβ.

(E and F) Histograms at left with 9,000 events displayed for each sample (WT, blue; *Ezh2*$^{\Delta SET/\Delta SET}$, red), and right panels show combined MFI results from five independent experiments.

All other data are representative of at least three independent experiments. **$p < 0.01$. See also Figure S6.

we observed a large increase in Gata3 expression in Ezh2-deficient Th1 cells (Figures 6E). Expression of T-bet was also higher in Ezh2-deficient compared to WT Th2 cells, and this was further enhanced by exposure to Th1 cell-inducing conditions (Figures 6F). Eomes was not expressed by Th2 cells after exposure to Th1 cell-inducing conditions (data not shown). Double staining of Gata3 with IL-4 showed a large increase in the number of Ezh2-deficient cells coexpressing Gata3 and IL-4 after Th2 cell-inducing secondary culture (Figure S6B). Ezh2 deficiency also enhanced the numbers of Th2 cells that coexpressed IFN-γ and T-bet after Th1 cell-inducing secondary culture (Figure S6C). These results show that both Th1 and Th2 cells lacking Ezh2 display considerably greater plasticity when compared to cells with intact Ezh2 function.

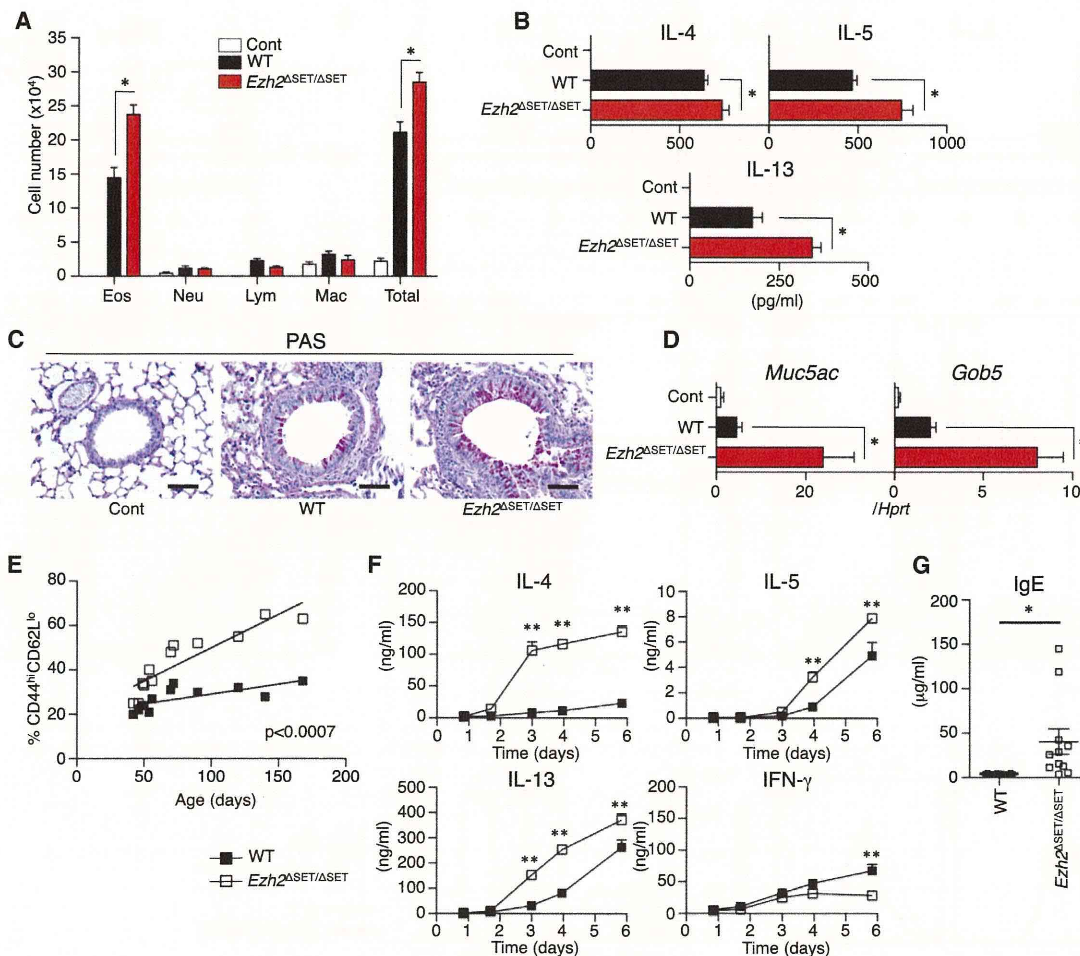

Figure 7. Th Cells Lacking Ezh2 Mediate Enhanced Type 2 Immune Responses In Vivo

(A–D) OVA-specific WT or *Ezh2*$^{\Delta SET/\Delta SET}$ Th2 cells were transferred to syngeneic WT C57BL/6 mice that were then challenged with an OVA aerosol. Total numbers of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym), and macrophages (Mac) recovered by bronchoalveolar lavage (BAL) (A) and indicated cytokines in cell-free BAL samples measured by ELISA (B). Representative images from lung tissue sections stained with periodic acid Schiff's reagent with mucus stained magenta; scale bars represent 60 μm (C). *Muc5ac* and *Gob5* mRNA expression in lung tissue measured by qRT-PCR (D).

(E–G) Untreated WT or *Ezh2*$^{\Delta SET/\Delta SET}$ mice analyzed for CD44^{hi}CD62L^{lo} spleen CD4$^+$ T cells by flow cytometry (E), and cytokine production by CD44^{hi}CD62L^{lo} spleen CD4$^+$ T cells stimulated with anti-TCRβ for the indicated times (F) and total serum IgE (G) measured by ELISA.

n = 5–6 mice/group (A–D); n = 11 mice/group (G); and n = 3 replicates (F) (all mean ± SEM). Data are representative of at least two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$. In (E), p value determined by analysis of covariance. See also Figure S7.

Loss of Ezh2 Results in Enhanced Asthma-like Pathology and Accumulation of Th2 Memory Phenotype Cells In Vivo

