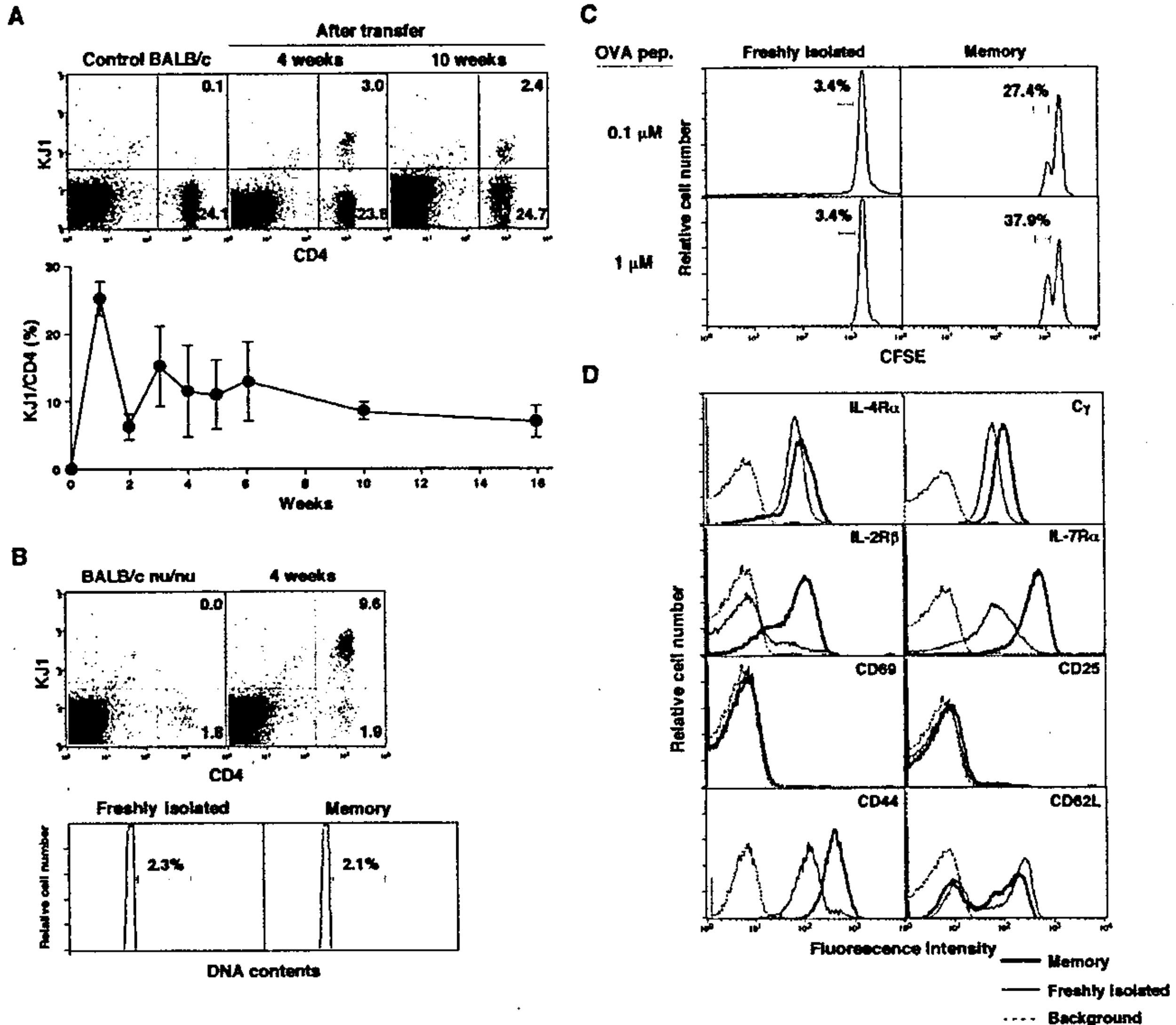

FIG. 1. **Generation and phenotypic characterization of OVA-specific memory Th2 cells.** *A*, kinetics of memory Th2 cell generation. Spleen cells were prepared from recipient BALB/c mice at the indicated time, and the number of recovered KJ1-positive cells was determined by the staining with KJ1 mAb. The typical staining patterns and the mean proportion of KJ1-positive cells in CD4 cells from three individual mice are shown. *B*, cell cycle analysis of memory Th2 cells. Effector Th2 cells were transferred into BALB/c *nu/nu* mice. Four weeks after cell transfer, memory Th2 cells were prepared, stained with PI, and analyzed by flow cytometry. Two independent experiments were done with similar results. *C*, memory Th2 cells rapidly proliferated in response to the antigen. Cells were labeled with CFSE and stimulated with OVA peptides (0.1 or 1 μM) plus APC for 16 h. Cell division of CFSE-labeled cells was analyzed by flow cytometry. The percentages of divided cells are shown in each panel. Two independent experiments were done with similar results. *D*, expression profiles of cell surface maker antigens in memory Th2 cells. Spleen cells from BALB/c *nu/nu* recipient mice (*memory*) and DO11.10 Tg mice (*freshly isolated*) were stained with KJ1 mAb and mAbs against indicated cell surface molecules. Staining profiles of electronically gated KJ1-positive cells are shown.

pressed in either memory or naïve populations. High-level expression of CD44 was observed in all recovered KJ1-positive cells. Finally, two subpopulations with high and low expression of CD62L were observed in memory Th2 cells as well as in naïve T cells.

Cytokine Production Profiles of in Vivo Generated Memory Th2 Cells—We examined the cytokine production profiles of *in vivo* generated memory Th2 cells recovered from BALB/c *nu/nu* recipient mice 4 weeks after cell transfer. Freshly isolated splenic KJ1$^+$ CD4 T cells from DO11.10 Tg mice (Fresh), *in vitro* newly generated effector Th2 cells by stimulation with OVA peptides for 5 days *in vitro* (Effector) and *in vivo* generated memory Th2 cells (memory) were re-stimulated with OVA peptide plus APC for 1–3 days. As shown in Fig. 2A, *in vivo* generated memory Th2 cells produced large amounts of Th2 cytokines (IL-4, IL-5, and IL-13). The levels were significantly higher than those of effector Th2 cells particularly on day 3. CD62L expression profiles and cytokine production of the recovered KJ1$^+$ cells were similar in both normal BALB/c and BALB/c *nu/nu* recipient mice (see Supplemental Fig. 1).

We also prepared *in vivo* generated Th1 memory cells to confirm the specificity of cytokine production of memory Th1 and Th2 cells. Splenic CD4 T cells from DO11.10 Tg mice were stimulated with OVA peptide plus APC under Th1- or Th2-skewed conditions for 5 days, and transferred into recipient BALB/c *nu/nu* mice. Four weeks after cell transfer, KJ1$^+$ cells were purified and re-stimulated with OVA peptide plus APC. As shown in Fig. 2B, memory Th2 cells produced large amounts

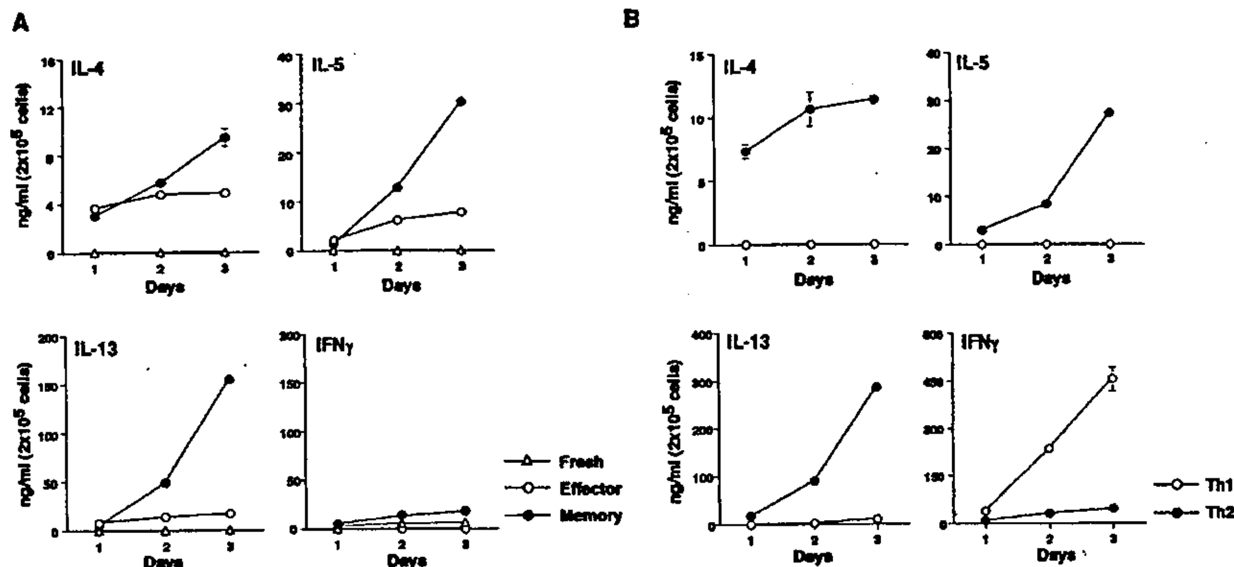

FIG. 2. **Cytokine production profiles of *in vivo* generated memory Th2 cells upon *in vitro* antigenic restimulation.** *A*, freshly isolated splenic KJ1$^+$ CD4 T cells from DO11.10 Tg mice (*Fresh*), *in vitro* newly generated effector Th2 cells by stimulation with OVA peptide for 5 days *in vitro* (*Effector*), and *in vivo* generated memory Th2 cells (*Memory*) were re-stimulated with OVA peptide plus APC for 1, 2, and 3 days. Purified KJ1-positive cells (2×10^5) were restimulated *in vitro* with 1 μM OVA peptide plus APC, and culture supernatants were collected at indicated times. The amounts of the indicated cytokines in the culture supernatant were assessed by ELISA. Four independent experiments with different T cell preparations were done with similar results. *B*, cytokine production profiles of *in vivo* generated memory Th1 and Th2 cells. CD4 T cells from DO11.10 Tg mice were stimulated with OVA peptide (1 μM) plus APC under the Th2-skewed condition or the Th1-skewed condition for 5 days. Then, the effector Th2 and Th1 cells (3×10^7) were transferred into BALB/c *nu/nu* mice intravenously. Four weeks after cell transfer, memory Th2 and Th1 cells were prepared and stimulated with OVA peptide antigens as in *A*. Two independent experiments with different T cell preparations were done with similar results.

of Th2 cytokines but not IFNγ, while memory Th1 cells produced large amounts of IFNγ but not Th2 cytokines. These results suggest that *in vivo* generated Th1 and Th2 memory cells preserved their original restricted cytokine production profiles. From these results, we decided to use KJ1$^+$ CD4 T cells recovered from BALB/c or BALB/c *nu/nu* recipient mice 4 weeks after cell transfer as *in vivo* generated memory CD4 T cells to investigate the molecular mechanisms that control the maintenance of memory Th2 cells.

Histone H3-K9/14 of the Th2 Cytokine Gene Loci Was Hyperacetylated in Memory Th2 Cells—We began with an assessment of the acetylation status of histones associated with the Th2 cytokine gene loci in freshly isolated *in vivo* generated memory Th2 cells. The KJ1$^+$ memory Th2 cells were isolated by cell sorting, and the acetylation levels of histone H3 (K9/14) was determined by ChIP assay as described previously (22). Histone H3-K9/14 associated with the *IL-4* and *IL-13*-related gene loci (CGRE, CNS1, V_A enhancer, IL-4p, and IL-13p) were hyperacetylated in both memory Th2 cells and effector Th2 cells compared with freshly isolated naïve DO11.10 TCR Tg CD4 T cells (Fig. 3*A*). The acetylation levels at the *IL-5* promoter were significantly lower in memory Th2 cells as compared with effector Th2 cells. No hyperacetylation in the *IFNγ* promoter was observed. A similar hyperacetylation pattern was observed in memory Th2 cells isolated 10 weeks after cell transfer (data not shown).

Next, *in vivo* generated memory Th1 and Th2 cells were prepared to examine the Th2-specific hyperacetylation (Fig. 3*B*). The levels of acetylation of the CGRE, CNS1, V_A enhancer, IL-4p and IL-13p region in memory Th2 cells were significantly higher than those of memory Th1 cells. Memory Th1 cells exhibited certain levels of acetylation of these regions. The acetylation levels of IL-5 in memory Th2 cells were equivalent to those of memory Th1 cells, but they were significantly higher than those of freshly prepared CD4T cells. Equivalent levels of acetylation in RAD50 promoter were seen. For the *IFNγ* promoter, there was no preferential increase in acetylation in the Th1 memory cells. We compared acetylation status of *IFNγ* promoter in effector and memory Th1 cells and found that significant levels of acetylation of the *IFNγ* promoter induced in effector Th1 cells were substantially decreased in memory Th1 cells (Supplemental Fig. 2). Taken together, these results suggest that memory Th1 and Th2 cells possess higher background levels of histone acetylation in all regions tested as compared with naïve T cells, and that Th2 memory cells preserved preferentially increased acetylation of histone H3-K9/14 in the *IL-4* and *IL-13* gene-related regions.

