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Received: 3 May 2023 | Revised: 12 July 2023 | Accepted: 28 September 2023

DOI: 10.1111/tan.15252

ORIGINAL ARTICLE

HLA Immune Response Genetics WILEY

Temporal regulation of transgene expression controlled by amino acid availability in human T cells

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T cell therapy strategies, from allogeneic stem cell transplantation toward genetically-modified T cells infusion, develop powerful anti-tumor effects but are often accompanied by side effects and their efficacy remains sometimes to be improved. It therefore appears important to provide a flexible and easily reversible gene expression regulation system to control T cells activity. We developed a gene expression regulation technology that exploits the physiological GCN2-ATF4 pathway's ability to induce gene expression in T cells in response to one essential amino acid deficiency. We first demonstrated the functionality of NUTRIREG in human T cells by transient expression of reporter genes. We then validated that NUTRIREG

Abbreviations: AA, amino acid; AARE, amino acid responsive element; Allo-HSCT, allogeneic hematopoietic stem cell transplantation; ASNS, asparagine synthetase; ATF4, activating transcription factor 4; bp, base pair; CAR-T, chimeric antigen receptor-T; CD, cluster of differentiation; cDNA, complementary DNA; CHOP, C/EBP homologous protein; Ctl, control; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EAA, essential amino acid; eGFP, enhanced green fluorescence protein; eIF2α, eukaryotic initiation factor 2 (α subunit); ELISA, enzyme-linked immunosorbant assay; FBS, fetal bovine serum; FoxP3, forkhead box P3; GCN2, general control nonrepressed 2; GCN2i, GCN2 inhibitor; GvHD, graft versus host disease; GvL, graft versus leukemia; GZMb, granzyme B; HRI, heme-regulated eIF2α kinase; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-γ; IgG, immunoglobulin G; IL, interleukin; INRAE, Institut National de Recherche pour l'Agriculture, l'alimentation et l'environnement; LAG-3, lymphocyte-activation gene 3; −Leu, leucine-free; LUC, luciferase; mRNA, messenger ribonucleic acid; NXG, NOD xenograft gamma strain; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PERK, pancreatic endoplasmic reticulum eIF2 kinase; PKR, protein kinase RNA-activated; RPMI, Roswell Park Memorial Institute medium; RT-qPCR, real time-quantitative polymerase chain reaction; TGF-β, transcription growth factor β; TK, thymidine-kinase; Tr1, type 1 regulatory t cells; TRB3, tribbles pseudokinase 3; tRNA, transfer ribonucleic acid.

Alain Bruhat and Paul Rouzaire should be considered as joint senior authors.

Funding information
UCA I-Site Emergence Program; Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE); the University Hospital of Clermont-Ferrand (DRCI); the startup NUTRITHERAGENE

can be used in human T cells to transiently express a therapeutic gene such as IL-10. Overall, our results represent a solid basis for the promising use of NUTRIREG to regulate transgene expression in human T cells in a reversible way, and more generally for numerous preventive or curative therapeutic possibilities in cellular immunotherapy strategies.

KEYWORDS
allo-HSCT, amino acid starvation, cellular immunotherapy, GCN2-ATF4 pathway, GvHD, transgene expression

1 | INTRODUCTION

Cellular immunotherapy strategies are currently growing for the treatment of cancer. Among these strategies, allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been the standard treatment of many hematological malignancies for years.[1] Allo-HSCT basically consists of the replacement of a patient's bone marrow with hematopoietic stem cells collected from a healthy donor, allowing hematopoietic and immune systems reconstitution but, above all, the anti-tumor effect of donor lymphocytes (Graft-vs.-Leukemia or GvL effect).[2] Unfortunately, this procedure is often accompanied by high toxicity and may lead to Graft versus Host Disease (GvHD) and infections, which limits its indications. These complications represent a major cause of morbidity and mortality, poor quality of life and an additional economic cost.[3,4] More recently, genetically modified T cells were introduced as a new approach for cancer treatment, such as CAR-T cells or TCR-T cells.[5,6] To date there are still many difficulties in controlling T cells once reinjected into the patient, either to increase their therapeutic action or to limit their toxicity. It therefore appears important to be able to provide a flexible and easily reversible gene expression regulation system to control the effects of these cells as simply as possible.

In this context, our results highlight the use in T cells of a gene expression regulatory technology called NUTRIREG that exploits an ubiquitous adaptive process known as the amino acid response pathway.[7] A diet deficient in one essential amino acid (EAA) leads to a rapid and sharp decrease of the limiting EAA blood concentration, triggering the adaptive GCN2-ATF4 pathway.[8–10] Firstly, GCN2 kinase is activated by uncharged tRNAs. Then, activated GCN2 phosphorylates the α-subunit of the eukaryotic Initiation Factor 2 (eIF2α), leading to upregulation of the Activating Transcription Factor 4 (ATF4) translation. ATF4 binds to amino acid responsive element (AARE) sequences on promoter sites to activate transcription of specific target genes involved in adaptation to cellular stress.[11–13] This GCN2-ATF4 pathway has therefore been harnessed to regulate transgene expression by NUTRIREG.[7,14] This technology is based on the association of (i) an artificial promoter (based on AARE sequences) strongly inducible by the lack of one EAA, which controls the expression of a transgene (AARE-Gene) with (ii) a diet devoid of one EAA. This leads to a sharp drop in the blood concentration of the limiting EAA and allows induction of the GCN2-ATF4 signaling pathway. Thus, after AARE-Gene plasmid delivery to the target tissue by a viral vector, the transgene expression can be induced by the consumption of the EAA-deficient diet. Interestingly, the pathway can be rapidly deactivated by consumption of balanced AA diet, providing a gene regulatory system that is easily reversible and free of adverse effects since it involves a physiological nutritional pathway. Proof of the functionality of this system has already been provided on mice, in different organs (liver, pancreas, brain, eye), but also in glioblastoma tumor cells thanks to the use of a pro-apoptotic gene.[7]