Finally, to investigate the physiological consequences of Ezh2-mediated regulation of Th cell function, in vitro generated ovalbumin (OVA)-specific Th2 cells (Figure S7A) were intravenously transferred into WT C57BL/6 mice, and these mice were then challenged with aerosolized OVA. Compared to mice that received WT cells, recipients of Ezh2-deficient cells displayed increased eosinophilic inflammation in the airways, as assessed by bronchoalveolar lavage (BAL) (Figure 7A). In addition, mice that received Ezh2-deficient cells had higher levels of the Th2 cytokines IL-4, IL-5, and IL-13 in BAL samples (Figure 7B), and these mice displayed enhanced pathology and mucus hyperproduction in the lungs and higher expression of mRNA for *Muc5ac* and *Clca3* (*Gob5*) (Figures 7C and 7D).

In untreated mice maintained under specific-pathogen-free (SPF) conditions, we observed a progressive increase in the proportion of effector memory phenotype (CD44^{hi}CD62L^{lo}) CD4$^+$ T cells that was greatly exaggerated in mice with CD4$^+$ T cell-specific Ezh2 deficiency and accounted for approximately 70% of the CD4$^+$ T cells in the spleens of these mice by 6 months of age (Figure 7E). Interestingly, purified Ezh2-deficient effector memory phenotype CD4$^+$ T cells from 12-week-old mice produced markedly increased amounts of the Th2 cytokines IL-4, IL-5, and IL-13 but not IFN-γ when compared to WT effector memory phenotype cells (Figures 7F and S7B). Large increases in IgE were also detected in serum from 6-month-old mice with CD4$^+$ T cell-specific Ezh2 deficiency (Figure 7G). Thus, Th2 cells lacking Ezh2 generate exaggerated type 2 biased immune responses in a mouse model of allergic asthma, and loss of Ezh2 function in CD4$^+$

T cells results in enhanced accumulation of memory type Th2 cells in vivo.

DISCUSSION

The ability of CD4$^+$ T cells to rapidly specialize into distinct subsets with defined functions is a critical aspect of immunoregulation. We have identified the polycomb protein Ezh2 as an essential regulator of Th1 and Th2 cell responses, both during differentiation and as a stabilizer of Th cell phenotype after specialization. Ezh2 directly bound and regulated correct expression of *Tbx21* and *Gata3* in developing Th1 and Th2 cells, and Ezh2 binding at these loci was accompanied by substantial H3K27me3. Ezh2 also constrained spontaneous generation of IFN-γ-producing cells, mainly by repressing expression of *Eomes*. In vivo, loss of Ezh2 enhanced Th2 cell-induced pathology in a model of allergic asthma and in untreated animals resulted in progressive accumulation of memory phenotype Th2 cells.