These results prompted us to examine whether a unique long-range Th2-specific histone hyperacetylation within the *IL-13* and *IL-4* loci (22) is preserved in memory Th2 cells. We analyzed the acetylation status of histone H3 in the *IL-13* and *IL-4* gene loci more precisely using 29 pairs of specific primers. Fig. 3*C* shows the actual ChIP assay PCR bands (Upper), the summary of relative band intensity (Ac-H3/Input DNA) and the ratios of acetylation intensity of effector and memory Th2 cells to that of freshly isolated CD4 T cells. The acetylation profiles induced in effector Th2 cells were maintained in memory Th2 cells with slightly decreased levels at the regions associated with IL-13. Furthermore, the boundary of Th2-specific hyperacetylation at the CGRE site was preserved in memory Th2 cells.

Histone H3 (K9/14) of the Th2 Cytokine Gene Loci Is Acetylated Equivalently in Effector and Central Memory Th2 Cells—Memory T cells can be subdivided into two distinct populations based on the expression level of CD62L (48). One is the effector memory T cell (CD44^{high}/CD62L^{low}) and the other is the central memory T cell (CD44^{high}/CD62L^{high}). The change in proportion of effector and central memory Th2 cells over time was assessed in our *in vivo* memory Th2 cell generation system (Supplemental Fig. 3*A*). The ratio (effector/central memory) in-

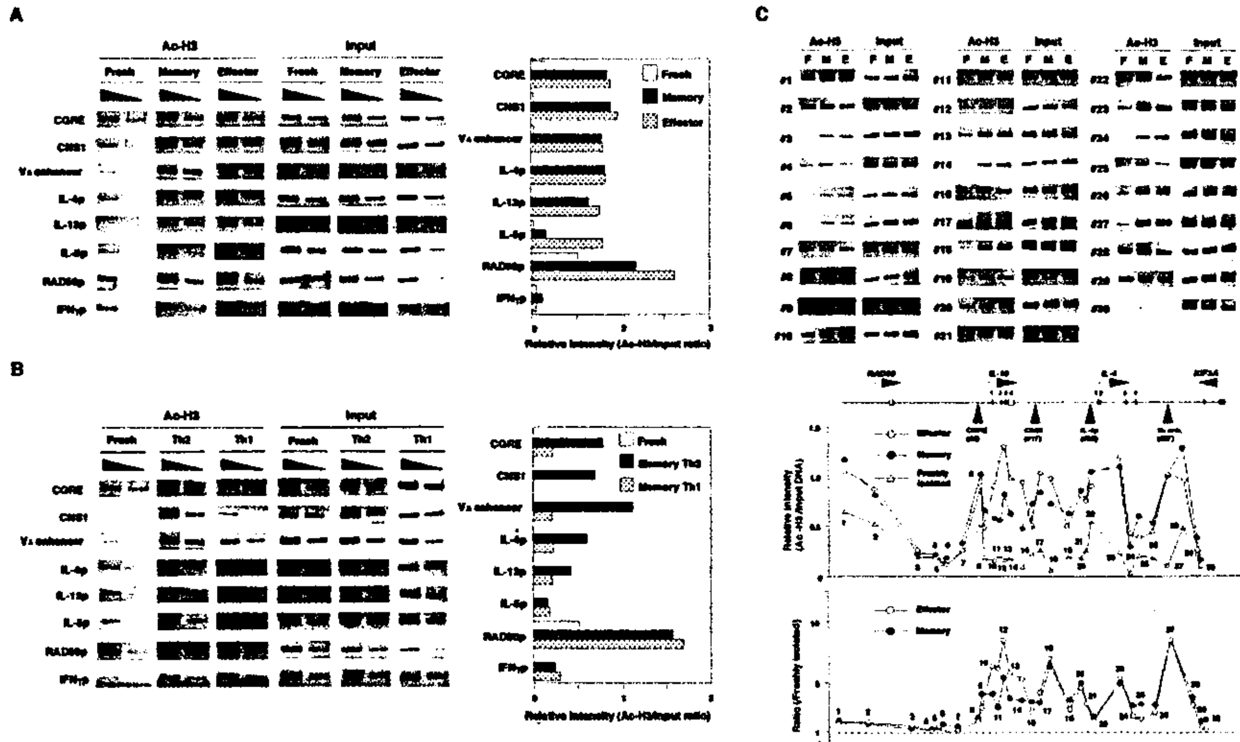

FIG. 3. **Acetylation status of histone H3-K9/14 in the Th2 cytokine gene loci in memory Th2 cells.** *A*, histone H3 (K9/14) acetylation of the Th2 cytokine gene loci in memory Th2 cells. *In vitro* differentiated effector Th2 cells were transferred into BALB/c *nu/nu* mice as in Fig. 1. Memory Th2 cells was prepared 4 weeks after cell transfer by sorting KJ1-positive cells. ChIP assay was performed with an anti-acetyl histone H3 (K9/14) antibody and the indicated specific primer pairs. PCR was performed with 3-fold serial dilution of template genomic DNA. Shown are the PCR product bands (*left*) and the relative intensity (*Ac-H3/Input*) for each primer pair (*right*). Three independent experiments with different T cell preparations were done with similar results. *B*, comparison of histone H3 acetylation of the Th2 cytokine gene loci between memory Th1 and Th2 cells. Shown are the PCR product bands (*left*) and the relative intensity (*Ac-H3/Input*) for each primer pair (*right*). Two independent experiments were performed with similar results. *C*, histone H3 hyperacetylation within the *IL-13* and *IL-4* loci in memory Th2 cells. Shown are the PCR product bands for each primer pair (*upper panel*), the relative intensity (*Ac-H3/Input*) (*middle panel*) and the Memory/Fresh or Effector/Fresh ratio (*lower panel*) of the band intensities. Three independent experiments with different T cell preparations were performed with similar results.

creased up to 4 weeks after cell transfer, and decreased thereafter. Phenotypic analysis revealed that these two memory Th2 subpopulations express similar levels of cytokine receptor components (IL-4Rα, Cγ, IL-2Rβ, and IL-7Rα) (Supplemental Fig. 3*B*). Both populations produced substantial amounts of IL-4, IL-5, and IL-13 with marginal production of IFNγ. The levels of IL-4 production were slightly but reproducibly higher in central memory Th2 cells, and those of IL-5 were higher in effector memory Th2 cells. The production of IL-13 was equivalent between these two subpopulations (Supplemental Fig. 3*C*, *lower left panel*).

Concurrently, we assessed the acetylation status of histone H3 (K9/14), and substantial and equivalent histone hyperacetylation of the IL-4 and IL-13-related regions were detected in these two subpopulations (Supplemental Fig. 3*D*). Similar results were obtained in effector and central memory Th2 cells 10 weeks after cell transfer (data not shown). These results would indicate that Th2-specific remodeled chromatin is preserved in both effector and central memory Th2 cells.

IL-4 Is Not Required for the Generation and the Maintenance of Memory Th2 Cells—IL-4 is a critical cytokine for the induction of chromatin remodeling of the Th2 cytokine gene loci during Th2 cell development. Consequently, we examined the requirement of IL-4 for the generation and the maintenance of memory Th2 cells. Splenic CD4 T cells from IL-4-deficient DO11.10 Tg mice with a BALB/c background were stimulated with OVA peptide and APC in the presence of exogenous IL-4 for 5 days. The effector Th2 cells from IL-4-deficient mice produced almost the same amounts of IL-5 and IL-13 compared with those from normal mice, and the acetylation status of the Th2 cytokine gene loci was almost equivalent (data not shown). Then, the cultured cells were transferred into recipient normal BALB/c mice. As shown in Fig. 4*A*, the numbers of KJ1$^+$CD4 T cells and the ratio of effector/central memory cells were similar between wild type (WT), IL-4$^{+/-}$ heterozygous (Hetero), and IL-4$^{-/-}$ homozygous deficient (KO) mice. Next, we used IL-4-deficient mice as hosts, and examined the generation of KJ1$^+$ cells. Equivalent levels of KJ1$^+$CD4 T cell generation were observed (Fig. 4*B*, *upper*). The ratio of effector/central memory cells was also similar regardless of the source donor cells or recipients, indicating the lack of dependence on IL-4 (Fig. 4*B*, *lower*).

The KJ1$^+$CD4 T cells generated in recipient mice shown in Fig. 4, *A* and *B* were purified by sorting, and their cytokine production profiles were determined by ELISA. The memory Th2 cells from IL-4-deficient mice produced equivalent amounts of IL-13, and slightly decreased levels of IL-5 (Fig. 4*C*). IFNγ production from IL-4-deficient memory Th2 cells was not robust but it was modestly increased (Fig. 4*C*, *extreme right panels*). IL-4 deficiency in the host mice did not affect the cytokine profiles of memory cells (Fig. 4*C*, *lower panels*). We assessed the acetylation status of histone H3 (K9/14) in the Th2 cytokine gene loci in the IL-4-deficient memory Th2 cells and found that the levels of acetylation in the *IL-4*-related gene loci (CGRE, CNS1, V_A enhancer, and IL-4p) were all equivalent among wild type and IL-4-deficient groups (Fig. 4*D*). The levels

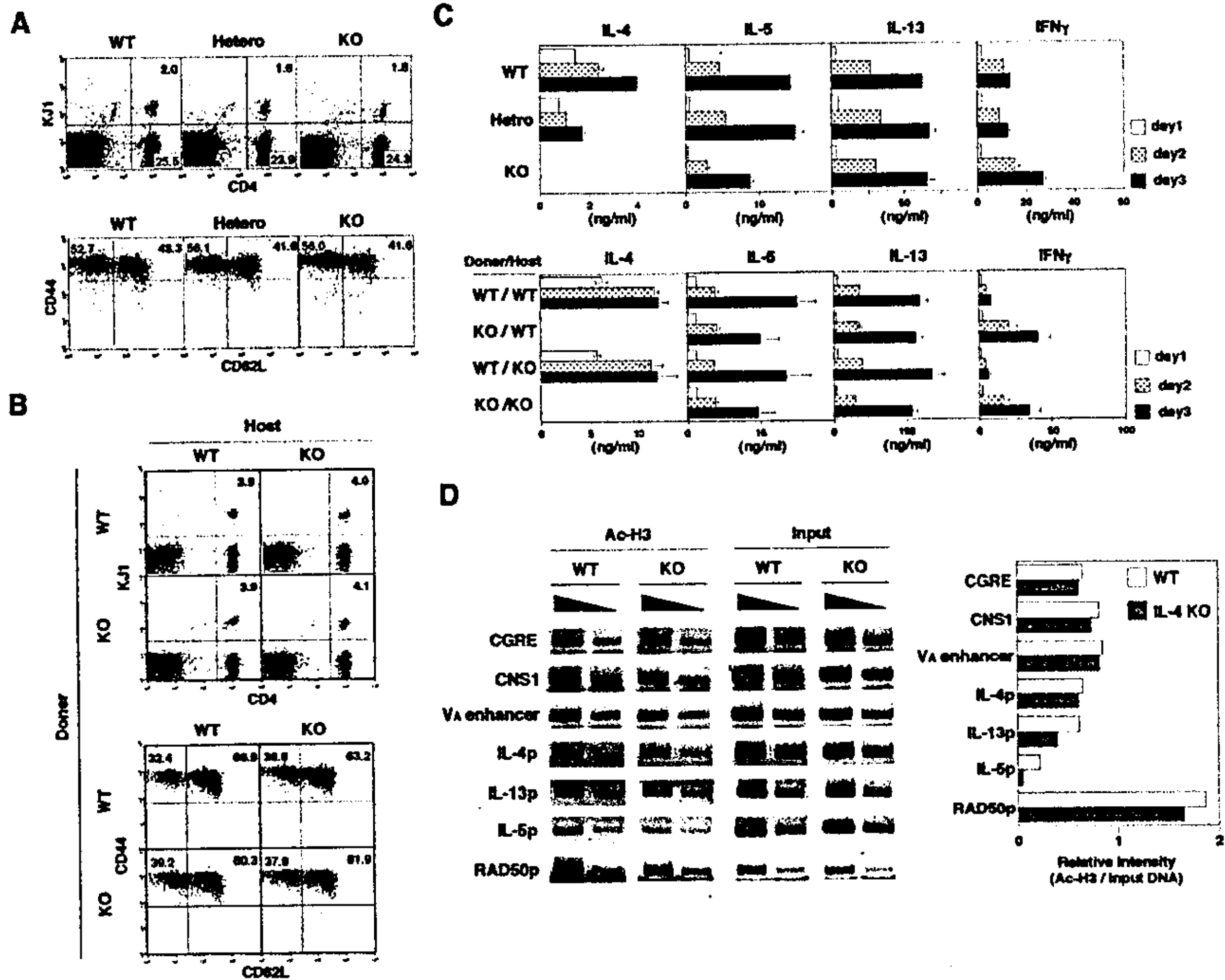

FIG. 4. **IL-4 is not required for the generation and the maintenance of memory Th2 cells.** *A*, *in vivo* generated Th2 cells were prepared from WT, IL-4$^{+/-}$ heterozygous (*Hetero*), and IL-4$^{-/-}$ homozygous-deficient (*KO*) mice 4 weeks after cell transfer into BALB/c mice. Representative staining profiles of CD4/KJ1 and CD62L/CD44 are shown with percentages in each quadrant. *B*, *in vivo* generated Th2 cells were prepared using WT or IL-4-deficient (KO) donor T cells, and WT and IL-4-deficient (KO) BALB/c recipient mice. *C*, effect of IL-4 deficiency on the cytokine production profiles of *in vivo* generated memory Th2 cells. Memory Th2 cells were generated as in *A* and *B*, restimulated with OVA peptide (1 μM), and the concentrations of cytokines in the culture supernatants were determined by ELISA. *D*, acetylation status of histone H3 of the Th2 cytokine gene loci in memory Th2 cells generated by transfer of IL-4-deficient effector Th2 cells. ChIP assay was performed as described in Fig. 3. PCR was performed with 3-fold serial dilution of template genomic DNA.

of acetylation at the *IL-13* promoter were slightly decreased in the absence of IL-4. The acetylation levels of the *IL-5* promoter was low in memory Th2 cells (see Fig. 3, *A* and *B*), and significantly lower in IL-4-deficient memory Th2 cells. These results indicate that IL-4 is not required for the generation of memory Th2 cells and the maintenance of the ability to produce Th2 cytokines. In addition, while IL-4 in T cells appears to play some specific role in the maintenance of acetylation at the *IL-5* gene locus, it does not affect the *IL-4*-related gene locus in memory Th2 cells. It may have some role in the maintenance of acetylation of the *IL-13*-related gene locus.

