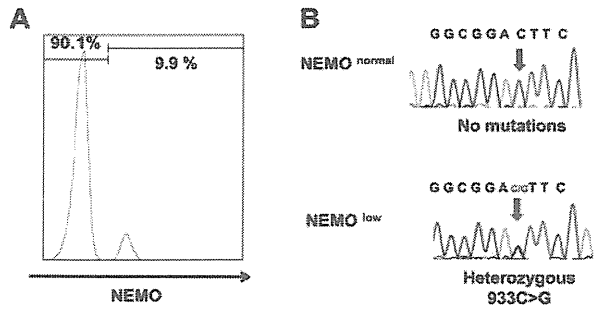

Figure 3. *NEMO* revertant T cells in patient 3. (A) Intracellular expression of NEMO in $CD8^-$ cells from patient 3. (B) Sequencing chromatograms of DNA from $NEMO^{normal}$ or $NEMO^{low}$ $CD8^+$ cells of patient 3. Arrows indicate the mutated base position at c. 931.

Similarly, there was an apparent high proportion of wild-type *NEMO* cDNA in monocytes and B cells from the mothers of patients 1/2, 8, and 10 (Table 5). These findings suggested a general selective advantage of $NEMO^{normal}$ cells over $NEMO^{low}$ cells in vivo, especially in T cells.

Proliferation capacity of $NEMO^{normal}$ and $NEMO^{low}$ T cells

T-cell proliferation stimulated by mitogens such as PHA is usually not reduced in XL-EDA-ID patients. However, the emergence of $NEMO^{normal}$ cells coincided with a reduction in mitogen-induced proliferation in patient 2. To further determine the effect of $NEMO^{normal}$ cells on mitogen-induced proliferation of peripheral T cells, the proportions of T cells carrying the wild-type and mutant were examined before and after PHA stimulation in XL-EDA-ID patients and their mothers (Table 6). In patients 2, 4, and 8, the percentage of the $NEMO^{normal}$ cells decreased after PHA stimulation, while $NEMO^{normal}$ cells prevailed in patient 9. In the mothers of patient 4 and 10, the percentage of $NEMO^{normal}$ cells increased after PHA stimulation, while the percentage of the $NEMO^{normal}$ cells decreased in the mother of patient 3. These results indicated that the NEMO mutation does not directly affect the mitogen-induced proliferation capacity of T cells and factors other than the *NEMO* genotype determine the proliferation capacity of $NEMO^{normal}$ and $NEMO^{low}$ T cells.

Discussion

Somatic reversion mosaicism has been described in several disorders affecting the hematopoietic system, the liver, and the skin.[23,26] Reports of somatic reversion cases have been particularly abundant in patients with immunodeficiency diseases, including Wiskott-Aldrich syndrome (WAS)[27] and SCID, which occur because of mutations in the interleukin receptor common γ chain,[28] CD3ζ,[29] *RAG-1*[30], and *ADA* genes.[31] Patients with somatic reversion mosaicism may present with significantly milder clinical phenotypes compared with nonrevertant patients with the same germline mutation, although this is not always the case.[26] One common feature in most cases where the somatic reversion mosaicism has been observed is a strong in vivo selective advantage of the revertant cells that express the wild-type gene product. One of the most intensively investigated diseases associated with somatic reversion mosaicism is WAS.[32-34] A report showed that up to 11% of WAS patients have presented with somatic reversion mosaicism.[33]

In our investigation, 9 of 10 XL-EDA-ID patients presented with somatic mosaicism. Two of the 9 were cases of reversion from a duplication mutation, while the others exhibited true back-reversion from a substitution or insertion mutation. This finding calls for caution when diagnosing XL-EDA-ID patients. Because the existence of a *NEMO* pseudogene makes it difficult to perform genetic analysis using genomic DNA, diagnosis of the disease is often confirmed by sequencing analysis of *NEMO* cDNA, and the presence of somatic mosaicism can cause misdiagnosis of XL-EDA-ID patients either when $NEMO^{normal}$ cells make up the majority of the patients' PBMCs or when the cDNA of the mutated *NEMO* gene cannot be amplified by PCR.[17] In fact, mutated *NEMO* cDNA could not be amplified from the PBMCs of patient 2 even when $NEMO^{normal}$ cells were absent (during early infancy), and only wild-type *NEMO* cDNA was amplified after the appearance of $NEMO^{normal}$ cells (data not shown), probably because of the instability of the mutated *NEMO* mRNA. Flow cytometric analysis of intracellular NEMO protein is of help in identifying the $NEMO^{low}$ cells in some patients, but the technique is not applicable when the *NEMO* mutation does not cause reduced expression of NEMO protein. Thus, some cases of XL-EDA-ID patients with reversion may be difficult to diagnose.

