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Natural killer cell memory precedes HLH in monozygotic twins discordant for chronic active Epstein-Barr virus disease.

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Abstract:

Severe mosquito bite allergy (SMBA) is a manifestation of chronic active Epstein-Barr virus (CAEBV) infection defined by necrotic ulceration of the stings. CAEBV with SMBA has a high mortality rate as most patients eventually develop fulminant and refractory hemophagocytic lymphohistiocytosis (HLH). However, how self-resolving SMBA escalates to systemic lethal HLH remains unclear. Through comprehensive immune profiling of a SMBA patient with CAEBV and her healthy monozygotic twin, we found that both twins were seropositive for EBV but showed high discordance in their circulating natural killer (NK) cells. The patient's EBV-infected NK cells displayed memory-like properties, including low CD16, high CD226 and induction of enhanced IFN γ production by IL-2 or IL-12. Her leukocytes also produced high levels of IL-2 and IL-12 when stimulated with salivary gland extract (SGE) specifically from *A. albopictus* mosquitoes, connected again with hyperproduction of IFN γ by her NK cells. Strikingly, pharmacological inhibition of STAT3 suppressed the NK memory-associated cytokine axis of IFN γ , IL-2 and IL-12 that is generated by *A. albopictus* SGE stimulation. Altogether, this study shows that NK memory is promoted during CAEBV with SMBA by repeated cytokine restimulation leading up to lethal HLH, and proposes STAT3 as a therapeutic target to halt its progression.

Conflict of interest: No COI declared

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Author contributions and disclosures: CKL and YZ are co-first authors and responsible for writing the initial draft of the manuscript. CKL coordinated scientific studies, collated experimental results and wrote the scientific portions of the manuscript. YZ was the patient's primary care physician who made initial observations, diagnosed and managed care of the patient. PLT was the physician who closely monitored the patient's condition and managed conditioning and preparation for HSCT. YZ, CHH, MLO, FY, MV, PPLN, SBN, TCQ and PLT were involved in the clinical care of the patient and contributed to the interpretation of clinical data. CKL, RH, WSX, BVA, SN, CYCL, AV, BW, JP and KCC contributed to in vitro experiments and data analysis and interpretation. CKL, YZ, RH, WXS, RH, SN, CHH, MLO and SBN contributed to the provision and editing of tables and figures. PLT, KCC and JEC were responsible for critical appraisal of the final manuscript. All coauthors reviewed and approved the final version of the submitted manuscript.

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To the editor:

Natural killer cell memory precedes HLH in monozygotic twins discordant for chronic active Epstein-Barr virus disease.

Running title: NK cell memory precedes CAEBV progression to HLH

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Ectopic infection of T and NK cells by Epstein-Barr virus (EBV) has been associated with lymphoproliferative disorders known as chronic active Epstein Barr Virus disease (CAEBV). CAEBV has predominantly been reported in East Asian countries, with sporadic cases in Latin America and the Mediterranean.¹⁻⁴ 30-70% of CAEBV patients comprise the NK-type and first present with severe mosquito bite allergy (SMBA), the local form of the disease.⁵⁻⁷ SMBA can evolve into the systemic form of CAEBV and then progress suddenly to aggressive malignancy or hemophagocytic lymphohistiocytosis (HLH) without identifiable biomarkers for onset.⁸ The only known curative treatment is hematopoietic stem cell transplantation (HSCT); otherwise, 50-58% of patients die within 10 to 15 years of diagnosis.^{6,8,9}

Clinical and immunological studies have revealed that both chronic EBV infection and the stimulation of immune cells from CAEBV patients with mosquito salivary gland extracts (SGE) may play key roles in the development of SMBA and life-threatening HLH.¹⁰ However, there are no clear biomarkers for disease severity or for progression to HLH. Here, we performed deep immunophenotyping on a pair of monozygotic (MZ) twins who were both seropositive for EBV but clinically discordant for SMBA and CAEBV and identified NK cell memory-associated cytokines as crucial mechanisms and biomarkers of disease progression.