Ezh2 bound strongly to *Tbx21* and *Gata3*, and these two master regulators displayed the highest levels of differential binding between Th1 and Th2 cells. In addition, we detected strong binding of Ezh2 to the gene *Eomes* that encodes another T-box transcription factor closely related to T-bet. Inactivation of Ezh2 resulted in a profound reduction in H3K27me3, confirming that in CD4$^+$ T cells functional Ezh2 is required for maintenance of H3K27me3 at each of these loci. However, it should be noted that although Ezh2 occupancy at genes encoding the Th1 and Th2 cytokines was at the lower limits of detection in both ChIP-PCR and ChIP-seq assays, we could still detect H3K27me3 at these loci that was lost in cells deficient in Ezh2. This may indicate that very low levels of Ezh2 binding could still have some control over H3K27me3 at these loci. Based on the results obtained with Th cells doubly deficient in Ezh2 together with T-bet or Ezh2 together with Eomes, Ezh2-mediated regulation of sensitivity to IL-12-induced Th1 cell polarization and spontaneous generation of IFN-γ-producing cells can be largely explained by the repression of *Tbx21* and *Eomes* expression. However, Th1 cells lacking T-bet could still produce appreciable amounts of IFN-γ, a result that has also been shown previously (Usui et al., 2006) and attributed to a decreased requirement for T-bet when IL-4 signals are neutralized. Likewise, Th cells doubly deficient in Ezh2 and T-bet could still produce significant amounts of IFN-γ, indicating that Ezh2 could also be involved in the regulation of other IFN-γ-inducing genes or the *Ifng* locus itself. It is also possible that Ezh2-mediated repression of *Gata3* together with repression of other loci such as the Th2 cytokine genes may contribute to the control of Th2 cell polarization. We also detected enhanced expression of c-Maf (encoded by *Maf*) in Ezh2-deficient Th cells. However, *Maf* does not appear to be a direct target of Ezh2, and no difference in Ezh2 binding at this locus was detected in Th1 compared to Th2 cells, indicating that *Maf* may be indirectly regulated in cells lacking Ezh2 function.

During differentiation of Ezh2-deficient Th cells, *Tbx21* and *Gata3* were more sensitive to upregulation in response to polarizing cytokines. This phenotype contrasts with that observed for T cells lacking DNA methyltransferase (*Dnmt1*), where dysregulated expression of IL-4 appears to be independent of Gata3 expression (Makar et al., 2003). Moreover, repression of cytokine production by Dnmt3a appears to depend on de novo methylation at the *Il4*, *Il13*, and *Ifng* loci (Thomas et al., 2012; Yu et al., 2012). In contrast, our data show that Ezh2-deficient cells maintain the ability to polarize into Th1 or Th2 cell subsets, accompanied by upregulation of the corresponding master regulator. Therefore, histone methyltransferases and DNA methyltransferases appear to control Th cell function through the regulation of distinct target genes. Interestingly, it was recently found that the SUV39H1-H3K9me3-HP1α pathway is also required to maintain Th2 cell stability and that loss of SUV39H1 changes the ratio of H3K9ac to H3K9me3 at the *Ifng* locus (Allan et al., 2012). In addition to directly binding to *Tbx21, Eomes*, and *Gata3*, we found that Ezh2 inhibited spontaneous accumulation of the HAT coactivator proteins p300 and CBP and of H3K9ac and H3K27ac at the *Tbx21* and *Eomes* loci.

