In Vivo Hematopoietic Stem Cell Engineering Restores the Function of NADPH Enzyme Complex in X-Linked Chronic Granulomatous Disease Model Mice

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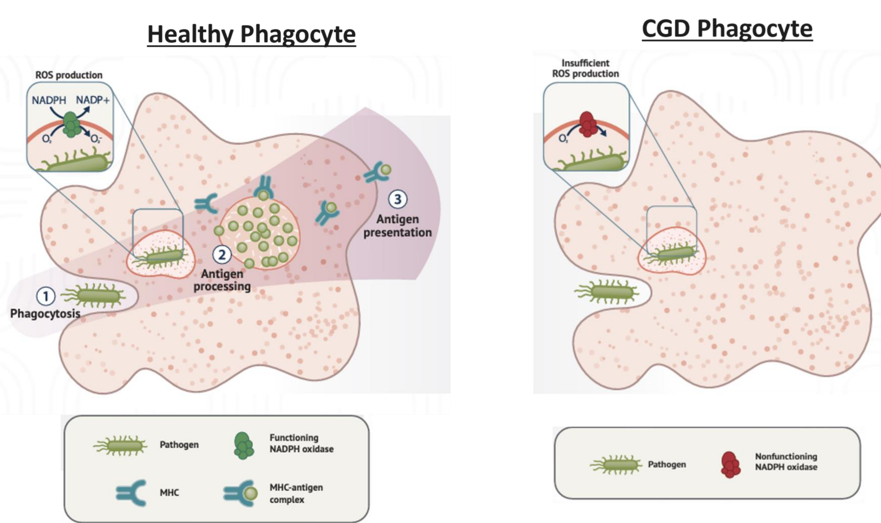
X-linked chronic granulomatous disease (X-CGD) is a rare primary immune deficiency disorder



CGD is a rare primary immune deficiency disorder caused by a defect in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex, with the most common mutations being in the X-linked gene, *CYBB*, affecting approximately 65-70% of patients with CGD. The inability to produce NADPH oxidase complex impairs the ability of phagocytic cells to eliminate bacterial and fungal pathogens with reactive oxygen species (1).

The Ensoma VLP Platform for *in vivo* HSC engineering

Helper-dependent Adenovirus (HDAd) 5/35++

Engineered capsid
Adenoviral vector built on evolved, high efficiency gene delivery vectors

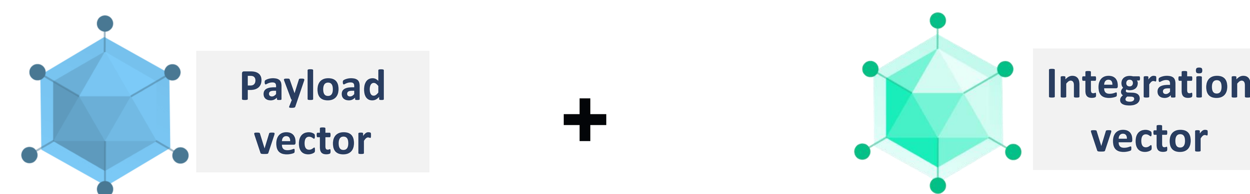
"Gutless" vector
Devoid of viral genes, resulting in low immunogenicity & high payload capacity

Hematopoietic Stem Cell (HSC) Tropism
Highly preferential transduction of primitive hematopoietic stem cells through Ad35++ fiber knob targeting human CD46 receptor

35 kB Payload Capacity
Enables multiplexed gene insertion controlled by distinct regulatory elements

EN-374 utilizes a dual vector system to enable durable neutrophil specific *CYBB* transgene expression and correct defective NADPH oxidase activity in X-CGD