The high frequency of somatic mosaicism observed in XL-EDA-ID patients indicates a strong in vivo selective advantage for $NEMO^{normal}$ cells, which express the wild-type gene product. Patient 2 presented with a high mutant T-cell count at birth that gradually decreased over time (Figure 1B). This finding indicates that wild-type NEMO expression is critical for the survival of certain cell lineages, including T cells, after birth. On the other hand, no $NEMO^{normal}$ monocytes and very few $NEMO^{normal}$ B cells were detected in the recruited XL-EDA-ID patients (Table 4). This specific feature is similar to other somatic reversion mosaicism seen in primary immunodeficiency patients[26] and indicates that the expression of NEMO is less critical for the survival of monocytes or B cells compared with that of T cells. There is also an apparent

Table 4. Analysis of *NEMO* gene mosaicism in various cell lineages for each patient

Patient	Mutation	Age at analysis	CD4, % (proportion)	CD8, % (proportion)	CD14, % (proportion)	CD19, % (proportion)
1	Duplication	2 y	90	100	0	4.0
2	Duplication	15 mo	45	66	0	4.0
3	D311E	3 y	2.4	9.9	0	1.2
4	A169P	12 y	0 (0/19)	24 (9/37)	0 (0/19)	0 (0/47)
5	L227P	3 y	0 (0/25)	0 (0/35)	0 (0/30)	0 (0/25)
6	R182P	4 y	18 (5/28)	17 (9/52)	0 (0/27)	0 (0/33)
7	R175P	6 y	0.4 (1/25)	39 (11/28)	0 (0/28)	0 (0/25)
8	Q348X	8 y	38 (6/16)	47 (9/19)	0 (0/33)	0 (0/25)
9	R175P	15 y	30 (9/30)	36 (12/33)	0 (0/23)	0 (0/14)
10	1167 ins C	9 mo	PBMC 9.3 (4/43)			

For patients 1 to 3, data represent the percentages of $NEMO^{normal}$ cells in each lineage, as assessed by flow cytometry. For patients 4 to 10, the ratio indicates the number of wild-type NEMO clones in various cell lineages as compared with the total number of clones analyzed, based on subcloning and sequencing analysis.

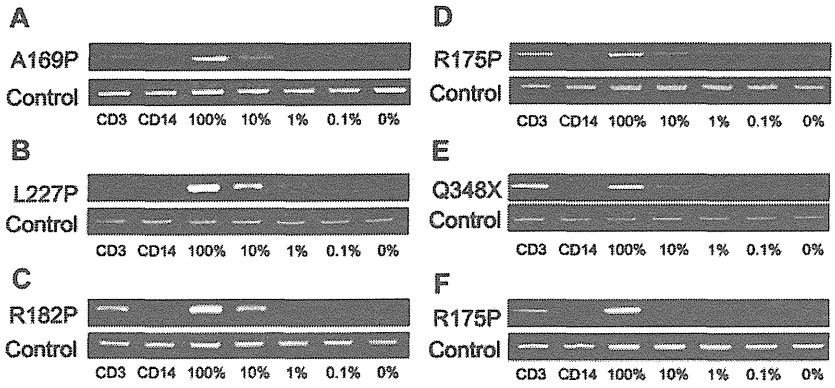

Figure 4. NEMO reversion selectively occurs in T cells of XL-EDA-ID patients. Allele-specific PCR for *NEMO* on $CD3^-$ or $CD14^+$ cells from (A) patient 4, (B) patient 5, (C) patient 6, (D) patient 7, (E) patient 8, and (F) patient 9. Numbers below each figure indicate the percentages of wild-type NEMO cDNA mixed with each mutant. Primers used in each PCR are shown on the left.