Memory Th2 Cells Express High Levels of GATA3 mRNA but Undetectable Amounts of GATA3 Protein—GATA3 is thought to be a master transcription factor and it is induced in developing Th2 cells in an IL-4- and STAT6-dependent manner. Since the Th2-specific acetylation profiles in the *IL-13* and *IL-4* gene loci were preserved in memory Th2 cell, we sought to examine the expression levels of GATA3 in memory Th2 cells. First, the expression of GATA3 mRNA was assessed by semi-quantitative RT-PCR analysis. The memory Th2 cells expressed substantial levels of GATA3 mRNA that were equivalent to those of effector Th2 cells (Fig. 5*A*). Two distinct promoters, *GATA3a* and original promoter *GATA3b* have been reported (49), and so we assessed the levels of mRNA of both sites in memory Th2 cells. GATA3a transcripts were detected only in the memory Th2 cells, although the levels were quite low when compared with GATA3b. The original *GATA3b* transcripts were detected in memory Th2 cells at equivalent levels to effector Th2 cells. The transcripts of the mature mRNA for IL-4, IL-5, and IL-13 were detected in effector Th2 cells but not in freshly isolated memory Th2 cells. Equivalent amounts of GATA3 mRNA were detected in wild-type and IL-4-deficient memory Th2 cells, suggesting that IL-4 is not required for the GATA3 transcription in memory Th2 cells (Fig. 5*B*). Similar results were obtained by real time PCR analyses (data not shown).

Next, the protein expression of GATA3 in memory Th2 cells was assessed by immunoblot analysis. Surprisingly, the expression levels of GATA3 protein in memory Th2 cells were very low (~1/10) and they were only equivalent to those of

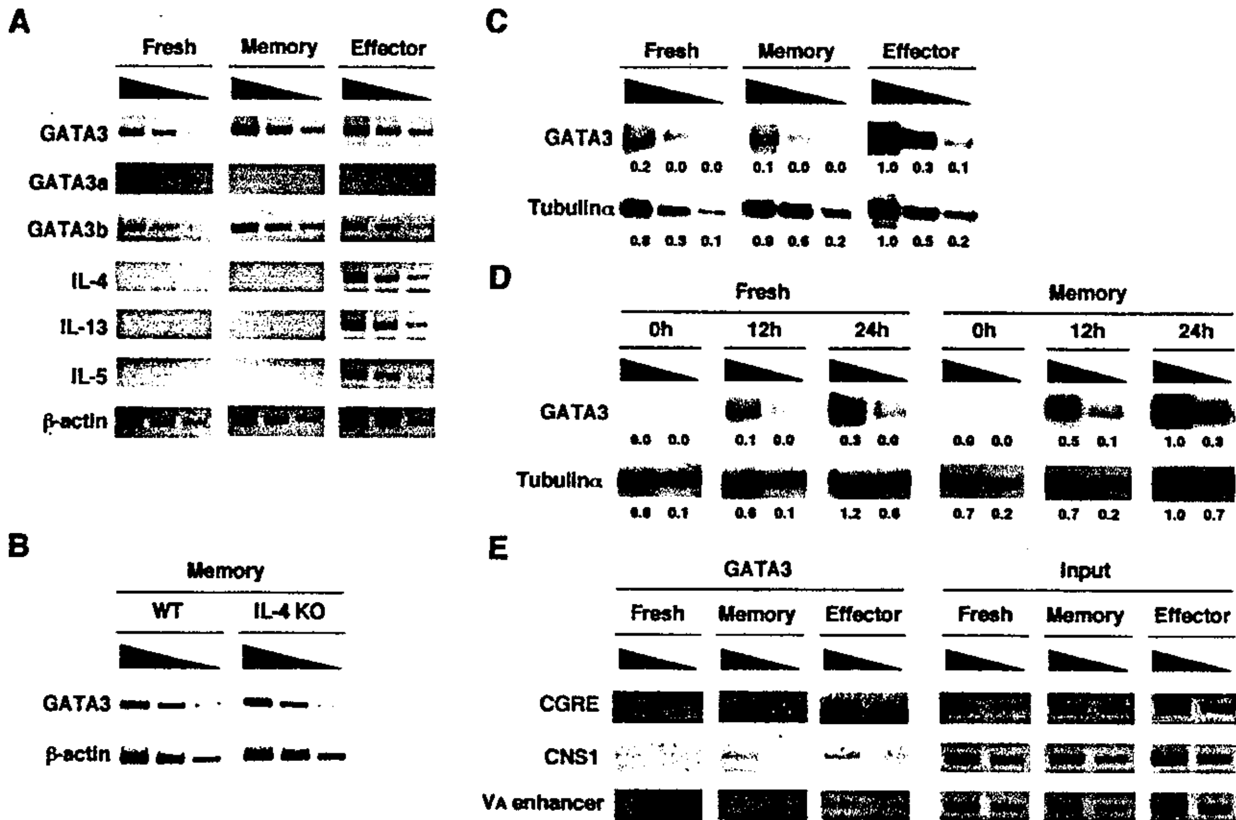

FIG. 5. **Expression of GATA3 mRNA and GATA3 protein in *in vivo* generated memory Th2 cells.** *A*, high level expression of GATA3 mRNA in memory Th2 cells. mRNA levels of GATA3, GATA3a, GATA3b, IL-4, IL-13, IL-5, and β-actin were determined by semiquantitative RT-PCR analysis with 3-fold serial dilutions of template cDNA. Shown are the representative PCR product bands of three independent experiments with memory Th2 cells generated in BALB/c *nu/nu* mice 4 weeks after cell transfer. *B*, IL-4-independent expression of GATA3 mRNA. The mRNA levels for GATA3 in IL-4-deficient memory Th2 cells were determined as described in *A*. *C*, expression of GATA3 protein in resting memory Th2 cells. Freshly prepared KJ1$^+$ CD4 T cells (*Fresh*), memory Th2 cells (*Memory*), and effector Th2 (*Effector*) were prepared as in *A*. The expression levels of GATA3 and tubulin-α protein were determined by immunoblotting. Arbitrary densitometric units are shown under each band. Three experiments were done with similar results. *D*, GATA3 protein induced in memory Th2 cells upon anti-TCR mAb restimulation. Freshly prepared KJ1$^+$ CD4 T cells and memory Th2 cells were stimulated with anti-TCR mAb under Th2-skewed conditions for indicated times. The expression levels of GATA3 and tubulin-α protein were examined by immunoblotting. *E*, GATA3 binding to the CGRE site was not detected in memory Th2 cells. ChIP assay using anti-GATA3 antibody was performed.

freshly prepared naïve CD4 T cells (Fig. 5*C*). However, 12 and 24 h after stimulation with anti-TCR mAb *in vitro*, memory Th2 cells expressed significantly higher amounts of GATA3 protein than freshly prepared naïve CD4 T cells (Fig. 5*D*). The efficient induction of GATA3 protein was also observed in IL-4-deficient memory Th2 cells upon anti-TCR mAb stimulation (data not shown). We reported previously the efficient binding of GATA3 protein to the CGRE regions in newly generated effector Th2 cells (22). Thus, we wanted to know whether the binding of GATA3 protein to the CGRE in memory Th2 cells in which histone hyperacetylation of the *IL-4* and *IL-13* gene loci was preserved. ChIP analyses with anti-GATA3 revealed that there was significant GATA3 binding to the CGRE region in effector Th2 cells but not in memory Th2 cells (Fig. 5*E*). No significant binding was observed at CNS1 and V_A enhancer regions in either memory or effector Th2 cells. Taken together, these results suggest that memory Th2 cells express substantial amounts of GATA3 mRNA although only marginal levels of GATA3 protein can be detected. Furthermore, histone hyperacetylation of the *IL-13* and *IL-4* gene loci appears to be maintained in a GATA3 protein expression-independent manner.

Intergenic Transcripts at the Downstream Region of the CNS1 Spanning to V_A Enhancer Site Are Preserved in Memory Th2 Cells—In our previous reports, we proposed a potential role of intergenic transcription for inducing long range histone hyperacetylation and the transactivation of the *IL-13* and *IL-4* gene loci (22, 25). Therefore, we assessed the intergenic transcripts of the *IL-13* and *IL-4* gene loci using 19 primer pairs (Fig. 6*A*). The ratios of band intensity (fresh/effector and memory/effector) in each group are summarized in the *lower panel* of Fig. 6*A*. In memory Th2 cells, substantial amounts of transcripts were detected in all regions that were tested, and their levels were essentially preserved at the downstream region of the CNS1 spanning to the V_A enhancer site. IL-4-deficient memory Th2 cells expressed equivalent amounts of intergenic transcripts at the CNS1, 18, V_A enhancer, and 28 sites as well. This indicates that IL-4 is not required for the intergenic transcription of these regions in memory Th2 cells (Fig. 6*B*).

Consequently, we assessed the changes in the intergenic transcript levels in memory Th2 cells after anti-TCR stimulation. The levels of intergenic transcripts upstream of the CNS1 region were increased substantially after anti-TCR stimulation, but those downstream of the CNS1 site remained unchanged (Fig. 6*C*). Also, there was no inhibition of the generation of intergenic transcripts in the presence of FK506, indicating that the intergenic transcripts were not dependent on the activation of calcineurin in memory Th2 cells. Under the same conditions, the mature IL-4 and IL-13 transcripts were

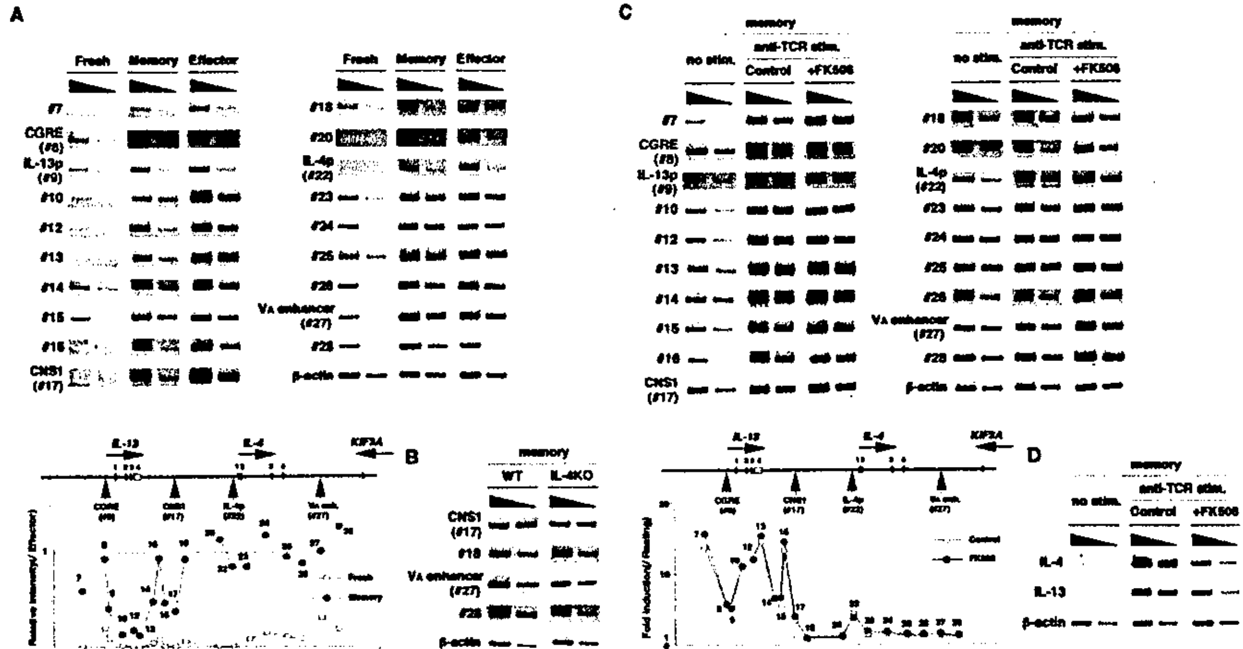

FIG. 6. **Intergenic transcripts are preserved in memory Th2 cells.** *A*, detection of non-coding intergenic transcripts of the *IL-13* and *IL-4* gene loci in memory Th2 cells. Freshly prepared KJ1$^+$ CD4 T cells (*Fresh*), memory Th2 cells (*Memory*), and effector Th2 (*Effector*) were prepared described in Fig. 5, and total RNA was prepared. To avoid contamination of genomic DNA, samples were treated with DNase I. Non-coding transcripts were determined by a semiquantitative RT-PCR analysis with 3-fold serial dilution of template cDNA. The ratios of band intensity of the fresh and memory cells to that of effector cells are depicted in the *lower panel*. Three independent experiments with different T cell preparations were performed with similar results. *B*, non-coding transcription in IL-4-deficient memory Th2 cells. The levels of intergenic transcription in IL-4-deficient memory Th2 cells were determined as described above. *C*, memory Th2 cells were prepared as in *A*, and stimulated with anti-TCR mAb for 48 h in the presence or absence of 100 nM of FK506. Non-coding transcripts were assessed as in *A*. The ratios of band intensity of cells after anti-TCR stimulation in the presence (*+FK506*) or absence (*Control*) of FK506 to that of before stimulation (*no stim.*) are summarized in the *lower panel*. *D*, levels of mature IL-4 and IL-13 transcripts in the cells as in *C*. Three independent experiments with different T cell preparations were performed with similar results.

induced by anti-TCR stimulation in memory Th2 cells, and these were found to be significantly inhibited in the presence of FK506 (Fig. 6*D*). These results suggest that the intergenic transcripts of the *IL-13* and *IL-4* gene loci were generated by a distinct signaling mechanism as compared with that for mature IL-13 and IL-4 mRNA.