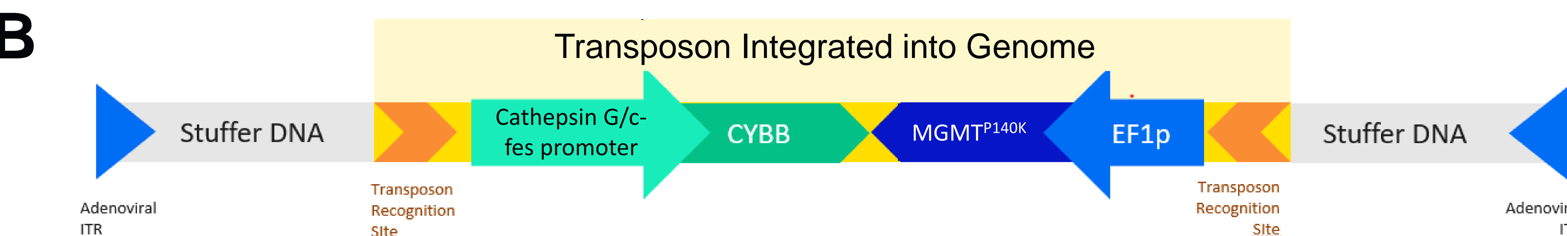
A



Contains the gene of interest, *CYBB* (under neutrophil specific promoter), and selection marker, MGMT^{P140K} (under ubiquitous promoter)

Contains a Sleeping Beauty 100x transposase and FLP recombinase used to mediate the integration of the CYBB and MGMT^{P140K} payload

B



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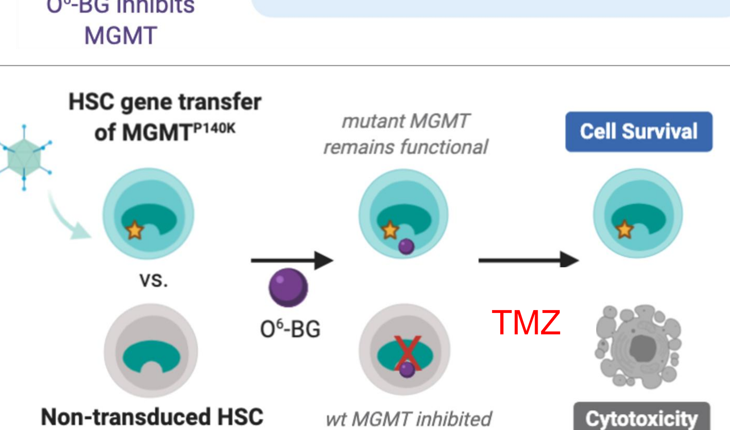
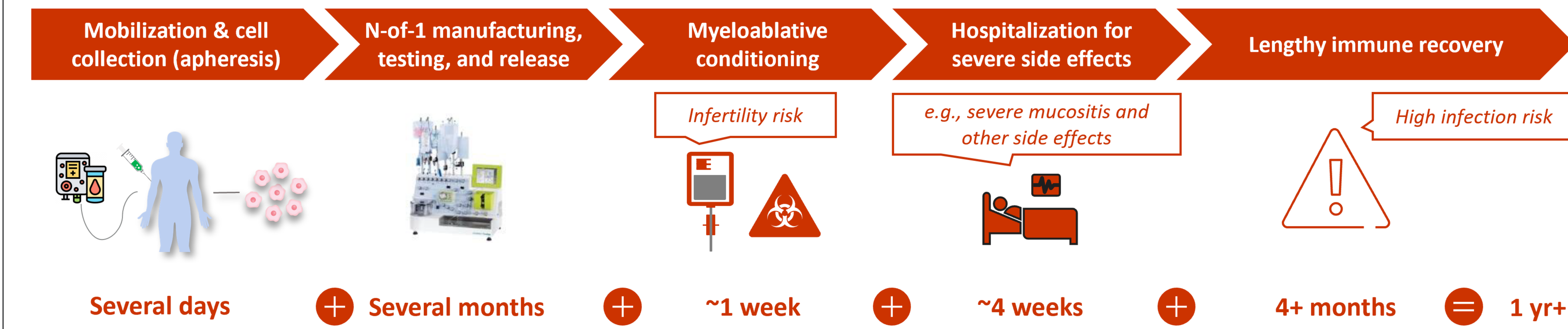