The patient and her MZ twin were born full-term to non-consanguineous Chinese Singaporean parents. She was healthy apart from SMBA starting age 3. Unfortunately, the patient progressed to fulminant, treatment-resistant HLH 9 months following referral at age 13, and succumbed to multi-organ failure 2 months after (Suppl Fig. 1). Targeted next generation sequencing panels for HLH and cancer did not reveal any candidate mutations and the family declined more comprehensive whole exome or genome sequencing. Therefore, we screened for clinical and immunological discordance 6 months after referral, between 2 SMBA reactions at 4 and 6.5 months after referral, but prior to fulfilling HLH criteria. Informed consent was obtained from the parents, and assent from the twins. Both twins had high serum levels of EBV VCA IgG, proving both had prior EBV exposure (Figure 1A). Despite similar counts of CD4⁺ and CD8⁺ T cells and CD20⁺ B cells, the patient had 10.6 times more NK cells than her healthy twin (Figure 1B).

We expanded NK cells from both twins *in vitro* to measure further phenotypic differences in 24 immunomodulatory receptors (Figure 1C; Supplementary Table 1).¹¹ Dimensionality reduction by t-distributed stochastic neighbor embedding (t-SNE), and cluster identification by phenograph showed nearly complete separation between the patient and her twin (Figure 1D). The patient's NK cells presented a hyperinflammatory phenotype: over 90% of them were CD226⁺ and they expressed significantly lower levels of the inhibitory receptors CD161, KIRs and TIM3 (Figure 1E and F) – all phenotypes previously ascribed to memory-like NK cells¹²⁻¹⁸. Thus, we examined whether the patient's PBMCs also showed memory-like IFN γ hypersecretion when stimulated by memory-priming cytokines IL-2, IL-12 and IL-18.¹⁶⁻¹⁸ IL-2 and IL-12 alone were sufficient to induce much higher levels of IFN γ secretion from the patient's PBMCs than the MZ twin's, while IL-18 had only a small effect (Figure 1G). Intracellular cytokine staining of patient's PBMC indicated that these IFN γ -producing cells were nearly all CD56^{dim} CD16^{dim} and contained preformed IFN γ protein before stimulation which was absent in her twin's NK cells (Figure 1H). In addition, the intracellular levels of IFN γ in NK cells were further increased when cells were stimulated with IFN α and IL-2, or PMA/ionomycin (Figure 1I).

Prior studies show that mosquito species differ in their ability to prime both NK cell lymphocytosis and CD4 T-cell proliferation in patients with SMBA¹⁹. We therefore hypothesized they also differ in ability to prime IFN γ production by the patient's memory NK cells. We stimulated the patient's

PBMCs with mosquito SGEs from two common mosquitoes in Singapore, *Aedes aegypti* and *Aedes albopictus*.²⁰ Either SGE alone induced high amounts of IFN γ from patient PBMCs, but we found a significantly stronger effect by *A. albopictus* (Figure 2A). Intracellular cytokine staining showed that IFN γ was produced mainly by NKs and CD4⁺ T cells of both twins; however, while the MZ twin's IFN γ was principally expressed by CD4⁺ T cells, the majority of the patient's IFN γ ⁺ PBMCs after stimulation with *A. albopictus* SGE were NK cells (Figure 2B). Because either IL-2 or IL-12 can cause IFN γ hyperproduction by the patient's PBMCs (Figure 1G), we hypothesized that they were induced by the *Aedes* SGEs and responsible for IFN γ production by the patient's NK cells. Stimulation of patient's PBMCs with SGE from either species produced high levels of IL-2, *A. albopictus* SGE more than *A. aegypti*. Meanwhile, IL-12 was induced uniquely by *A. albopictus* SGE (Figure 2C). The patient's NK cells also increased CD25 expression and, thus, sensitivity to IL-2 in response to *A. albopictus* SGE, but not *A. aegypti* (Figure 2D).