Histone Methylation (H3-K4) in the Long Range Region of the IL-13 and IL-4 Gene Loci Is Totally Preserved in Memory Th2 Cells—It has been reported that the methylation of histone H3-K4 is well correlated with active chromatin in transcription and some specific role in the maintenance of H3-K9/14 acetylation in mammalian systems (50). Consequently, we analyzed the methylation status of histone H3-K4 of the *IL-4* and *IL-13* gene loci in fresh DO11.10 Tg KJ1$^+$ CD4 T cells (F), memory (M), and effector (E) Th2 cells using a series of primer pairs and anti-di- and tri-methyl histone specific Abs (Fig. 7*A*). The relative intensity profiles are depicted in Fig. 7*B*. The relative levels of di (Me2)- or tri (Me3)-methylation at histone H3-K4 of the *IL-4* and *IL-13* gene loci were low in fresh CD4 T cells, but there was substantial methylation at the site in memory Th2 cells and effector Th2 cells to almost equivalent levels. These results suggest that histone methylation (H3-K4) in the long range region of the *IL-13* and *IL-4* gene loci is totally preserved in memory Th2 cells.

Memory Th2 Cell-specific Accumulation of Pol II Complex at Specific Intergenic Regions (CNS1, IL-4p, and V_A Enhancer)—Because some of the histone methyltransferase for H3-K4 functionally interacted with Pol II (26), ChIP assay with anti-Pol II Ab was performed. Interestingly, strong bindings of Pol II to specific regions, *i.e.* CNS1 (17), *IL-4* promoter (22), and V_A enhancer (27) sites, and a weak binding to the CGRE site (8) were observed in memory Th2 cells (Fig. 7*C*). In addition, weak binding of Pol II was observed at almost all regions of the *IL-4* and *IL-13* gene loci in effector Th2 cells. Although nineteen regions throughout the *IL-13* and *IL-4* gene loci were analyzed, the strong binding of Pol II was observed at only these three sites in memory Th2 cells (Fig. 7*C*). We also tested additional 12 sites within the region, but found no additional strong sites (data not shown). The binding of TFIIB was observed at the same three strong binding sites for Pol II (CNS1, IL-4p, and V_A enhancer; Fig. 7*D*). These results indicate that highly restricted accumulation of Pol II complex to specific sites is unique to memory Th2 cells, and may play a role in the maintenance of intergenic transcription and histone methylation (H3-K4) in the *IL-13* and *IL-4* gene loci in memory Th2 cells.

DISCUSSION

In the present study, we investigated the molecular basis for the maintenance of Th2 cytokine production in memory Th2 cells using *in vivo* generated OVA-specific memory Th2 cells. These memory Th2 cells appeared to have typical memory Th2 cell phenotypes as evidenced by the prompt proliferation upon restimulation with a low dose antigen (Fig. 1*C*) and the production of large amounts of Th2-specific cytokines (Fig. 2).

It is known that IL-4-induced STAT6 activation and the subsequent induction of GATA3 protein are essential for chromatin remodeling including histone hyperacetylation in developing Th2 cells (22). In developed Th2 cells, the production of IL-4 and IL-13 is not dependent on IL-4 (51, 52). Here, we assessed the role for IL-4 in the generation and the maintenance of memory Th2 cells, and found that IL-4 is dispensable (Fig. 4). In addition, the expression of GATA3 protein may not

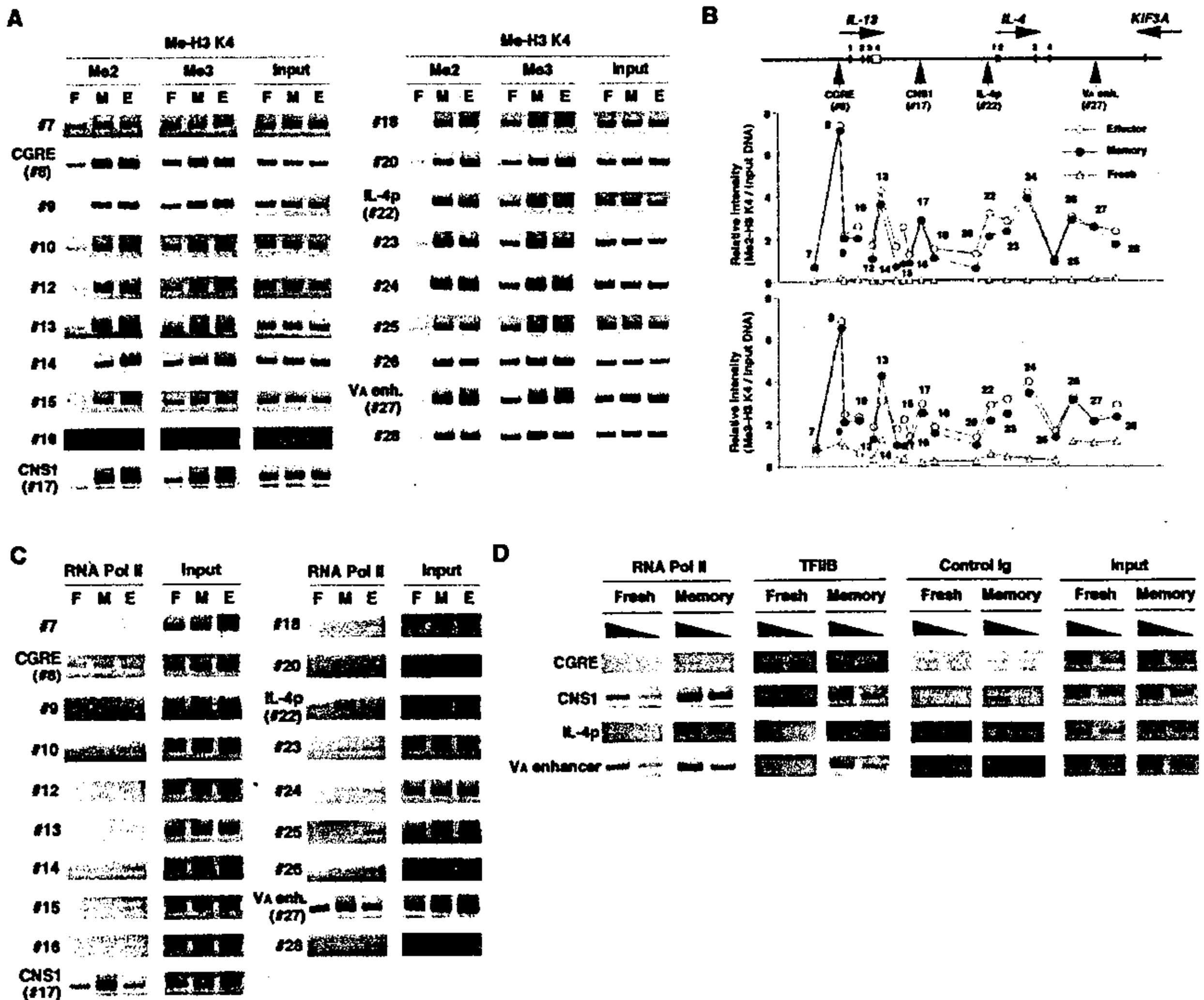

FIG. 7. **Histone methylation (H3-K4) in the long-range region of the *IL-13* and *IL-4* gene loci and accumulation of Pol II complex in memory Th2 cells.** *A* and *B*, histone H3 (*K4*) methylation of the *IL-13* and *IL-4* gene loci in memory Th2 cells. Anti-dimethyl- or anti-trimethyl-histone H3 (K4) antibodies and the indicated specific primer pairs were used. Shown are the PCR product bands for each primer pair (*A*) and Me2/Input (*B*, *upper*) and Me3/Input ratio (*B*, *lower*). Three independent experiments were done with similar results. *C*, binding of Pol II to highly restricted sites of the *IL-4* and *IL-13* gene loci in memory Th2 cells. ChIP assay was performed using anti-Pol II antisera and indicated specific primer pairs. *D*, ChIP assay was performed using anti-Pol II and anti-TFIIB antisera. PCR was performed with 3-fold serial dilution of template genomic DNA.

have an important role in the process because the protein expression level of GATA3 was marginal at best in the memory Th2 cells (Fig. 5*C*). However, our study revealed that the Th2-specific remodeled chromatin in the *IL-13* and *IL-4* gene loci was preserved in memory Th2 cells (Figs. 3 and 7, *A* and *B*). The upstream boundary of the hyperacetylation at the CGRE site and the levels of acetylation in each region assessed by a series of primer pairs were almost perfectly maintained (Fig. 3*C*). Thus, the molecular mechanisms that govern the maintenance of the remodeled chromatin in the *IL-13* and *IL-4* gene loci in memory Th2 cells appear to be distinct from those for the induction of chromatin remodeling.

As for the mechanisms responsible for the maintenance of remodeled chromatin, the transcriptional events including continuous intergenic transcription may play an important role (Fig. 6). The non-coding transcription of the *IL-13* and *IL-4* gene loci, particularly that of downstream regions of CNS1 was well preserved in memory Th2 cells, and it was insensitive to FK506 (Fig. 6*C*), suggesting that the non-coding regions are transcribed by a distinct mechanism from that for mature mRNA for IL-4 and IL-13. Interestingly, we identified highly restricted unique accumulation of Pol II complex at three intergenic regions (CNS1, IL-4p, and V_A enhancer) (Fig. 7, *C* and *D*). These are located in the region where the intergenic transcripts were perfectly preserved in memory Th2 cells (downstream of the CNS1 site), and thus this could account for the continuous generation of high level intergenic transcripts observed in the region. Similar highly restricted localization of Pol II within a locus control region was reported in the β-globin gene (53). As Pol II is known to associate with histone-modifying enzymes (26), Pol II localization within a locus control region may have also a specific role in histone modification, such as H3-K4 methylation and H3-K9/14 acetylation.

Site-specific histone methylation appears to play also an important role in transcriptional regulation (29). Methylation of H3-K4 disrupts binding of the nucleosome remodeling and deacetylase (NuRD) complex to H3 tails, thereby preventing targeted histone deacetylation catalyzed by the NuRD complex

(54, 55). The SET domain of MLL, a human homolog of *Drosophila trithorax*, is reported to be an H3-K4-specific methyltransferase, and the disruption of MLL SET domain reduced histone acetylation levels of the Hox *c8* gene locus in mouse embryo fibroblasts (50, 56). More recently, several groups have demonstrated that the yeast Set1 and Set2 H3-K4-specific methyltransferase complexes interact with Pol II (26). Thus, it is also probable that methylation of histone H3-K4 residues is important for the maintenance of the intergenic transcripts. Taken together, although we do not know the precise role of the accumulation of Pol II in a certain restricted regions at this time, intergenic transcription, methylation of histone H3-K4, and hyperacetylation of histone H3-K9/14 appear to be critical active events for maintaining the histone modification of the *IL-4* and *IL-13* gene loci in memory Th2 cells.

In contrast to the *IL-13* and *IL-4* gene loci, the level of histone hyperacetylation of the *IL-5* gene locus was dramatically decreased in memory Th2 cells as compared with those of effector Th2 cells (Fig. 3*A*). Also IL-4 dependence was observed in the histone hyperacetylation of the *IL-5* gene locus (Fig. 4*D*). Furthermore, di- and tri-methylation of H3-K4 was not observed at the *IL-5* locus in memory Th2 cells.[2] However, although the production of IL-5 after antigenic restimulation was slightly decreased in IL-4-deficient memory Th2 cells, substantial amounts of IL-5 were produced upon restimulation (Fig. 4C). The acetylation levels of histone H3 in the *IL-5* locus were rapidly increased after TCR restimulation in memory Th2 cells.[2] The kinetics of induction of histone acetylation of the *IL-5* gene locus appeared to correlate with the kinetics of the expression levels of GATA3 protein after anti-TCR stimulation (shown in Fig. 5*D*), suggesting that histone acetylation of the *IL-5* gene locus in memory Th2 cells remained highly dependent on GATA3. This suggests that the control mechanisms for the transcriptional memory of the *IL-5* gene are clearly distinct from that of the *IL-4* and *IL-13* gene loci. Similarly, hyperacetylation of the *IFNγ* promoter region was not preserved in memory Th1 cells (Fig. 3*B* and Supplemental Fig. 2). Further investigation is required to address the precise mechanisms that control the maintenance of remodeled chromatin of the *IL-5* and *IFNγ* gene loci in memory T cells.