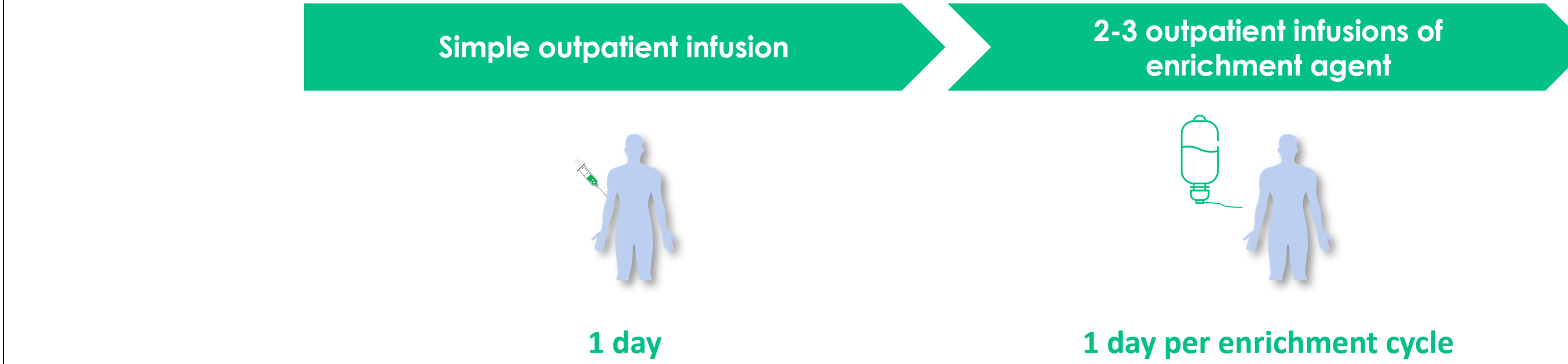
Figure 1 A) EN-374 consists of two HDAd vectors. In cells co-transduced by both vectors, the transposon is excised and integrated into the genomic DNA. **B)** The payload vector expresses a functional *CYBB* gene under the control of Cathepsin G/c-fes promoter, previously shown to regulate tissue-specific transgene expression and function in neutrophils (2,3) and MGMT^{P140K}, a selective marker, under the control of a ubiquitous promoter, EF1α, to confer resistance to a regimen of O^{6}BG and Temozolomide (TMZ). **C)** TMZ, an alkylating agent, induces apoptosis by alkylating guanine at the O^6 position in DNA, initiating a double-strand break and cell cycle arrest. The DNA repair enzyme, MGMT, can rapidly remove the damaging alkyl groups from the O^6 position of guanine and repair the DNA. O^{6}BG inhibits WT MGMT, however MGMT^{P140K} is resistant to O^{6}BG. Thus, the expression of MGMT^{P140K} in gene modified HSCs confers protection against the effects of O^{6}BG/TMZ and thereby allows for selective enrichment of modified HSCs.