concordance between the degree of the disruption of *NEMO* gene and the proportion of reverted $NEMO^{normal}$ cells compared with $NEMO^{low}$ cells. The high proportion of reverted T cells seen in patients 1 and 2 as well as in patient 8 was associated with a highly disruptive mutation of the *NEMO* gene (ie, a duplication mutation in patients 1 and 2, and a truncation mutation in patient 8). In addition, the highly selective X-chromosome inactivation observed in the mothers of XL-EDA-ID patients indicated a strong selective advantage for $NEMO^{normal}$ cells over $NEMO^{low}$ cells. It is also noteworthy that reverted T cells were not detected in patient 5, who carried an L227P mutation that was not localized to either of the functional domains in the NEMO protein. Other reported cases with the same mutation presented with polysaccharide-specific humoral immunodeficiency and autoinflammatory diseases, but were spared complications such as cellular immunodeficiency and susceptibility to *Mycobacterium* (similar to patient 5).[4,8] This may reflect the fact that the L227P mutation in NEMO has less influence on T-cell growth than NEMO mutations that occur in functional domains, and suggests that reversion of the mutation has little impact on T-cell survival. Although the number of cases in our study is limited, it appears that the more disruptive NEMO mutations favor the survival of $NEMO^{normal}$ cells after reversion and X-chromosome inactivation.

Regarding the gradual decline in the number of NEMO-deficient T cells, one candidate trigger could be infection. Because the dominance of the memory phenotype and the skewed TCR repertoire among $CD8^+$ T cells in $NEMO^{normal}$ cells were observed in both patients 1 and 2 (Figure 1C and Mizukami et al[18]), continuous infection of pyogenic bacteria in patient 1 and *M szulgai* in patient 2 could be a reason for the emergence of $NEMO^{normal}$ cells and the elimination of $NEMO^{low}$ cells. The decrease in $NEMO^{normal}$ cells and restoration of $NEMO^{low}$ cells after anti-mycobacterial therapy in patient 2 support this hypothesis. In the case of patient 1, the predominance of $NEMO^{normal}$ T cells with an effector/memory phenotype at diagnosis (Table 4 and Mizukami et al[18]) is likely to be the result of chronic infection, and it is possible that $NEMO^{low}$ cells were predominant during his early infancy. Because some reports have indicated that TNF-α–induced programmed cell death of several cell types, including a human T-cell line, was enhanced by hypomorphic *NEMO* mutations,[12,35] and considering our finding that the levels of TNF-α expressed in revertant T cells were similar to levels in healthy control T cells in vitro (Figure 1F), TNF-α produced from these cells in response to infection could be involved in mutant T-cell elimination.

Unexpectedly, T-cell proliferation in patient 2 was equivalent to that of normal controls at the age of 2 months and was reduced after $NEMO^{normal}$ T cells increased (Figure 1B; Table 3). This finding indicates that the $NEMO^{low}$ T cells did not have intrinsically impaired mitogen-induced proliferation. One reasonable explanation for the reduced proliferation observed after the increase in $NEMO^{normal}$ T cells is a reduction in the absolute number of T cells (naive T cells in particular), probably because of the infection.

Table 5. Expression of mutant NEMO in various cell lineages for the mother of each XL-EDA-ID patient

Sample	Mutation	Analysis	Subtype	Mutant type, % (proportion)
Mother of patients 1 and 2	Duplication	FACS	CD3	0
			CD14	0
			CD19	0
Mother of patient 3	D311E	FACS	CD3	13
			$CD3^-$	54
		Subcloning	CD3	22 (6/27)
			$CD3^-$	55 (12/22)
Mother of patient 4	A169P	Subcloning	CD3	52 (11/21)
			CD14	58 (11/19)
			CD19	42 (5/12)
Mother of patient 8	Q348X	Subcloning	CD3	0 (0/26)
			CD14	17 (3/18)
			CD19	0 (0/18)
Mother of patient 10	1167insC	Subcloning	CD3	18 (7/39)
			CD14	12 (5/43)
			CD19	27 (12/44)

Data are shown as either the percentages of $NEMO^{normal}$ cells, as assessed by flow cytometry, or as the ratio of clones containing wild-type NEMO to the total number of clones, as analyzed by subcloning and sequencing analysis.