Another unexpected but interesting result is that substantial levels of mRNA of GATA3 were detected in the freshly isolated memory Th2 cells (Fig. 5*A*). The transcription of GATA3 is maintained in the absence of IL-4 (Fig. 5*B*). These results indicated that the transcriptional induction of GATA3 in memory Th2 cells is independent on IL-4. Murphy and co-workers (57) reported that the expression of GATA3 is controlled by autoactivation. Two distinct promoters (*GATA3a* and *GATA3b*) control the expression of GATA3 (49). A newly identified promoter *GATA3a* is suggested to be responsible for GATA3-dependent *GATA3* transcription (GATA3 autoactivation). It is possible that the IL-4-independent transcription of *GATA3* in memory Th2 cells is mediated by GATA3 autoactivation. However, only trace levels of transcripts from the GATA3a were detected in memory Th2 cells (Fig. 5*A*) and the protein expression of GATA3 was marginal (Fig. 5*C*). Thus, the maintenance of GATA3 transcription in memory Th2 cells may not be explained by the action of the newly identified *GATA3a* promoter. Although the mechanism to account for the high level transcription of the *GATA3* gene in memory Th2 cells is not completely known, it appears to be clear that chromatin remodeling of the *GATA3* gene locus is induced during the Th2 cell differentiation and that it is maintained in the memory Th2 cells in an IL-4-independent manner. Furthermore, the protein expression level of GATA3 in memory Th2 cells was very low and comparable to those of naïve T cells (Fig. 5*C*), suggesting the presence of post-transcriptional regulation of GATA3 in memory Th2 cells. Following anti-TCR mAb restimulation of memory Th2 cells, the GATA3 protein expression is rapidly induced (Fig. 5*D*). This may account for the great amounts of Th2 cytokine production including IL-13 and IL-5 (Fig. 2), whose transcription is highly sensitive to GATA3 (58, 59).

Only weak association of Pol II was observed at the CGRE site, 71 bp of CGRE at 1.6-kbp upstream of the IL-13 locus exon 1 (Fig. 7*C*). We previously proposed that the CGRE plays a crucial role for GATA3-mediated targeting and downstream spreading of core histone hyperacetylation within the *IL-13* and *IL-4* gene loci in developing Th2 cells (22). The binding of Pol II to this site is dependent on GATA3 (22). Since GATA3 protein was not highly expressed in resting memory Th2 cells, Pol II may fail to associate CGRE site. However, histone H3-K4 was highly methylated at the CGRE site (Fig. 7*B*), suggesting that unique molecular events in chromatin of this particular region are taking place.

Memory CD4 T cells can be subdivided into two distinct subsets based on the expression level of CD62L (48). The $CD62L^{low}$ memory subset (effector memory) functionally resembles to effector cells that exhibit hyperresponsiveness to anti-CD3 and antigenic stimuli, high proliferative capacity, and rapid activation kinetics. The $CD62L^{high}$ memory subset (central memory) exhibits hyporeponsiveness to anti-CD3 and antigen stimuli, lower proliferative capacity, and slower activation kinetics (60). We have confirmed that the proliferative activity of the effector memory Th2 cells is higher than that of $CD62L^{high}$ central memory population.[2] We observed the $CD62L^{low}$ effector memory Th2 cells produced higher levels of IL-5 compared with $CD62L^{high}$ central memory Th2 cells in response to antigens, whereas IL-4 production was slightly lower and IL-13 production was equivalent (Supplemental Fig. 3*C*). Interestingly, the levels of histone H3-K9/14 acetylation of the *Th2* cytokine gene loci were equivalent between these two subpopulations (Supplemental Fig. 3). Although the acetylation status of histones in the *IL-13* and *IL-4* gene loci is not different, it will be of interest to explore the difference in the chromatin status of effector and central memory T cells.

In summary, memory Th2 cells maintain a unique Th2-specifc remodeled chromatin in the *IL-4* and *IL-13* gene loci, characterized by H3-K9/14 hyperacetylation and H3-K4 methylation associated with non-coding transcription and unique RNA Pol II accumulation in an IL-4-independent manner. The maintenance of the remodeled chromatin structure in the *IL-13* and *IL-4* gene loci in memory Th2 cells appears to be mediated by active molecular events that are distinct from those that operate during the induction of chromatin remodeling in developing Th2 cells.

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REFERENCES

1. Mosmann, T. R., and Coffman, R. L. (1989) *Adv. Immunol.* 46, 111–147
2. Seder, R. A., and Paul, W. E. (1994) *Annu. Rev. Immunol.* 12, 635–673
3. Reiner, S. L., and Locksley, R. M. (1995) *Annu. Rev. Immunol.* 13, 151–177
4. Abbas, A. K., Murphy, K. M., and Sher, A. (1996) *Nature* 383, 787–793
5. Constant, S. L., and Bottomly, K. (1997) *Annu. Rev. Immunol.* 15, 297–322
6. O'Garra, A. (1998) *Immunity* 8, 275–283
7. Gately, M. K., Renzetti, L. M., Magram, J., Stern, A. S., Adorini, L., Gubler, U., and Presky, D. H. (1998) *Annu. Rev. Immunol.* 16, 495–521
8. Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J., and Paul, W. E. (1999) *Annu. Rev. Immunol.* 17, 701–738
9. Murphy, K. M., Ouyang, W., Farrar, J. D., Yang, J., Ranganath, S., Asnagli, H., Afkarian, M., and Murphy, T. L. (2000) *Annu. Rev. Immunol.* 18, 451–494
10. Yamashita, M., Hashimoto, K., Kimura, M., Kubo, M., Tada, T., and

[2] T. Nakayama and M. Yamashita, unpublished observation.

Nakayama, T. (1998) *Int. Immunol.* **10**, 577–591
11. Yamashita, M., Kimura, M., Kubo, M., Shimizu, C., Tada, T., Perlmutter, R. M., and Nakayama, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 1024–1029
12. Yamashita, M., Katsumata, M., Iwashima, M., Kimura, M., Shimizu, C., Kamata, T., Shin, T., Seki, N., Suzuki, S., Taniguchi, M., and Nakayama, T. (2000) *J. Exp. Med.* **191**, 1869–1879
13. Grogan, J. L., and Locksley, R. M. (2002) *Curr. Opin. Immunol.* **14**, 366–372
14. Murphy, K. M., and Reiner, S. L. (2002) *Nat. Rev. Immunol.* **2**, 933–944
15. Zhang, D. H., Cohn, L., Ray, P., Bottomly, K., and Ray, A. (1997) *J. Biol. Chem.* **272**, 21597–21603
16. Zheng, W., and Flavell, R. A. (1997) *Cell* **89**, 587–596
17. Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D., Murphy, T. L., Sha, W. C., and Murphy, K. M. (1998) *Immunity* **9**, 745–755
18. Lee, H. J., Takemoto, N., Kurata, H., Kamogawa, Y., Miyatake, S., O'Garra, A., and Arai, N. (2000) *J. Exp. Med.* **192**, 105–115
19. Lohning, M., Richter, A., and Radbruch, A. (2002) *Adv. Immunol.* **80**, 115–181
20. Ansel, K. M., Lee, D. U., and Rao, A. (2003) *Nat. Immunol.* **4**, 616–623
21. Turner, B. M. (2002) *Cell* **111**, 285–291
22. Yamashita, M., Ukai-Tadenuma, M., Kimura, M., Omori, M., Inami, M., Taniguchi, M., and Nakayama, T. (2002) *J. Biol. Chem.* **277**, 42399–42408
23. Avni, O., Lee, D., Macian, F., Szabo, S. J., Glimcher, L. H., and Rao, A. (2002) *Nat. Immunol.* **3**, 643–651
24. Fields, P. E., Kim, S. T., and Flavell, R. A. (2002) *J. Immunol.* **169**, 647–650
25. Omori, M., Yamashita, M., Inami, M., Ukai-Tadenuma, M., Kimura, M., Nigo, Y., Hosokawa, H., Hasegawa, A., Taniguchi, M., and Nakayama, T. (2003) *Immunity* **19**, 281–294
26. Hampsey, M., and Reinberg, D. (2003) *Cell* **113**, 429–432
27. Lachner, M., and Jenuwein, T. (2002) *Curr. Opin. Cell Biol.* **14**, 286–298
28. Sims, R. J., 3rd, Nishioka, K., and Reinberg, D. (2003) *Trends Genet.* **19**, 629–639
29. Kouzarides, T. (2002) *Curr. Opin. Genet. Dev.* **12**, 198–209
30. Swain, S. L. (1994) *Immunity* **1**, 543–552
31. Sprent, J., and Surh, C. D. (2002) *Annu. Rev. Immunol.* **20**, 551–579
32. Swain, S. L., Hu, H., and Huston, G. (1999) *Science* **286**, 1381–1383
33. Tan, J. T., Ernst, B., Kieper, W. C., LeRoy, E., Sprent, J., and Surh, C. D. (2002) *J. Exp. Med.* **195**, 1523–1532
34. Polic, B., Kunkel, D., Scheffold, A., and Rajewsky, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8744–8749
35. Seddon, B., and Zamoyska, R. (2002) *J. Immunol.* **169**, 2997–3005
36. Dorfman, J. R., Stefanova, I., Yasutomo, K., and Germain, R. N. (2000) *Nat. Immunol.* **1**, 329–335
37. Lantz, O., Grandjean, I., Matzinger, P., and Di Santo, J. P. (2000) *Nat. Immunol.* **1**, 54–58
38. Jameson, S. C. (2002) *Nat. Rev. Immunol.* **2**, 547–556
39. Schluns, K. S., and Lefrancois, L. (2003) *Nat. Rev. Immunol.* **3**, 269–279
40. Kondrack, R. M., Harbertson, J., Tan, J. T., McBreen, M. E., Surh, C. D., and Bradley, L. M. (2003) *J. Exp. Med.* **198**, 1797–1806
41. Li, J., Huston, G., and Swain, S. L. (2003) *J. Exp. Med.* **198**, 1807–1815
42. Seddon, B., Tomlinson, P., and Zamoyska, R. (2003) *Nat. Immunol.* **4**, 680–686
43. Wu, C. Y., Kirman, J. R., Rotte, M. J., Davey, D. F., Perfetto, S. P., Rhee, E. G., Freidag, B. L., Hill, B. J., Douek, D. C., and Seder, R. A. (2002) *Nat. Immunol.* **3**, 852–858
44. Kopf, M., Le Gros, G., Bachmann, M., Lamers, M. C., Bluethmann, H., and Kohler, G. (1993) *Nature* **362**, 245–248
45. Murphy, K. M., Heimberger, A. B., and Loh, D. Y. (1990) *Science* **250**, 1720–1723
46. Kimura, M., Koseki, Y., Yamashita, M., Watanabe, N., Shimizu, C., Katsumoto, T., Kitamura, T., Taniguchi, M., Koseki, H., and Nakayama, T. (2001) *Immunity* **15**, 275–287
47. Rogers, P. R., Dubey, C., and Swain, S. L. (2000) *J. Immunol.* **164**, 2338–2346
48. Hengel, R. L., Thaker, V., Pavlick, M. V., Metcalf, J. A., Dennis, G., Jr., Yang, J., Lempicki, R. A., Sereti, I., and Lane, H. C. (2003) *J. Immunol.* **170**, 28–32
49. Asnagli, H., Afkarian, M., and Murphy, K. M. (2002) *J. Immunol.* **168**, 4268–4271
50. Milne, T. A., Briggs, S. D., Brock, H. W., Martin, M. E., Gibbs, D., Allis, C. D., and Hess, J. L. (2002) *Mol. Cell* **10**, 1107–1117
51. Hu-Li, J., Huang, H., Ryan, J., and Paul, W. E. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3189–3194
52. Huang, H., Hu-Li, J., Chen, H., Ben-Sasson, S. Z., and Paul, W. E. (1997) *J. Immunol.* **159**, 3731–3738
53. Johnson, K. D., Grass, J. A., Park, C., Im, H., Choi, K., and Bresnick, E. H. (2003) *Mol. Cell. Biol.* **23**, 6484–6493
54. Nishioka, K., Chuikov, S., Sarma, K., Erdjument-Bromage, H., Allis, C. D., Tempst, P., and Reinberg, D. (2002) *Genes Dev.* **16**, 479–489
55. Zegerman, P., Canas, B., Pappin, D., and Kouzarides, T. (2002) *J. Biol. Chem.* **277**, 11621–11624
56. Nakamura, T., Mori, T., Tada, S., Krajewski, W., Rozovskaia, T., Wassell, R., Dubois, G., Mazo, A., Croce, C. M., and Canaani, E. (2002) *Mol. Cell* **10**, 1119–1128
57. Ouyang, W., Lohning, M., Gao, Z., Assenmacher, M., Ranganath, S., Radbruch, A., and Murphy, K. M. (2000) *Immunity* **12**, 27–37
58. Zhang, D. H., Yang, L., and Ray, A. (1998) *J. Immunol.* **161**, 3817–3821
59. Lavenu-Bombled, C., Trainor, C. D., Makeh, I., Romeo, P. H., and Max-Audit, I. (2002) *J. Biol. Chem.* **277**, 18313–18321
60. Ahmadzadeh, M., Hussain, S. F., and Farber, D. L. (2001) *J. Immunol.* **166**, 926–935

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Treatment options for children with allergic rhinitis

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Summary
Allergen avoidance, pharmacotherapy, immunotherapy and surgical procedures are indicated for the treatment of allergic rhinitis (AR) in children. Antihistamines are the usual pharmacotherapeutic option; however, second-generation antihistamines are of limited availability in Japan. Immunotherapy is the only strategy that can offer remission or cure of paediatric AR.