Ezh2 does not appear to play a major role in the repression of other Th cell differentiation programs. Th17 cell polarization and stable repression of Th17 cell differentiation are less sensitive to loss of Ezh2, and Th9 cell and iTreg cell differentiation is actually inhibited in the absence of Ezh2. Impaired differentiation of Ezh2-deficient Th9 and iTreg cells may be due to the inability of these cells to repress IFN-γ, leaving open the possibility that Ezh2 may have important as yet undiscovered effects on differentiation of these cell types. In contrast to *Tbx21*, *Eomes*, and *Gata3*, Ezh2 does not appear to target the master regulators of other lineages, specifically *Rorc* (Th17 cells), *Foxp3* (iTreg cells), or *Sfpi1* or *Irf4* (Th9 cells). Eomes may control IFN-γ expression by CD4$^+$ T cells under some circumstances (Suto et al., 2006; Tofukuji et al., 2012; Yang et al., 2008), but Eomes is not highly expressed in IL-12-induced Th1 cells. Our data indicate that Ezh2-mediated repression of *Eomes* is essential to inhibit spontaneous expression of IFN-γ production when not directly inhibited by other Th cell differentiation programs. It will therefore be important to further investigate the functional role for Ezh2-mediated repression of *Eomes* in other Th cell subsets.

We also found that Ezh2-deficient Th cells were capable of causing enhanced airway inflammation and pathology and that loss of Ezh2 function in CD4$^+$ T cells resulted in progressive accumulation of Th2 cytokine-producing cells with a memory phenotype over the life of the animal, accompanied by strong increases in circulating IgE. Several other lines of mice with mutations in genes encoding signaling molecules in T cells have previously been reported to display similar increases in CD44^{hi} cells with a Th2 cell phenotype and increases in type 2 inflammation in vivo. However, in these cases, severe alterations in thymic development (Aguado et al., 2002; Ji et al., 2007) or lymphocyte proliferation (Ranger et al., 1998) are evident. Although the exact mechanisms underlying the progressive Th2 cell-biased phenotype of SPF-maintained mice with CD4$^+$ cell-specific deletion of Ezh2 remain unclear, it will be important to further explore the function of Ezh2 in other Th cell lineages, for example iTreg cells, as well as at other Ezh2 target genes that may contribute to Th2 cell-mediated inflammation.

In summary, we found that Ezh2 binds strongly to the master regulators of Th1 and Th2 cell differentiation *Tbx21* and *Gata3*, respectively, and its presence is linked to the maintenance of the H3K27me3 mark at these loci. Ezh2 is essential for controlling differentiation, cytokine production, and maintenance of polarization of CD4$^+$ Th cells. We also identified a pathway

regulating IFN-γ production in CD4+ T cells: in the absence of polarizing signals, Ezh2 suppresses spontaneous generation of IFN-γ-producing cells via inhibition of Eomes expression. The findings presented herein provide a dramatic example of the importance of Ezh2 in establishing and maintaining phenotypic identity and indicate that without the ability to actively suppress alternative fates via chromatin modifications, dysregulated immune responses ensue.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were from CLEA Co. Mice with loxp sites (fl) flanking the SET domain of *Ezh2* ($Ezh2^{SET-fl/SET-fl}$) were generated as described (Hirabayashi et al., 2009) and backcrossed with C57BL/6 mice for ten generations. $Eomes^{Exon1-fl/Exon1-fl}$ mice were kindly provided by S. Reiner (Columbia University) (Intlekofer et al., 2008). $Eomes^{Exon1-fl/Exon1-fl}$ mice and $Tbx21^{-/-}$ mice (Szabo et al., 2002) were crossed with $Ezh2^{SET-fl/SET-fl}$ mice in our laboratory and bred with mice expressing transgenes for Cre recombinase. CD4-Cre mice were purchased from Taconic and OX40-Cre mice were kindly provided by N. Killeen (UCSF) (Klinger et al., 2009). For antigen-specific activation of CD4+ T cells, mice expressing the OTII-TCRαβ specific for residues 323–339 of the ovalbumin protein were used. All wild-type control mice used were sex-matched littermates. Controls were either $Ezh2^{SET-fl/SET-fl}$ Cre-negative or $Ezh2^{wt/wt}$ Cre-positive with similar experimental results obtained irrespective of Cre transgene expression. All mice were maintained under specific-pathogen-free conditions and animal care was conducted in accordance with the guidelines of Chiba University.

CD4+ T Cell Cultures

Naive ($CD44^{lo}CD62L^{hi}$) CD4+ T cell purification from spleens of mice and culture conditions for Th cell differentiation are described in the Supplemental Experimental Procedures.

Immunofluorescent Staining for Flow-Cytometric Analysis

The antibodies used for detection of surface molecules and intracellular staining are listed in the Supplemental Experimental Procedures. Flow cytometry data was acquired on a FACSCalibur or FACSCanto flow cytometer and results analyzed with FlowJo software (Tree Star).