Fulminant HLH is accompanied by a pro-inflammatory cytokine storm.²¹ To assess whether there are further similarities in cytokine production between SMBA and fulminant HLH, we performed multiplexed ELISAs on patient PBMCs stimulated with the SGEs. *A. albopictus* SGE universally induced higher levels of secreted IL-2Ra, a diagnostic criterion of HLH; pro-inflammatory cytokines and chemokines; and type 2 immunity-associated cytokines, with increasing responsiveness over time (Figure 2E). As these cytokines are common in HLH-associated cytokine storms, they may have heralded the patient's progression to HLH.²¹

The above analyses suggest that persistent induction of IL-2 and IL-12 by the *Aedes* SGEs drives SMBA and CAEBV progression by promoting functional memory in the patient's infected NK cells. Thus, blocking IL-2 and IL-12 production may present new therapeutic opportunities to ameliorate disease. We examined whether we could abolish production of these cytokines through inhibition of STAT3, STAT5, and NF κ B, which have previously been shown to promote the production of these important cytokines. The patient's PBMCs were stimulated with *Aedes* SGE after pre-incubation with the IKK2 inhibitor ML-120B, STAT3 inhibitor S3I-201 and STAT5 inhibitor. S3I-201 abolished production of IL-2 and also fully blocked IL-12 and IFN γ production, whilst only partial suppressive affects were observed when using ML-120B or the STAT5 inhibitor (Figure 2F to I).

Discordance of infection and immunophenotype between the MZ twins supports the notion that SMBA is not predominantly driven by inborn errors of immunity. Given that the patient's SMBA started when she was a young child and became more severe over time, with larger lesions and higher fevers, it is likely that NK memory priming began at a young age and developed gradually through the course of her life, amplified by multiple rounds of stimulation by IL-12 and IL-2 during SMBA reactions. *A. albopictus* SGE induces both IL-2 and IL-12 from the patient's PBMCs, while *A. aegypti* only induces IL-2 (Figure 2C). Mechanistically, this suggests that *A. albopictus* is a stronger and more versatile agonist of the patient's NK memory response, as both cytokines prime robust IFN γ production and NK memory (Figure 2A).^{16,17,22} Such differences in cytokine induction may also underlie species selectivity of reactions reported in other CAEBV patients with SMBA.¹⁹ Supporting the dependence of memory NK responses on IL-2 and IL-12, STAT3 inhibition completely abrogated IFN γ together with IL-2 and IL-12 from patient PBMCs stimulated with *A. albopictus* SGE (Figure 2F to I). STAT3 underlies many complex mechanisms of both tumorigenesis and NK cell immune function; our findings demonstrate it may also be a suitable target for prevention of NK memory priming as a third pathogenic aspect to CAEBV.²³ Our results highlight that it is crucial to initiate early treatment of CAEBV with HSCT or cytokine inhibitors, before EBV infected NK cells establish a memory population, and suggest that further investigation into the effects of memory-priming cytokines and STAT3 inhibitors may present new therapeutic opportunities.

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Authorship Contributions

CKL and YZ are co-first authors and responsible for writing the initial draft of the manuscript. CKL coordinated scientific studies, collated experimental results and wrote the scientific portions of the manuscript. YZ was the patient's primary care physician who made initial observations, diagnosed and managed care of the patient. PLT was the physician who closely monitored the patient's condition and managed conditioning and preparation for HSCT. YZ, CHH, MLO, FY, MV, PPLN, SBN, TCQ and PLT were involved in the clinical care of the patient and contributed to the interpretation of clinical data. CKL, RH, WSX, BVA, SN, CYCL, AV, BW, JP and KCC contributed to in vitro experiments and data analysis and interpretation. CKL, YZ, RH, WXS, RH, SN, CHH, MLO and SBN contributed to the provision and editing of tables and figures. PLT, KCC and JEC were responsible for critical appraisal of the final manuscript. All coauthors reviewed and approved the final version of the submitted manuscript.

Conflict of Interest Disclosure

CKL is currently an employee of MiroBio Limited, Oxford, UK, now a part of Gilead Sciences, Foster City, CA. JEC has an appointment as Chief Scientific Officer at the Parker Institute of Cancer Immunotherapy, San Francisco, CA. Both appointments bear no contribution or relation to this work, and therefore no competing financial interest. All other authors declare no conflict of interest.