Keywords Allergen avoidance, immunotherapy, laser surgery, pharmacotherapy, second-generation antihistamines, topical corticosteroids

Introduction

The principles of treatment of allergic rhinitis (AR) in children are similar to those applied in adult patients. Current practical guidelines for the treatment of AR in Japan [1] recommend allergen avoidance measures, pharmacotherapy, immunotherapy and surgical procedures. The goals of therapy, dependent on the patient's disease profile, include elimination or reduction of symptoms so as not to disturb the patient's daily life, with little need for rescue medication; control of symptoms with low frequency or short duration of exacerbations; and absence of triggering of symptoms by nasal allergen provocation.

Children with AR usually present with the following characteristics: males are more affected than females, and paediatric AR patients probably have symptoms throughout the year due to house dust mite allergen. In addition, the child's nose is often anatomically narrow and easily obstructed with congestion, leading to nasal blockage.

Treatment

A range of treatments has been developed for AR in children. Good doctor communication with patients and their guardians is essential. Elimination and avoidance of allergen, pharmacotherapy, allergen-specific immunotherapy and surgical procedures are recommended management strategies. Treatment options for allergic rhinitis are summarized below [1]:

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1 Communication with patients;
2 Elimination and avoidance of allergens:
   - mites: cleaning, dehumidification, impermeable covers, etc.
   - pollen: wearing masks, glasses, etc.
3 Pharmacotherapy:
   - antihistamines: first-generation, second-generation (topical, oral)
   - antileukotrienes, antithromboxane A_2 (oral)
   - mast cell stabilizers (topical, oral)
   - corticosteroids (topical, oral)
   - autonomotropic agents (alpha-adrenergic, anticholinergic)
4 Specific immunotherapy (conventional, rush)
5 Surgical procedures:
   - cautery: electrical, laser, chemical (trichloroacetate);
   - resection: deviatomy, conchotomy, submucosal turbinectomy; and
   - lateral posterior inferior nerve neurectomy, vidian neurectomy, etc.

Allergen avoidance

The first step towards controlling AR is to reduce or avoid exposure to allergen. The Japanese practical guidelines [1] include useful tips on how to take measures to reduce house dust mite and pet allergens in the home.

Pharmacotherapy

Medical therapy is recommended according to the severity and type of AR in Japanese patients. Table 1 shows some treatment choices for AR in adults. In moderate cases with symptoms of sneezing and runny nose, second-generation antihistamines, mast cell stabilizers and intranasal corticosteroids are recommended. Concomitant topical corticosteroids may be used with antihistamines or mast cell

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Table 1. Treatment of allergic rhinitis in adults. Medicines are stepped down when nasal symptoms are well controlled after several months of treatment (adapted with permission from Baba et al. [1])

	Severity				
	Mild	Moderate		Severe	
		Symptoms: sneezing and rhinorrhea	Symptoms: nasal obstruction	Symptoms: Sneezing and rhinorrhea	Symptoms: nasal obstruction
Treatment	1. Second-generation antihistamines	1. Second-generation antihistamines	1. Antileukotrienes	Topical steroids + second-generation antihistamines	Topical steroids + antileukotrienes or anti-TXA_2
	2. Mast cell stablilizers	2. Mast cell stabilizers	2. Anti-TXA_2		
		3. Topical steroids	3. Topical steroids		Topical decongestants for < 5–7 consecutive days as necessary
	Either 1 or 2	Either 1, 2 or 3 Combine 3 with either 1 or 2 if necessary	Either 1, 2 or 3 Combine 3 with either 1 or 2 if necessary		
			Surgical procedures		
		Specific immunotherapy			
	Elimination and avoidance of allergen				

stabilizers if necessary. In contrast, in moderate cases with nasal blockage antileukotriene or antithromboxane A_2 is recommended. Intranasal corticosteroids are the first-choice recommendation in severe cases. In addition, second-generation antihistamines are prescribed in patients with sneezing and runny nose, while antileukotriene or antithromboxane A_2 are indications for those with nasal blockage.

The anti-allergic medicines, including Th2 cytokine blocker, available in Japan are listed in Table 2. However, not all the medications available for the management of AR in adults are indicated for use in children. Among second-generation antihistamines only ketotifen and mequitazine are currently available for use in paediatric patients in Japan.

Mast cell stabilizers such as cromoglycate disodium, tranilast and pemirolast are approved for use in children in Japan. Cromolyn sodium is applied topically; tranilast and pemirolast are administered orally.

Intranasal corticosteroids are highly effective in relieving AR symptoms such as sneezing, rhinorrhoea and nasal

Table 2. Anti-allergic medicines for allergic rhinitis in Japan

	Generic name (Brand name)	Administration
Antihistamines (second-generation)	Ketotifen (Zaditen)*	Oral, topical
	Oxatomide (Celtect)	Oral
	Azelastine (Azeptin)	Oral
	Mequitazine (Zesulan, Nipolazine)*	Oral
	Emedastine difumarate (Daren, Remicut)	Oral
	Epinastine hydrochloride (Alesion)	Oral
	Ebastine (Ebastel)	Oral
	Cetirizine hydrochloride (Zyrtec)	Oral
	Levocabastine hydrochloride (Livostin)	Topical
	Bepotastine besilate (Talion)	Oral
	Fexofenadine (Allegra)	Oral
	Olopatadine (Allelock)	Oral
	Loratadine (Claritin)	Oral
Antileukotrienes	Pranlukast hydrate (Onon)	Oral
Antithromboxane A_2	Ramatroban (Baynas)	Oral
Mast cell stablilizers	Sodium cromoglycate (Intal)*	Topical
	Tranilast (Rizaben)*	Oral
	Amlexanox (Solfa)	Oral, topical
	Pemirolast potassium (Alegysal, Pemilaston)*	Oral
Th2 cytokine inhibitors	Suplatast tosilate (IPD)	Oral

*Available for children.

blockage. In Japan, beclomethasone dipropionate (for children aged ≥ 6 years) and fluticasone propionate (≥ 5 years) are currently available.

In children with AR complicated with asthma and in moderate or severe cases of adult AR of nasal blockage type, antileukotriene or antithromboxane A_2 are recommended.

Immunotherapy

Double-blind placebo-controlled studies have suggested that immunotherapy may be efficacious against allergies towards bee venom, pollens, mites, cat dander, and molds [2]. Ohashi et al. [3] reported that 5–10 years' immunotherapy in children may lead to suppression of interleukin (IL)-4 and specific IgE antibodies. Conventional immunotherapy requires frequent injections up to maintenance doses, with some minor reported risk of anaphylactic reactions. However, this therapy is currently the only means offering long-term remission or cure of AR.

Surgery

Surgical procedures in the AR setting are recommended only in certain patients such as those with severe nasal obstruction due to inferior turbinate hypertrophy and/or nasal septum deviation. However, such procedures are very limited in children for anatomical reasons. Of the available methods, laser surgery is easily conducted in children. Kubo has shown good relative effectiveness of laser surgery in children aged < 12 years compared with adults in terms of better improvement of nasal symptoms after 2 years of surgery (N. Kubo, personal communication 2003). However, the precise mechanism for this is not clear.

Conclusions

The first step in controlling nasal symptoms is to eliminate or avoid environmental allergens. Pharmacotherapy should be based on the severity and type of AR. Antihistamines control sneezing and rhinorrhoea effectively but are less effective against nasal blockage. However, these medications currently are of only limited availability for children in Japan. Topical corticosteroids are highly effective in controlling most nasal symptoms and are available for use in children aged ≥ 5 years. Immunotherapy is the only measure that currently might offer remission or cure of AR and thus should be considered in appropriate cases. Further research aimed at minimizing the risk of systemic adverse reactions to conventional immunotherapy will help popularize the use of this option.

References

1 Baba K, Konno A, Takenaka H., eds. Practical Guideline for the Treatment of Allergic Rhinitis – Perennial Rhinitis and Pollinosis, 4th edn. Tokyo: Life Sciences, 2003

2 Bousquet J, Lockey R, Malling HJ et al. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol 1998; **102**:558–62.

3 Ohashi Y, Nakai Y, Tanaka A et al. Serological study of the working mechanisms of immunotherapy for children with perennial allergic rhinitis. Arch Otolaryngol Head Neck Surg 1998; **124**: 1337–46.

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Influence of viral infection on the development of nasal hypersensitivity

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Summary

Background The underlying relationship between viral infections and allergic diseases of the upper respiratory tract has not been well clarified.

Methods In order to clarify the relationship between viral infection and nasal hypersensitivity, mice were sensitized with ovalbumin (OVA) and then infected intranasally with respiratory syncytial virus (RSV), after which their nasal sensitivity to histamine or antigen was examined.

Results Non-sensitized mice showed transient mild nasal hypersensitivity following nasal administration of histamine after intranasal RSV inoculation. In mice sensitized with OVA, RSV infection significantly exaggerated their nasal hypersensitivity to histamine and OVA. Treatment of these mice with a neurokinin (NK)-1/NK-2 receptor antagonist, but not with anti-IL-5 antibodies, reduced their hypersensitivity. The infiltration of nasal mucosa with eosinophils was temporarily associated with accelerated rate of RSV elimination in these animals.

Conclusion RSV infection induced transient nasal hypersensitivity. Several mechanisms, including impairment of nasal epithelial cells are thought to mediate this effect. In allergen-sensitized mice, RSV inoculation strongly enhanced nasal hypersensitivity.

Keywords histamine, nasal hypersensitivity, RSV

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Q1

Introduction

Recent epidemiological evidence has suggested that acute respiratory viral infections exacerbate the symptoms of pre-existing reactive airway diseases and is the most important trigger of acute asthmatic attacks [1–4]. Viruses, rather than bacteria, cause most acute respiratory tract infections, and asthma attacks in children are often preceded by viral infection [5–7].

The nasal cavity is often the first target of invading viruses, because it is the point of entry into the respiratory tract. The common cold is the most widespread viral infectious condition and is usually caused by viruses such as rhinoviruses, parainfluenza viruses, influenza viruses, adenoviruses and respiratory syncytial virus (RSV) [8, 9]. However, the relationship between viral infections and allergic diseases in the upper respiratory tract has not been well defined. The results from studies that have examined the influence of atopy on the development of the symptom after viral infections are controversial [10–13]. Bardin et al. [11] observed more severe cold symptoms in atopic subjects than in non-atopic subjects after experimental rhinovirus infection. However, in another study, augmented nasal allergic inflammation induced by antigen provocation before viral inoculation did not result in a worsening of cold symptoms [12]. The effects of the common cold on nasal hypersensitivity or allergic rhinitis have not been clearly established.

Nasal responses to viral infection are thought to differ depending on the viral species. Although rhinoviruses causes little damage to epithelial cells in the respiratory tract, RSV induces marked cytopathic effects [13]. RSV is an RNA virus infection which usually results in common cold symptoms, although progression to lower respiratory tract symptoms, the most common being bronchiolitis, frequently occurs in infants. RSV causes about 60% of the bronchitis cases in children [14, 15]. In prospective studies, as many as 75–90% of infants with a clinical diagnosis of bronchiolitis subsequently developed recurrent episodes of wheezing suggestive of childhood asthma and experienced airway histamine or methacholine hypersensitivity which persisted for several years [16–22].

In the present study, we have shown that RSV infection contributes to the exacerbation of nasal hypersensitivity in an allergic rhinitis mouse model.

Materials and Methods

Animals

Eight-week-old male C57BL/6 mice (Nippon Clea, Shizuoka, Japan) that were raised on ovalbumin (OVA)-free chow were

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used in this study. Hartley strain guinea-pigs (Nippon Clea) were also used to measure passive cutaneous anaphylaxis (PCA). The use of these laboratory animals was approved by the local Animal Ethics Committee (Yamanashi Medical University) and the experiments were conducted in conformity with the guidelines of the committee.

Experimental infection with respiratory syncytial virus

The long strain of RSV (prototype RSV group A strain) was grown in HEp-2 cells in minimal essential medium (MEM) supplemented with 2% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics. RSV was partially purified by polyethylene glycol precipitation, followed by centrifugation in a 35–65% discontinuous sucrose gradient, as described elsewhere [23]. RSV (1×10^6 plaque-forming units (PFU)) in a volume of 20 μL was administered intranasally to mice. Uninfected Hep-2 cells were processed similarly and used as controls.

Virus assay

Lungs and nasal tissues were collected and homogenized in MEM containing 2% FCS and were stored at −70 °C until they were assayed. RSV was assayed by the plaque method using HEp-2 cells in 24-well microplates. The overlay for the plaque assay consisted of MEM supplemented with 2% FCS, antibiotics and 1% methylcellulose. Plates were incubated for 7 days at 37 °C. After the methylcellulose was removed, the plaques were fixed with 10% formaldehyde and stained with 0.1% crystal violet.

Evaluation of sensitivity to histamine in nasal mucosa

One microlitre of various concentrations of histamine, diluted in phosphate-buffered saline (PBS), was administered into each nostril of the experimental mice. The number of nasal rubbing attacks that occurred during the ensuing 10 min was then counted.