Enzyme-Linked Immunosorbent Assay

A standard sandwich ELISA protocol was used to measure the concentrations of cytokines in cell-free culture supernatants and BAL fluid samples. Antibody pairs are listed in the Supplemental Experimental Procedures.

Knockdown Analysis

For knockdown of Gata3, the Mouse T cell Nucleofector Kit (Amaxa) was used according to the manufacturer's protocol. Th2 cells were transfected with 675 pmole of control random siRNA (AM4635) or siRNA for *Gata3* (s66482) from Applied Biosystems and cultured for 24 hr before analysis.

Quantitative Reverse Transcription-Polymerase Chain Reaction

RT-PCR was performed by standard protocols. A detailed description is included in the Supplemental Experimental Procedures. Probes and primers are listed in Table S3.

Microarray Data Collection and Analysis

Total cellular RNA was extracted with TRIzol reagent (Invitrogen). RNA was labeled with a 3′ IVT Express kit (Affymetrix) and hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) according to the manufacturer's protocols. Expression values were determined with GeneChip Operating Software (GCOS) software (Affymetrix).

Chromatin Immunoprecipitation

ChIP assays were performed as described in the Supplemental Experimental Procedures. Polyclonal anti-Ezh2 (pAB-039-50, Diagenode), anti-H3K27me3 (07-446, Millipore), anti-H3K9-Ac (06-599, Millipore), anti-H3K27ac (ab4729, Abcam), anti-p300 (N-15 sc-548, Santa Cruz), and anti-CBP (ab2832, Abcam) were used for immunoprecipitation. Probes and primers are listed in Table S3. Enrichment was calculated with the following formula: (specific antibody ChIP − control Ig ChIP)/input DNA. The highest enrichment for each antibody was set to 10 with all other positions calculated as a function of this value.

ChIP-Sequence

Data acquisition for ChIP-sequence was performed as described previously (Kanai et al., 2011). Read sequences were aligned to the mm9 mouse reference genome (University of California, Santa Cruz [UCSC], July 2007). Enrichment values were calculated for intervals from −5 kb to +3 kb relative to the transcriptional start site of each gene. A detailed description of the analysis protocol is included in the Supplemental Experimental Procedures.

Mouse Model of Allergic Asthma

Th2 cell polarized Ezh2 WT or OX40-Cre-induced $Ezh2^{\Delta SET/\Delta SET}$ OT-II CD4+ cells (2×10^6) were intravenously transferred to syngeneic C57BL/6 mice. These mice were then challenged 1 and 3 days later with aerosolized OVA (10 mg/ml) for 30 min. BAL was performed either 12 hr (for ELISA) or 48 hr (for analysis of airway inflammatory cells) after the last allergen challenge. mRNA and histological analysis was performed 48 hr after the last allergen challenge. A detailed description is included in the Supplemental Experimental Procedures.

Statistical Analysis

Unless otherwise indicated, p values were calculated with Student's t tests or ANOVA with Bonferroni's post-tests when multiple comparisons were performed.

ACCESSION NUMBERS

ChIP-seq and microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession numbers GSE51079 and GSE50729, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.09.012.

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V. 資　　料

厚生労働省　臨床研究中核病院整備事業

医師主導治験

Crow-Fukase症候群に対するサリドマイドの試験（寛解導入試験）

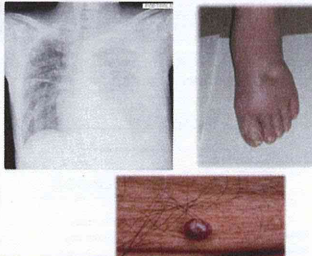
プロジェクト責任者/桑原聡　千葉大学医学部附属病院　神経内科　教授

開発試験物	サリドマイド（サレド®カプセル　藤本製薬（株））
対象疾患	Crow-Fukase症候群
開発の最終目標	製造販売承認（適応拡大）
プロジェクトの出口	医師主導治験の終了（千葉大学） 製造販売承認事項一部変更承認申請（藤本製薬（株））
現時点での到達点	症例登録中　5/5-10例
作用機序	サリドマイドの形質細胞への直接効果、VEGF産生抑制効果
市場での位置づけ	高齢者に対する治療、自己末梢血幹細胞移植療法前の寛解導入療法