Ensoma's outpatient *in vivo* gene therapy patient journey for X-CGD

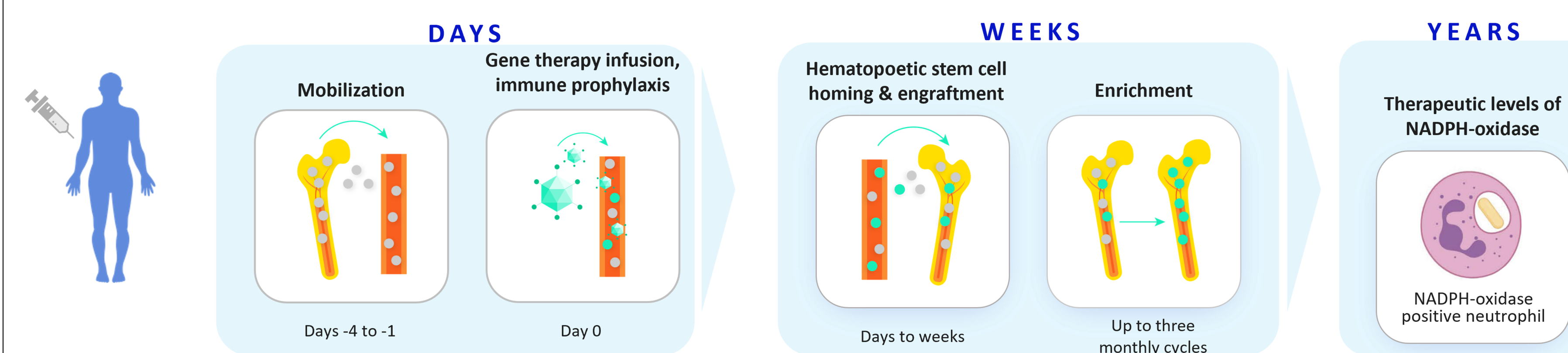
Ex Vivo Patient Journey



Our Vision: *In Vivo* Patient Journey



Ensoma's approach to *in vivo* modification of HSCs for X-CGD



HSCs are mobilized to the peripheral circulation to facilitate vector transduction. At the peak of HSC mobilization, the clinical product, EN-374, is infused intravenously to transduce the mobilized HSCs. Prior to EN-374 administration, an immune prophylaxis regimen is given to attenuate potential innate immune activation to HDAd. Gene-modified HSCs in the peripheral circulation then home back to the bone marrow shortly after transduction, leading to long-term engraftment. Gene-modified HSCs are then enriched to attain therapeutic levels by administration of O6BG and TMZ.

Neutrophil population recovers in X-CGD mice treated with EN-374 and O6BG/TMZ enrichment