Experimental protocol for sensitization with ovalbumin

Mice were immunized with 10 μg OVA (grade V, Sigma Chemical Co., St Louis, MO, USA) intraperitoneally with alum once a week for 4 weeks. Heat-killed bordetella pertussis (1×10^8 bacterial units) was used as an adjuvant in the first immunization. Five days after the last immunization, the mice were either inoculated with RSV or sham-infected with sonicated non-RSV-infected HEp-2 cells. Two micrograms OVA in 2 μL PBS was administered intranasally for 5 consecutive days after the inoculation. Sensitized mice were divided into the following experimental groups and treated as follows. Group 1 consisted of 30 mice treated with a neutralizing IL-5 antibody or a neurokinin (NK)-1/NK-2 antagonist. A rat neutralizing monoclonal antibody (mAb) directed against mouse IL-5 (PharMingen, San Diego, CA, USA) and a control isotype mouse IgGI mAb (PharMingen) were used. Antibodies were injected intraperitoneally twice a week at a dose of 0.1 mg for 1 week before RSV inoculation, and were administered intranasally for 5 consecutive days after inoculation. Group 2 consisted of 10 OVA-sensitized mice who received 0.04 μg of the NK-1/NK-2 antagonist [24] FK224 (Fujisawa Co Ltd, Japan) intranasally for 5 consecutive days after RSV inoculation. On the day following the last nasal administration of OVA, the nasal rubbing attacks were counted for 10 min. The sensitivity of the mice to histamine was examined 24 h later in a similar manner.

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Treatment of ovalbumin-sensitized mice with a neutralizing anti-interferon-γ monoclonal antibody or with interferon-γ

OVA-sensitized mice received 0.1 mg of anti-IFN-γ neutralizing mAb (PharMingen) or control mAb intraperitoneally twice a week and then intranasally for 5 consecutive days before nasal provocation with OVA. Other OVA-sensitized mice were administered 1 μg of IFN-γ (PharMingen) intranasally for 5 consecutive days before provocation with OVA.

Detection of ovalbumin-specific immunoglobulin E antibody

OVA-specific IgE antibodies were detected by PCA [25]. Briefly, 100 μL of undiluted and twofold diluted serum samples were injected intradermally into the dorsal skin of shaved guinea-pigs. Three days later, the animals were challenged intravenously with 1 mg OVA together with 1% Evans blue. A blue lesion of a diameter greater than 5 mm, as determined 30 min after the challenge, was considered to be positive. PCA titres were expressed as the reciprocal of the highest dilution giving a positive reaction.

Histological examination

On the 4th day after RSV inoculation the mice were killed by CO_2 overdose. The heads of the mice were detached along the line between the upper and lower jaws, and they were then fixed in formalin and decalcified. The section of the nasal cavity anterior to the eyeball was examined and processed for paraffin sectioning. Tissue sections were stained with PAS and the number of infiltrating eosinophils in the whole nasal septum mucosa of each section was determined.

Fluorescence-activated cell sorting analysis

Nasal mucosal tissue from the above mice was cut into small pieces, which were then teased gently through a nylon mesh using frost glass slides. The disrupted mucosa was then suspended in RPMI-1640 containing 10% FCS, penicillin (100 units/mL) and streptomycin (100 μg/mL). After washing twice with medium, $CD3^+$ T cells were purified in 0.2 mL of RPMI-1640 using magnetic beads (Dynal, Great Neck, NY, USA). Following purification, the medium was supplemented with 10% FCS. 10^6 nasal $CD3^+$ T cells collected from seven RSV-infected OVA-sensitized mice or from non-infected OVA-sensitized mice were stained with fluorescein-conjugated anti-CD4 antibody (PharMingen) and fixed overnight with 4% paraformaldehyde (Sigma Chemical Co). The fixed cells were permeabilized by incubation in PBS with 1% bovine serum albumin and 2% saponin (Sigma Chemical Co) for 10 min. A phycoerythrin-conjugated anti-IFN-γ antibody (PharMingen) or an anti-IL-5 antibody (PharMingen), diluted to 20 μg/mL in PBS, was then added. After a 30 min incubation, the cells were washed with PBS and were

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analyzed using a FACScan (Becton Dickinson, Fullerton, CA, USA).

Statistical analysis

Comparisons between groups were evaluated using Student's *t* test and Wilcoxon's test.

Results

Viral replication and nasal histamine sensitivity

After nasal inoculation with 10^6 PFU of RSV, mild replication of RSV in the respiratory tract was observed with peak levels occurring in the lung on day 4 and the levels then declined until day 7 as shown previously [26]. RSV was recovered from the nasal mucosa for 12 days after inoculation.

Non-specific stimulation of the nasal mucosa of mice also resulted in nasal rubbings. The number of nasal rubbing attacks observed in 20 normal mice following nasal installation of 2 μL PBS was 9.4 ± 2.9 (mean ± SD). Thus, the lowest histamine concentration administered intranasally in a volume of 2 μL that was needed to induce more than 20 nasal rubbing attacks was defined as the threshold level of nasal histamine hypersensitivity. After RSV inoculation, the threshold decreased and reached its lowest on day 4. It returned to normal by day 14 (Fig. 1(a)).

Influence of respiratory syncytial virus infection on ovalbumin-sensitized mice

The threshold of nasal hypersensitivity to histamine decreased in OVA-sensitized mice and RSV infection in OVA-sensitized mice induced a dramatic enhancement of nasal sensitivity to histamine (Fig. 1(b)). The threshold of nasal hypersensitivity to histamine observed in RSV-infected mice increased gradually after the last nasal administration of OVA and 14 days later, it was the same as that of non-infected mice (data not shown). Fluorescence-activated cell sorting analysis of nasal mucosal T lymphocytes in the RSV-infected OVA-sensitized mice not only revealed an increased expression of IFN-γ, but also of IL-5 (Table 1). Anti-IL-5 treatment of RSV-infected OVA-sensitized mice using neutralizing antibodies reduced the histamine sensitivity in some degree ($P<0.05$) and the treatment with an NK-1/NK-2 antagonist resulted in a marked reduction ($P<0.001$) of the sensitivity (Fig. 1(b)).

After OVA nasal provocation the frequency of nasal rubbing attacks dramatically increased in RSV-infected OVA-sensitized mice, compared with non-infected sensitized mice (Fig. 2). However, anti-IL-5 treatment of RSV-infected OVA-sensitized mice did not significantly improve nasal symptoms after OVA administration. On the other hand, an NK-1/NK-2 antagonist resulted in a significant improvement (Fig. 2).

The number of eosinophils in the nasal mucosa was markedly increased in RSV-infected OVA-sensitized mice compared with those in non-infected OVA-sensitized mice (Fig. 3). The PCA titre, on the other hand, was not significantly different between the two groups (mean ± SD; 21.1 ± 21.0 in infected sensitized mice, 16.6 ± 11.4 in non-infected sensitized mice). Anti-IL-5 treatment of RSV-infected OVA-sensitized mice significantly reduced the number of infiltrated eosinophils, however, the treatment with an NK-1/NK-2 antagonist had no effect on eosinophil infiltration.

The nasal administration of IFN-γ to OVA-sensitized mice increased the number of nasal eosinophils, but had no effect on nasal symptoms (Fig. 4). Treatment with anti-IFN-γ neutralizing antibodies did not affect nasal symptoms or eosinophil infiltration (Fig. 4).

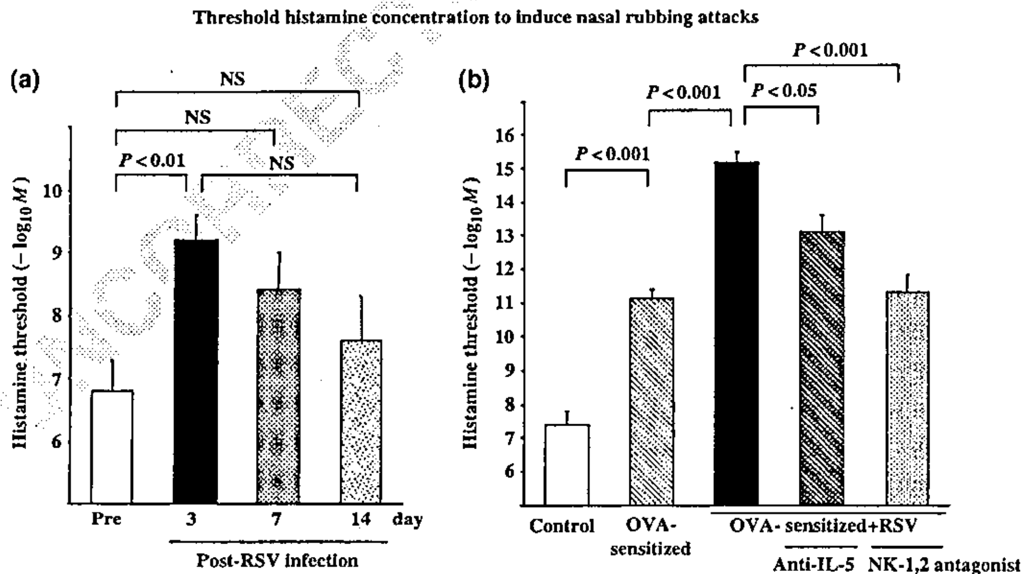

Fig. 1. Threshold histamine concentration needed to induce nasal rubbing attacks in respiratory syncytial virus (RSV)-infected non-sensitized mice (a) and in ovalbumin (OVA)-sensitized mice (b). After RSV inoculation, the threshold decreased transiently and reached its lowest on day 4. Although the threshold decreased in OVA-sensitized mice, RSV infection in OVA-sensitized mice induced a dramatic reduction of the threshold. The treatment with neurokinin (NK)-1/NK-2 receptor antagonist but not with anti-IL-5 neutralizing antibodies improved the reduction. Non-OVA-sensitized mice were used as controls.

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Table 1. IL-5 and IFN-γ expression of nasal mucosal T lymphocytes from OVA-sensitized mice*

	RSV-infected mice (%)	Sham-infected mice (%)
IL-5	11.9	6.2
IFN-γ	17.4	11.4

OVA, ovalbumin; RSV, respiratory syncytial virus.
*Mean of two groups and each group consisted of T lymphocytes collected from nasal mucosa of seven mice.

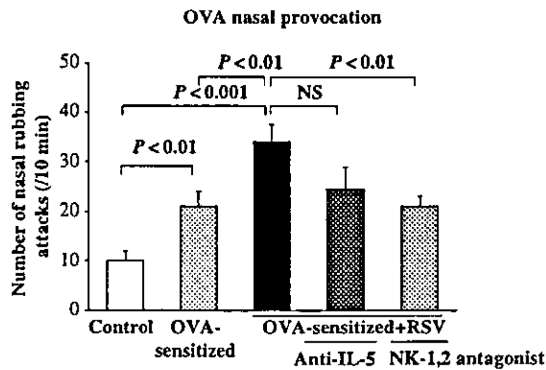

Fig. 2. The number of nasal rubbing attacks in ovalbumin (OVA)-sensitized mice following OVA provocation. Respiratory syncytial virus (RSV) infection in OVA-sensitized mice induced a dramatic enhancement of number of attacks. The anti-IL-5 treatment reduced the enhancement in some degree and the topical administration of the neurokinin-1/NK-2 receptor antagonist did more.

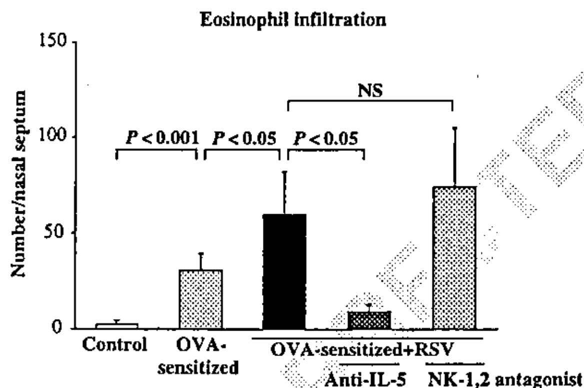

Fig. 3. The number of eosinophils in the nasal mucosa. Respiratory syncytial virus (RSV) infection markedly increased the eosinophil infiltration in ovalbumin (OVA)-sensitized mice. The treatment with anti-IL-5 antibodies reduced the number significantly but not with neurokinin-1/NK-2 receptor antagonists.

RSV replication on day 4 was significantly reduced in OVA-sensitized mice. However, the use of anti-IL-5 did not exhibit any influence on viral replication and no reduction in viral shedding was observed in anti-IL-5-treated OVA-sensitized mice (Fig. 5).

Discussion

The above studies were designed to examine the mechanism of nasal hypersensitivity observed during viral infections. A murine RSV infection model was used in which the quantitative analysis of nasal rubbing attacks was evaluated as a measure of nasal hypersensitivity. Sneezes in mice are not clearly distinguishable as in humans and are difficult to quantify precisely. The evaluation of nasal obstruction is also difficult, because mice cannot survive by breathing orally. BALB/c mice are known to be sensitive to allergic reactions [27], particularly in the lower respiratory tract, although their nasal reactivity to histamine and other antigens is quite low (data not shown). While C57BL/6 mice are known to mount a Th1 dominant immune response [28], IgE production is inducible in these animals if the correct adjuvant, such as alum, is used, and nasal hypersensitivity can be observed after the topical administration of histamine or antigens. In light of the above and because RSV replication in the nose of BALB/c mice is tolerated well by these animals, we chose to use C57BL/6 mice in our study.

The observations summarized in this report suggest that experimentally induced infection with RSV results in significant enhancement of nasal sensitivity to OVA and histamine in previously sensitized animals. OVA-sensitized animals also exhibited increased expression of IL-5 and IFN-γ and pronounced accumulation of eosinophils in the nasal mucosa after RSV infection.