Crow-Fukase症候群
（POEMS症候群）

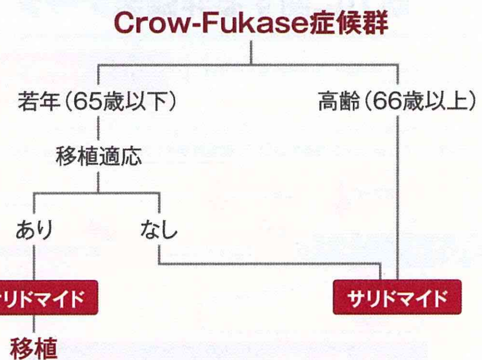
形質細胞腫に関連して上昇するVEGF（血管内皮増殖因子）により多彩な症状を呈する全身性疾患

P：polyneuropathy（多発性神経炎）
O：organomegaly（臓器腫大）
E：endocrinopathy（内分泌異常）
M：M-protein（M蛋白）
S：skin changes（皮膚症状）

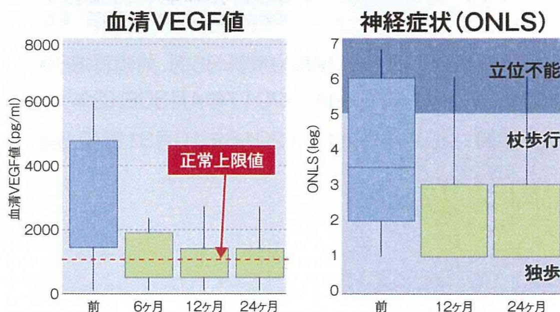


全国患者数　推定340名　日本に多い

Crow-Fukase症候群の治療方針



先行臨床試験成績
自験データ



試験の概要

- 研究期間　2013/7/4~2016/8/31（登録期間:2013/7/4~2016/1/31）
- 目標症例数　5~10例
- 実施医療機関　千葉大学医学部附属病院

CHIBA UNIVERSITY　千葉大学医学部附属病院

●問い合わせ先
担当PL 片山　TEL 043-226-2737　FAX 043-226-2735
E-mail jpost.k@gmail.com

CCRC

厚生労働省　臨床研究中核病院整備事業

医師主導治験

重症川崎病に対するシクロスポリンの試験

プロジェクト責任者/羽田明　医学研究院公衆衛生学　附属病院遺伝子診療部　教授

開発試験物	シクロスポリン（ネオーラル®　ノバルティスファーマ（株））
対象疾患	重症川崎病
開発の最終目標	製造販売承認（適応拡大）
プロジェクトの出口	医師主導治験の終了（千葉大） 製造販売承認事項一部変更承認申請（ノバルティスファーマ（株））
出口に至る主なハードル	症例リクルート・治験開始・申請
出口に至る現時点での到達点	医師主導治験　準備中 ・スタートアップミーティング・・・・・ 2013年11月30日 ・心エコー講習会・・・・・・・・・・・・・ 2014年2月8日 ・各施設のIRB　・・・・・・・・・・・・・ 2014年2月～3月 ・治験届提出　・・・・・・・・・・・・・・・・ 2014年4月16日 ・治験開始　・・・・・・・・・・・・・・・・・・ 2014年5月1日

効力に関する非臨床データ

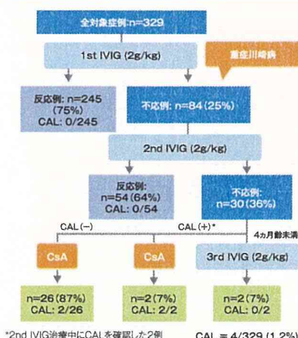
- Inositol 1,4,5-trisphosphate 3-kinase C (ITPKC)
- Caspase3 (CASP3)

免疫細胞の過剰な活性化を抑制している

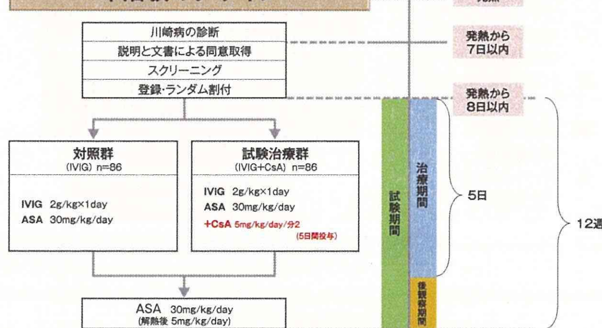
川崎病の患児、特に重症例では、ITPKC, CASP3の発現が低下するSNPの型が有意に多い。
⇒ ITPKC, Caspase3の機能が低下、炎症性サイトカインの過剰産生が起こる（仮説）。