Table 6. Expression of mutant NEMO in CD3-positive cells and PHA blasts

Sample	Mutations	Analysis	Subtype	Mutant type, % (proportion)
Mother of patient 3	D311E	FACS	CD3	13
			PHA blast	47
		Subcloning	CD3	22 (6/27)
			PHA blast	48 (11/23)
Mother of patient 4	A169P	Subcloning	CD3	52 (11/21)
			PHA blast	18 (9/49)
Mother of patient 8	Q348X	Subcloning	CD3	0 (0/26)
			PHA blast	0 (0/21)
Mother of patient 10	1167insC	Subcloning	CD3	18 (7/39)
			PHA blast	9 (4/43)
Patient 2	Duplication	FACS	CD3	73
			PHA blast	93
Patient 4	A169P	Subcloning	CD3	79 (19/24)
			PHA blast	100 (37/37)
Patient 8	Q348X	Subcloning	CD3	56 (18/32)
			PHA blast	100 (16/16)
Patient 9	R175P	Subcloning	CD3	87 (34/39)
			PHA blast	0 (0/28)

PHA blasts were obtained by incubating PBMCs with PHA and soluble IL-2 for 7 days. Data are shown as either the percentages of $NEMO^{normal}$ cells, as assessed by flow cytometry, or as the ratio of clones containing wild-type NEMO to the total number of clones, as analyzed by subcloning and sequencing analysis.

Therefore, to identify other mechanisms underlying reduced T-cell proliferation, the impact of *NEMO* mutation on PHA-induced T-cell proliferation was indirectly examined in vitro by comparing the response of $NEMO^{normal}$ and $NEMO^{low}$ cells derived from XL-EDA-ID patients and their mothers. After PHA stimulation and proliferation, the proportion of $NEMO^{low}$ T cells increased in patients 2, 4, and 8, while the opposite result was observed in patient 9 and in the mother of patient 4 (Table 6). Although the precise mechanism is unclear, a reduction in the proportion of $NEMO^{normal}$ cells after PHA stimulation would reflect the lower proliferative capacity of $NEMO^{normal}$ cells compared with that of $NEMO^{low}$ cells, which may be another explanation for the reduced T-cell proliferation observed in patient 2 at 23 months of age when $NEMO^{normal}$ T cells were dominant. In the reports on reversion mosaicism of *IL2RG* gene mutations,[28,36] the restoration of T-cell function and clinical symptoms varied among patients. Therefore, other factors besides the genotype of the mutations, such as the developmental stage where reversion occurred and the frequency of reversion, affect the clinical impact of somatic mosaicism of *NEMO* gene mutations.

In this study, the effect of somatic mosaicism of the *NEMO* gene on clinical phenotype could not be fully evaluated. However, cytokines produced by revertant T cells could influence the development of clinical symptoms of XL-EDA-ID, such as inflammatory bowel disease. In a mouse model, intestinal epithelial cell-specific inhibition of NF-κB through the conditional ablation of NEMO resulted in the development of chronic bowel inflammation sensitized intestinal epithelial cells to TNF-α–induced apoptosis.[37] In this model, the first phase of intestinal inflammation was initiated by epithelial cell death and was followed by a second phase of TNF-α–induced intestinal inflammation, the latter being dependent on T cells. Another report showed that HSCT in XL-EDA-ID patients exacerbated the patients' inflammatory bowel disease.[38] Indeed, in patient 4, the percentage of reverted T cells was reduced after repeated administrations of anti-TNFα blocking Ab, and the symptoms of inflammatory bowel disease improved.[18] Considering this evidence, somatic mosaicism in T cells might be an important factor leading to inflammatory disease in XL-EDA-ID patients with defective NF-κB activation. However, our study did not show a tight association between inflammatory bowel disease and somatic mosaicism, and further investigation is needed to determine whether the $NEMO^{normal}$ T cells play a role in inflammatory processes in XL-EDA-ID.

In conclusion, this study has identified a high frequency of somatic mosaicism in XL-EDA-ID patients, particularly in T cells, and has revealed important insights into human T-cell immunobiology in XL-EDA-ID. Although we could not demonstrate the clinical impact of somatic mosaicism in XL-EDA-ID patients, our findings suggest that care is required when making molecular diagnoses of XL-EDA-ID, and might shed light on the mechanisms underlying the variability in the clinical manifestation of XL-ED-ID and facilitate the search for appropriate treatments.

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Authorship

Contribution: Tomoki Kawai wrote the manuscript and performed research; R.N., T.Y., T.N., and T.H. edited the manuscript and supervised this work; K.I., Y.M., N.T., H.S., M.S., and Y.T. cultured cells; and T. Mizukami, H.N., Y.K., A.Y., T. Murata, S.S., E.I., H.A., Toshinao Kawai, C.I., S.O., and M.K. treated patients and analyzed data.

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Correspondence: Ryuta Nishikomori, MD, PhD, Department of Pediatrics, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan; e-mail: rnishiko@kuhp.kyoto-u.ac.jp.

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