The mechanisms underlying the development of hypersensitivity states after viral infections such as RSV have not been clinically defined. It is possible that viral infection-associated mucosal damage; recruitment of mast cells, eosinophils and other cellular mediators of hypersensitivity; and activation of cholenergic, adrenergic or non-adrenergic non-cholinergic neurogenic mechanisms may play an important role in the development of mucosal hypersensitivity states [29–31].

In the present studies, pre-treatment with anti-IL-5 resulted in significant decrease in the accumulation of eosinophils. However, such treatment did not influence the degree of viral induced hypersensitivity. In fact, anti-IL-5 treatment was associated with decreased viral elimination in the nasal cavity, and as a result eosinophils may be associated with accelerated RSV elimination. It has been shown that eosinophil cationic protein and eosinophil-derived neurotoxin may act as rebonuclease-dependent antiviral agents [32]. In the present studies, it is interesting to note that use of IFN-γ was associated with increasing eosinophil counts but did not influence nasal hypersensitivity reactions. Thus, although eosinophils may play an important role in viral induced allergic inflammation [33, 34], eosinophils did not seem to contribute to nasal hypersensitivity to OVA in the current experimental setting. IFN-γ is a classical Th1 cytokine that has been shown to reduce allergic reactions when administered during sensitization [35]. However, treatment of OVA-sensitized animals with anti-IFN-γ neutralizing antibody did not decrease nasal sensitivity to OVA during RSV infection.

The observation of particular interest in the current studies is the significant reduction of nasal hypersensitivity detected after the use of NK-1/NK-2 antagonists, although such treatment did not influence eosinophil counts. Recently, it has been shown that infection with RSV frequently is associated with activation of NK receptor sites [36–38]. Tachykinin family of neuropeptides such as substance P have been shown to exhibit strong blinding affinity for NK receptors especially NK-1. Such receptor-neuropeptide interactions are associated

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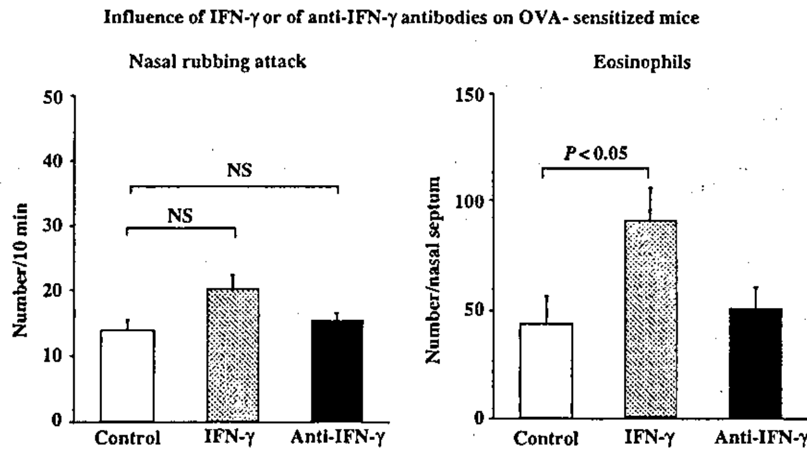

Fig. 4. Influence of IFN-γ and anti-IFN-γ antibodies on ovalbumin (OVA)-sensitized mice. The nasal administration of IFN-γ increased the number of eosinophils, but did not affect the nasal symptoms. Anti-IFN-γ treatment had no effect on either nasal symptoms or eosinophil numbers.

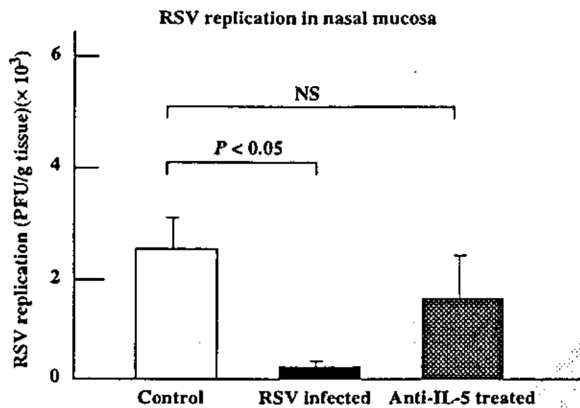

Fig. 5. Respiratory syncytial virus (RSV) replication in the nasal mucosa on day 4 after RSV inoculation. Replication was reduced in ovalbumin (OVA)-sensitized mice, but this reduction was abolished in anti-IL-5-treated OVA-sensitized mice. Non-OVA-sensitized mice were used as controls.

with a wide variety of biologic inflammatory effects, including changes in vascular permeability, mucous secretion, leucocyte chemotaxis and bronchoconstriction [39–41]. It is thus suggested that RSV-associated increase in allergic nasal hypersensitivity to OVA and possibly to other allergens may in part be related to activation of neuropeptide receptors during acute viral infection of the nasal mucosa.

It is possible that increased eosinophil recruitment is mediated by chemokines induced by IFN-γ. Recently induction of eotaxin 3 and IP-10 by IFN-γ in mucosal cell cultures has been demonstrated after experimental RSV infection in *in vivo* settings [42–44]. Based on these reports and the present studies, it is proposed that a possible relationship exists between IFN-γ and induction, recruitment and/or activation of eosinophils in allergic sensitization in the nasal mucosa during viral infections.

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References

1 Green RM, Custovic A, Sanderson G, Hunter J, Johnston SL, Woodcock A. Synergism between allergens and viruses and risk of hospital admission with asthma: case-control study. BMJ 2002; 324:1–5.

2 Message SD, Johnston SL. Viruses in asthma. Br Med Bull 2002; 61:29–43.

3 Gern JE. Viral and bacterial infections in the development and progression of asthma. J Allergy Clin Immunol 2000; 105: s497–502.

4 Johnston SL, Plattemore PK, Sanderson G et al. The relationship between upper respiratory infections and hospital admissions for asthma: a time-trend analysis. Am J Respir Crit Care Med 1996; 154:654–60.

5 Tuffaha A, Gern JE, Lemanske Jr RF. The role of respiratory viruses in acute and chronic asthma. Clin Infect Chest Med 2000; 21:289–300.

6 Schwarze J, Gelfand EW. The role of viruses in development or exacerbation of atopic asthma. Clin Infect Chest Med 2000; 21:279–87.

7 Johnston SL, Pattemore PK, Sanderson G et al. Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. BMJ 1995; 310:1225–9.

8 Makela MJ, Puhakka T, Ruuskanen O et al. Viruses and bacteria in the ethiology of the common cold. J Clin Microbiol 1998; 36:539–42.

9 Irwin RS, Madison JM. The diagnosis and treatment of cough. N Engl J Med 2000; 343:1715–21.

10 Doyle WJ, Skoner DP, Fireman P et al. Rhinovirus 39 infection in allergic and non-allergic subjects. J Allergy Clin Immu[illegible] 89:968–78.

11 Bardin PG, Fraenkel DJ, Sanderson G et al. Amplified rhinovirus colds in atopic subjects. Clin Exp Allergy 1994; 24:457–64.

12 Avila PC, Abisheganaden JA, Wong H et al. Effects of allergic inflammation of the nasal mucosa on the severity rhinovirus 16 cold. J Allergy Clin Immunol 2000; 105:923–32.

13 Folkerts G, Busse WW, Nikamp FP, Sorkness R, Gern JE. Virus-induced airway hyperresponsiveness and asthma. Am J Respir Crit Care Med 1998; 157:1708–720.

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14 Van Schaik SM, Welliver RC, Kimpen JK. Novel pathways in the pathogenesis of respiratory syncytial virus disease. Pediatr Pulmonol 2000; 30:131–8.
15 Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980–1999. JAMA 1999; 282:1440–6.
16 Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. Am J Respir Crit Care Med 2000; 161:1501–7.
17 Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B, Bjorksten B. Asthma and immunoglobulin E antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. Pediatrics 1995; 95:500–5.
18 Hall CB, Hall WJ, Gala CL, MaGill FB, Leddy JP. Long-term prospective study in childrem after respiratory syncytial virus infection. J Pediatr 1984; 105:358–64.
19 Eisen AH, Bacal HL. The relationship of acute bronchiolitis to bronchial asthma-a 4 to 14-year follow up. Pediatrics 1963; 31: 859–61.
20 Sims DG, Downham MAPS, Gardner PS, Webb JKG, Weighman D. Study of 8-year-old children with a history of respiratory syncytial virus bronchiolitis in infancy. BMJ 1978; 1:11–4.
21 Welliver RC, Duffy L. The relationship of RSV-specific immunoglobulin E antibody responses in infancy, recurrent wheezing, and pulmonary function at age 7–8 years. Pediatr Pulmonol 1993; 15:19–27.
22 Pullan CR, Hey EM. Wheezing, asthma, and pulmonary dysfunction 10 years after infection with respiratory syncytial virus in infancy. Br Med J 1982; 284:1665–9.
23 Ueba O. Respiratory syncytial virus 1 concentration and purification of the infectious virus. Acta Med Okayama 1978; 32:265–72.
24 Morimoto H, Murai M, Maeda Y et al. FK224, a novel cyclopeptide substance P antagonist with NK1 and NK2 receptor selectivity. J Pharmacol Exp Ther 1992; 262:398–402.
25 Mota I, Wong D. Homologous and heterologous passive cutaneous anaphylactic activity of mouse antisea during the course of immunization. Life Sci 1969; 8:813–20.
26 Matsuoka T, Okamoto Y, Matsuzaki Z et al. Characteristics of immunity induced by viral antigen or conferred by antibody via different administration routes. Clin Exp Immunol 2002; 130: 386–92.
27 Sato M, Iwakabe K, Ohta A, Sekimoto M, Kimura S, Nishimura T. Self-priming cell culture system for monitoring genetically controlled spontaneous cytokine-producing ability in mice. Immunol Lett 1999; 70:173–8.
28 Shankar AH, Titus RG. T cell and non-T cell compartments can independently determine resistance to Leishmania major. J Exp Med 1995; 181:845–55.
29 Fryer AD, Jacoby DB. Parainfluenza virus infection damages inhibitory M_2 muscarinic receptors on pulmonary parasympathetic nerves in the guinea-pig. Br J Pharmacol 1991; 102:267–71.
30 Jacoby DB, Fryer AD. Interaction of viral infections with muscarinic receptors. Clin Exp Allergy 1999; 29: 59–64.
31 Adamko DJ, Yost BL, Gleich GJ, Fryer AD, Jacoby DB. Ovalbumin sensitization changes the inflammatory responses to subsequent parainfluenza infection: eosinophils mediate airway hyperresponsiveness, M_2 muscarinic receptor dysfunction, and antiviral effects. J Exp Med 1999; 190:1465–77.
32 Domachowske JB, Dyer KD, Adams AG, Leto TL, Rosenberg HF. Eosinophil cationic protein1 R Nase 3 is anather RNase A-family ribonuclease with direct antivial activity. Nucleic Acids Res 1998; 26:5327–32.
33 Kay AB. Modulation of eosinophil function in vitro. Clin Exp Allergy 1990; 20:31–4.
34 Terada N, Konno A, Tada H, Shirotori K, Ishikawa K, Togawa K. The effect of recombinant human interleukin-5 on eosinophil accumulation and degranulation in human nasal mucosa. J Allergy Clin Immunol 1992; 90:160–8.
35 Young HA, Hardy KJ. Role of interferon-gamma in immune cell regulation. J Leukocyte Biol 1995; 58:373–81.
36 Piedimonte G, Rodriguez MM, King KA, McLean S, Jiang X. Respiratory syncytial virus upregulates expression of the substance P receptor in rat lung. Am J Physiol 1999; 277:L831–40.
37 King KA, Hu C, Rodriguez MM, Jiang X, Piedimonte G. Exaggerated neurogenic inflammation and substance P receptor upregulation in RSV-infected weanling rate. Am J Respir Cell Mol Biol 2001; 24:101–7.
38 Tripp RA, Barskey A, Goss L, Anderson LJ. Substance P receptor expression on lymphocytes is associated with the immune response to respiratory syncytial virus infection. J Neuroimmunol 2002; 129:141–53.
39 Konno A, Numata T, Terada N, Hanazawa T, Nagata H, Motosugi H. Role of substance P in the vascular response of nasal mucosa in nasal allergy. Ann Otol Rhinol Laryngol 1996; 105:648–53.
40 Gungor A, Baroody FM, Naclerio RM, White SR, Corey JP. Decreased neuropetide release may play a role in the pathogenesis of nasal polyps. Otolaryngol Head Neck Surg 1999; 121: 585–90.
41 Okamoto Y, Shirotori K, Kudo K et al. Cytokine expression after the topical administration of substance P to human nasal mucosa. J Immunol 1993; 151:4391–8.
42 Yamamoto S, Kobayashi I, Tsuji K et al. Upregulation of IL-4R by IFN-γ: enhanced IL-4 induced eotaxin-3 production in airway epithelium. Am J Respir Cell Mol Biol 2004.
43 Azuma MA, Szczepanik M, Tsuji RF et al. Early delaved-type hypersensitivity eosinophil infiltrares depend on T helper 2 cytokines and interferon-γ via CXCR3 chemokines. Immunology 2004; 111:306–17.
44 Li H, Chunsong H, Guobin C et al. Highly up-regulated CXCR2 expression on eosinophils in mice infected with *Schistosoma japonicum*. Immunology 2004; 111:107–17.

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