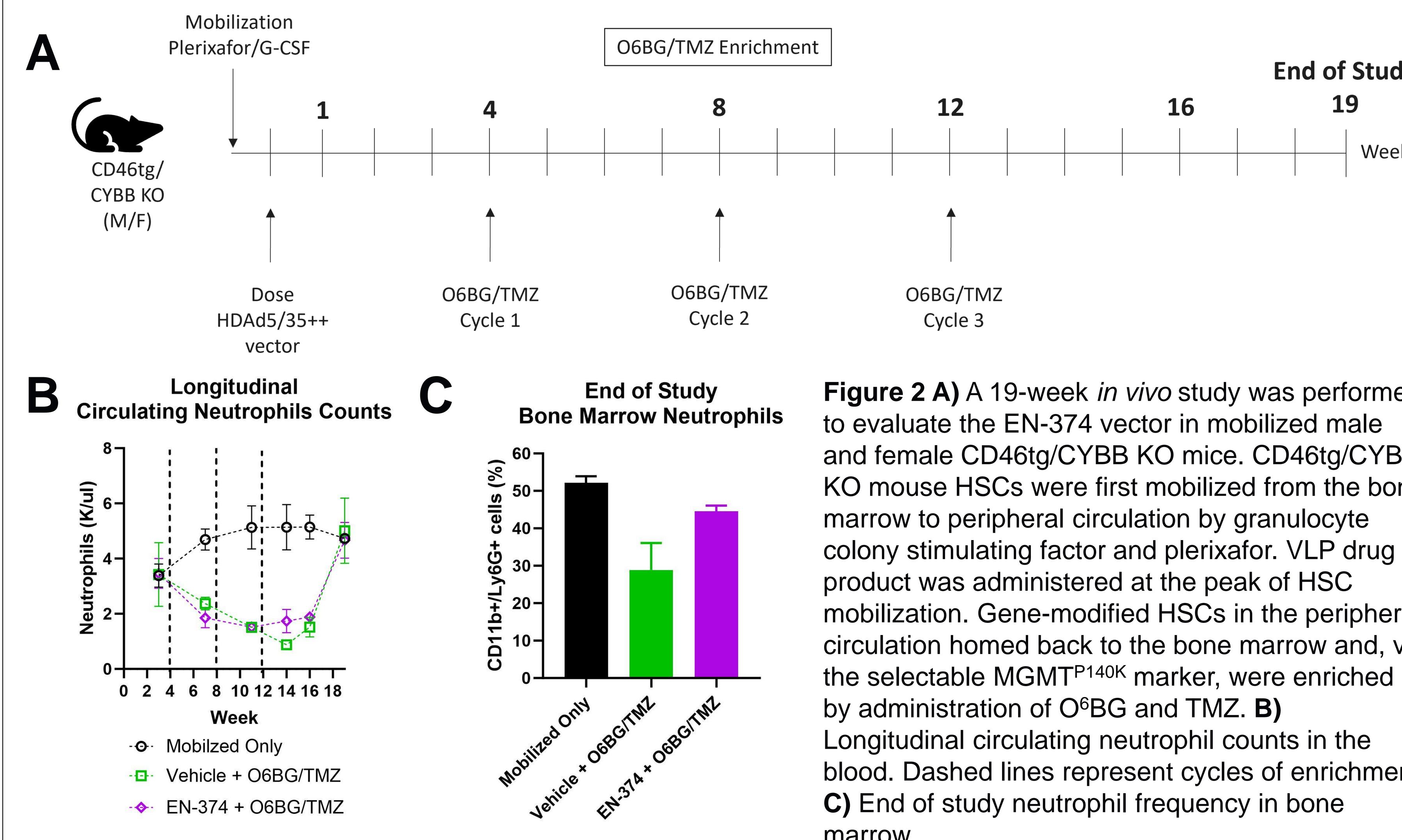


Figure 2 A) A 19-week *in vivo* study was performed to evaluate the EN-374 vector in mobilized male and female CD46tg/CYBB KO mice. CD46tg/CYBB KO mouse HSCs were first mobilized from the bone marrow to peripheral circulation by granulocyte colony stimulating factor and plerixafor. VLP drug product was administered at the peak of HSC mobilization. Gene-modified HSCs in the peripheral circulation homed back to the bone marrow and, via the selectable MGMT^{P140K} marker, were enriched by administration of O^{6}BG and TMZ. **B)** Longitudinal circulating neutrophil counts in the blood. Dashed lines represent cycles of enrichment. **C)** End of study neutrophil frequency in bone marrow.

EN-374 restores CYBB Expression and NADPH oxidase activity in X-CGD disease model mice