Figure Legends

Figure 1: Patient with SMBA and CAEBV progressing to lethal HLH shows expansion of hyperinflammatory, memory NK cells absent in her monozygotic (MZ) twin.

(A) Titers of reactive IgG antibodies against EBV VCA and Ea antigens in sera from patient and MZ twin 6 months after referral. (B) CD3⁺, CD4⁺, CD8⁺ and CD56⁺CD16 lymphocyte counts from the patient and her twin 6 months after referral. (C) Procedure for *in vitro* expansion of NK cells from PBMCs. (D) t-SNE clustering of expanded NK cells from patient and her MZ twin based on expression of 24 NK cell activating and inhibitory receptors. Left, clusters labelled by donor: red, patient; orange, MZ twin. Right, phenograph of further subpopulations based on detailed phenotype. (E) Surface receptor expression of NK cell receptors in each phenograph subpopulation, as a heatmap of Z-scores of mean fluorescence intensity of each receptor. (F) Frequency of NK cells from MZ twin and patient in each phenograph subpopulation. (G) Concentration of IFN γ produced by PBMCs after stimulation with IL-2 (80 IU/ml), IL-12 (100 μ g/ml), IL-18 (100 μ g/ml) or IL-12 with IL-18. (H) Intracellular levels of IFN γ among total CD56^{dim}CD16^{dim} NK cells from the patient and her MZ twin when unstimulated (H₂O), or when stimulated with IFN α (1000 IU/ml) + IL-2 or PMA (5 ng/ml)/ionomycin (1 μ g/ml). Left, histogram of IFN γ staining normalized to mode of cell counts. Right, percentage of cells gated IFN γ ⁺ among CD56^{dim}CD16^{dim} NK cells. (I) Percentage of CD56^{dim}CD16^{dim} cells among total IFN γ ⁺ PBMCs from the patient and her MZ twin when unstimulated (H₂O) or stimulated with IFN α + IL-2 or PMA/ionomycin. Data are shown from single replicates due to limited amounts of primary sample.

Figure 2: *Aedes albopictus* SGE glands induce pro-inflammatory cytokine production by CAEBV patient's PBMCs which can be suppressed via STAT3.

(A) Concentration of IFN γ in supernatant of PBMCs from the patient and her MZ twin after 16 h stimulation with SGE from *A. aegypti* or *A. albopictus* (25 μ g/ml). (B) Percentage of each leukocyte population among IFN γ ⁺ PBMCs from the patient and her MZ twin stained after 4 h stimulation with SGE from *A. aegypti* or *A. albopictus*. (C) Concentrations of IL-2 and IL-12 in supernatant of PBMCs from the patient, her MZ twin and URHCs after 16 h stimulation with SGE from *A. aegypti* or *A. albopictus*. (D) Expression of CD25 on NK cells from the patient, her MZ twin and URHCs after 16 h stimulation with SGE from *A. aegypti* or *A. albopictus*. (E) Heat maps showing relative concentrations of IFN γ , IL-12p70, IL-2, soluble IL-2R (sIL-2R), IL-6, IL-8, TNF α , CCL3, CXCL10, GM-CSF, IL-5 and IL-13 after 16 h stimulation with SGE from *A. aegypti* or *A. albopictus*, from patient and her MZ twin at 6 months after referral (left), and patient and 2 unrelated healthy controls (URHC) at 9 months after referral (right). Data are presented as a heat map, scaled relative to maximum concentration for each cytokine. (F to H) Concentrations of IL-2 (F), IL-12 (G) and IFN γ (H) in supernatants of PBMCs from the patient and her MZ twin, preincubated with S3I-201 (200 μ M), STAT5 inhibitor (200 μ M) or ML-120B (10 μ M), and then stimulated with *Aedes* SGEs for 16h. (I) Relative concentrations of cytokines in the supernatant of patient PBMCs preincubated with S3I-201, STAT5 inhibitor or ML-120B, then stimulated with *Aedes* SGEs for 16h. Data are presented as a heat map, scaled relative to maximum concentration for each cytokine. Data are shown from single replicates due to limited amounts of primary sample.