Onouchi Y, Hata A, et al., Nat. Genet., 2008
Onouchi Y, Hata A, et al., Hum. Mol. Genet., 2010
Onouchi Y, Hata A, et al., Pharmacogenomics J., 2011

先行臨床試験成績



実施の概要



- 実施予定施設：約20施設　·千葉大学医学部附属病院　·東京女子医科大学八千代医療センター　·和歌山県立医科大学附属病院　など…
- 目標被験者数：172名（試験治療群:86例、対照群:86例）
- 試験期間：2014年5月1日～2017年4月30日（3年）
- 登録期間：2014年5月1日～2016年10月31日（2.5年）

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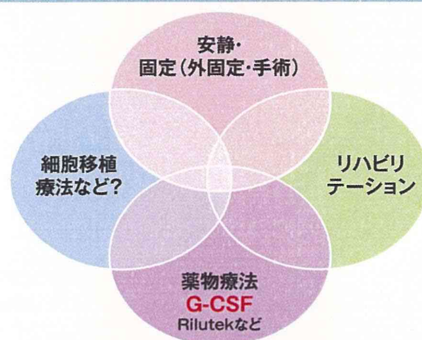
先進医療B

急性脊髄損傷に対するG-CSFを用いた試験

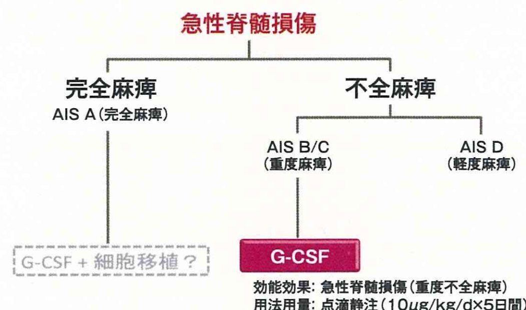
プロジェクト責任者/高橋和久　千葉大学医学部附属病院　整形外科　教授

開発試験物	顆粒球コロニー刺激因子 （フィルグラスチム:商品名グラン®　協和発酵キリン（株））
対象疾患	急性脊髄損傷
開発の最終目標	製造販売承認（適応拡大）
プロジェクトの出口	医師主導治験の終了（千葉大学） 製造販売承認事項一部変更承認申請（協和発酵キリン（株））
現時点での到達点	先進医療B申請中
作用機序	神経保護作用
市場での位置づけ	標準的治療はリハビリと手術。標準的薬物療法を目指す。

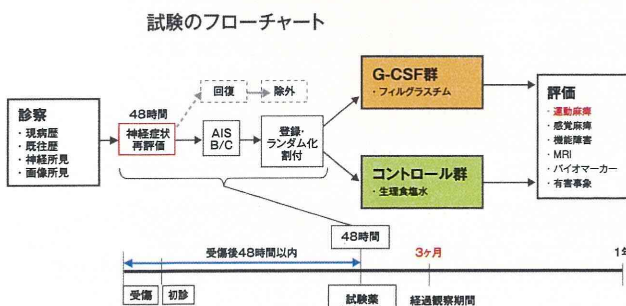
脊椎損傷の治療法



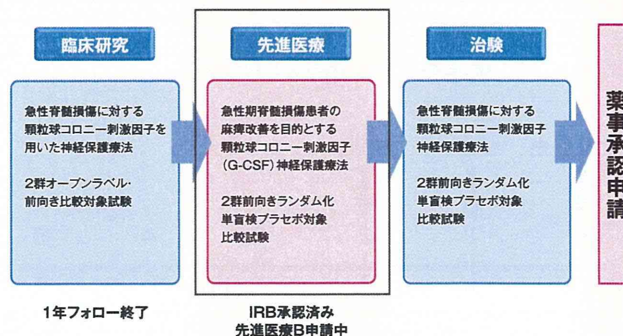
製品化（標準治療化）イメージ



試験デザイン



開発スケジュール（ロードマップ）



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