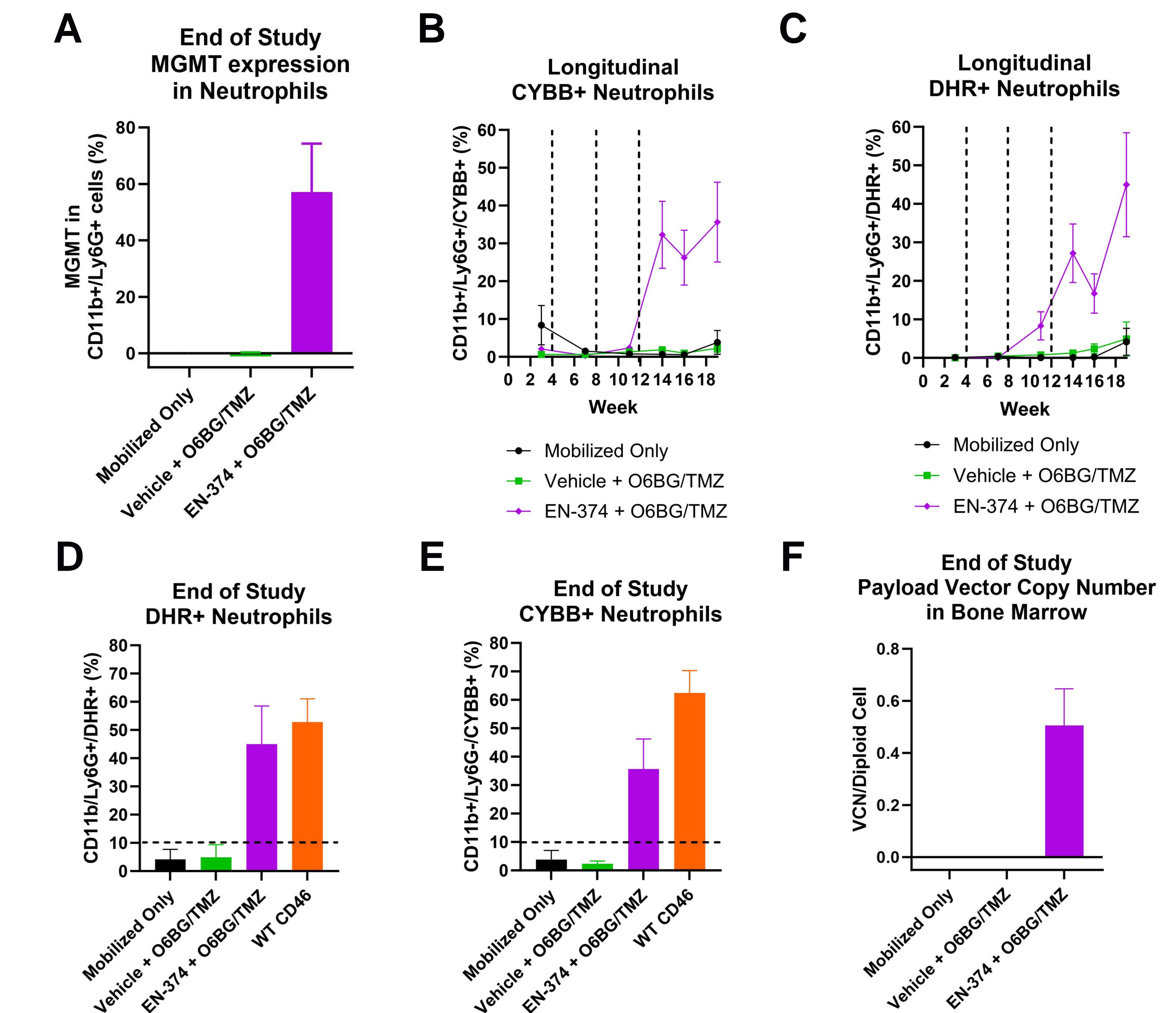


Figure 3 A) The expression of MGMT^{P140K} protein in EN-374 is driven by a ubiquitous promoter and by using an anti-human MGMT specific antibody, gene marking in pan-lineages can be assessed. In this study MGMT gene marking was measured in circulating neutrophils is assessed by flow cytometry. CYBB is expressed in terminally differentiated neutrophils and the dihydrorhodamine (DHR) assay is a flow cytometry-based method to measure NADPH oxidase activity in circulating neutrophils that are stimulated. Longitudinal **B)** CYBB protein and **C)** DHR activity in circulating peripheral neutrophils. Vertical dashed lines indicate cycles of enrichment. End of study **D)** CYBB protein and **E)** DHR activity in circulating neutrophils. Horizontal dashed line indicates 10% therapeutic threshold. Restoring more than 10% of neutrophils with NADPH oxidase activity has been shown to confer clinically meaningful improvements in infection outcomes in patients with X-CGD. **F)** End of study vector copy number (VCN) of payload in bulk bone marrow. Together these data demonstrate compelling proof-of-concept for efficient *in vivo* engineering of HSCs that leads to the expression of functional CYBB at therapeutic levels in X-CGD disease model mice.

Conclusions

- Ensoma has developed an *in vivo* approach to genetically modify HSCs designed to provide durable correction and overcome the current limitations with allogeneic HSC transplant and *ex vivo* gene therapy.
- The development candidate, EN-374, is intended to modify HSCs *in vivo* to express the functional *CYBB* gene and restore ≥10% of neutrophils with NADPH oxidase activity to confer clinically meaningful improvements in infection outcomes in male patients with X-CGD
- This study sets the foundation for the first *in vivo* HSC gene therapy to reach the clinic and can be applied to a range of genetic disorders which thus far have only been addressed with *ex vivo* gene therapies that involve significant patient burden and manufacturing limitations.

References and Key Founder Publications

Cited References:
1) https://www.cgdpathways.com
2) Santilli et al. Mol Ther, 2011.
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Key Founder Publications:
1. Adair et al. JCI, 2014.
2. Jansen et al. Cancer Gene Ther, 2002
3. Li et al. Mol Ther Methods Clin Dev, 2021.
4. Richter et al. Blood, 2016.
5. Wang et al Exp Hematol, 2008.
6. Wang et al Mol Ther Methods Clin Dev, 2018.