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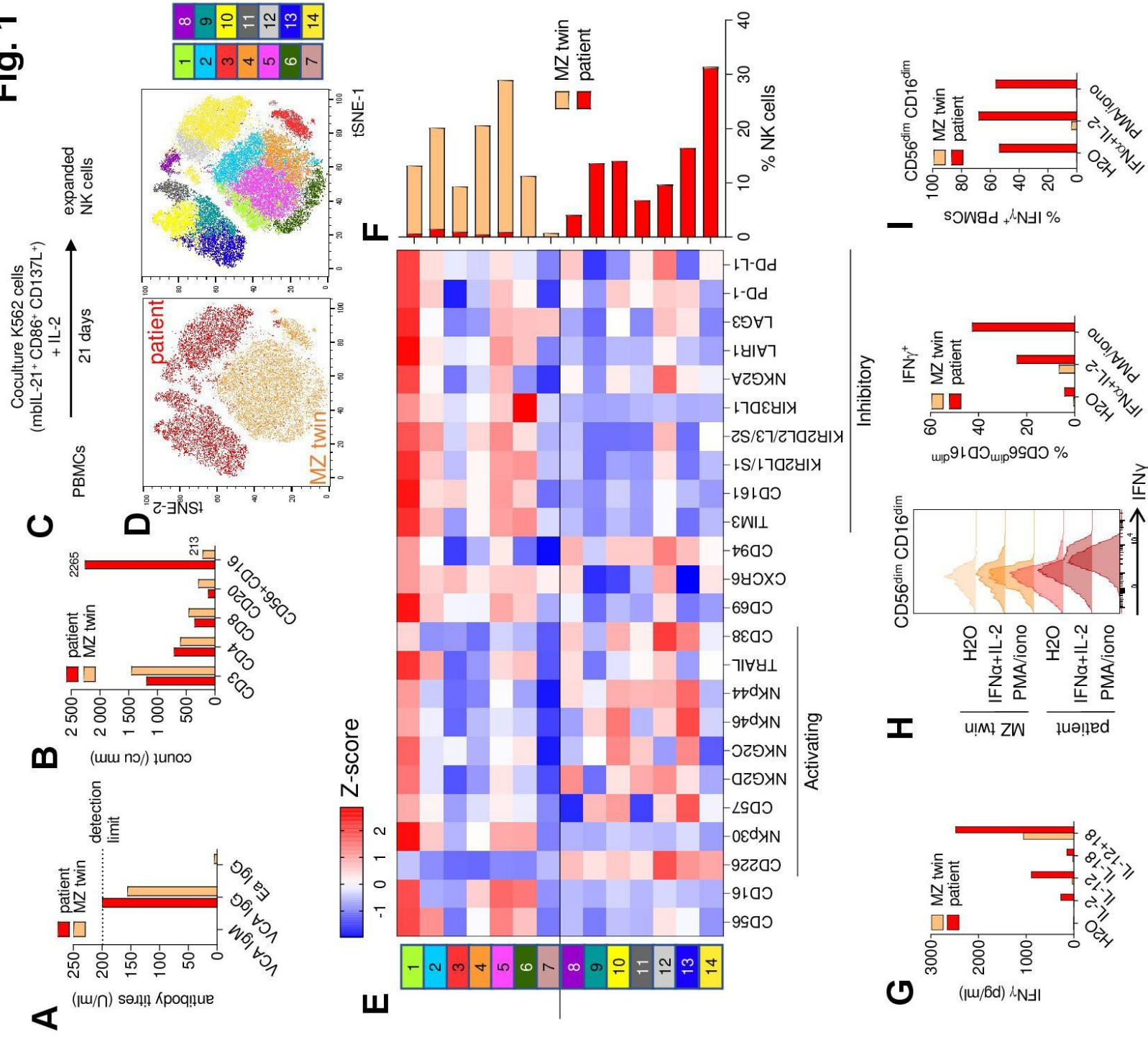
Fig. 1

Fig. 2