In order to use the NUTRIREG technology in human T cells allowing a therapeutic application in cellular immunotherapy, we first validated that the GCN2-ATF4 pathway is inducible by an EAA starvation in this cell type. We then validated the in vitro functionality of NUTRIREG in human T cells using reporter genes. Finally, as an example of application of NUTRIREG in T cell therapy, we then demonstrated that our technology could be used to control the expression of a therapeutic gene known to prevent GvHD after allo-HSCT. In this perspective, we showed that transient expression of IL-10 in human T cells via NUTRIREG was able to direct the immune response towards a Tr1 type response and validated the proof of concept of in vivo nutritional induction of IL-10 in human T cells using immunodeficient mice.

2 | MATERIALS AND METHODS

2.1 | Preparation of human primary T cells

PBMCs were purified from buffy coats collected from healthy donors by the French Blood Establishment (convention 19–088) using Cytiva Ficoll-Paque™ PLUS (Fisher) and used fresh or frozen at −80°C. In a second step, human T cells were isolated from PBMCs using the human Pan T Cell Isolation Kit (Miltenyi Biotec, Germany) according to

the manufacturer's instructions. Isolated T cells were then resuspended in RPMI 1640 medium (PAN BIOTECH) at a density of 1–2 × 10^6 cells/ml.

2.2 | T cell activation

Purified human T cells were activated using human T Cell Activation/Expansion Kit (Miltenyi Biotec, Germany). Ratio of MACSiBead™ particles per cell was 1:2. RPMI medium containing 10% heat inactivated FBS was supplemented with human IL-2 IS (Miltenyi Biotec, Germany) at 50 IU/mL.

2.3 | T cell culture and treatments

Activated human T cells were incubated in 10% FBS-RPMI medium supplemented with IL-2 at 37°C and 5% CO2 for up to 14 days of expansion. When indicated, Dulbecco's Modified Eagle's Medium (DMEM) (with 10% of dialyzed calf serum) lacking one EAA was used (GENAXXON bioscience). A control medium was simultaneously reconstituted by adding all nine EAAs. RPMI cultured human T cells were collected when needed and washed in PBS with centrifugation at room temperature at 300g for 5 min. Cells were then resuspended either in reconstituted DMEM control or in DMEM lacking one EAA, at a density of 1 × 10^6 cells/mL. DMEM was freshly supplemented with IL-2 (50 IU/mL) (or not, in experiments without IL-2). GCN2 inhibitor (GCN2i) (TAP20) was previously described[15,16] and generously provided by Merck KGaA. The compound was resuspended in DMSO and used at a final concentration of 2.5 μM.

2.4 | Plasmid constructions and lentiviral production

2XAARE-TRB3-TK-eGFP, 2XAARE-TRB3-TK-LUC and 2XAARE-TRB3-TK-IL-10 plasmids contain two copies of the AAREs from the *TRB3* gene followed by cDNA sequences encoding eGFP, luciferase, or the human IL-10 (Interleukin-10). Plasmids and lentiviruses were produced by VectorBuilder (Chicago, USA).

2.5 | Lentiviral transduction

Primary human T cells were transduced 24 h after activation. Cell suspension was centrifuged at 300g for 5 min and cell pellet was resuspended in fresh RPMI supplemented with IL-2 at a density of 1 × 10^6 cells/mL. Lentiviral suspension was then added to cells with a multiplicity of infection of 5–10. Cell suspensions were maintained for experimental purposes until 8 days after activation.

2.6 | Immunoblot analysis and antibodies

Western blot analysis was performed as previously described.[17] ATF4 (# 11815), GCN2 (# 3302), eIF2α (# 9722) and normal rabbit IgG (# 7074) antibodies were purchased from Cell Signaling Technology. Antibodies recognizing phosphorylated GCN2 (ab75836), and phosphorylated eIF2α (ab32157) were from Abcam (Cambridge, UK) and the antibody recognizing actin (sc-47778) was from Santa Cruz. CD4-PerCP-Vio700 (REA623), CD8-VioBlue (REA734), CD45-APC-Vio770 (REA747), CD69-APC (REA824), CD25-PE (REA570), CD223-APC (REA351), CD49b-FITC (REA188) and IL-10-PE (REA842) antibodies were purchased from Miltenyi Biotec (Germany).

2.7 | Flow cytometry

For cell surface staining, human T cells were resuspended in 100 μL of PBS and incubated with antibodies in the dark at 4°C for 15 min. For intracellular analysis of human IL-10, staining was performed with the MACS Inside Stain Kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions.

2.8 | ELISA assay

IL-10 was measured (i) in the media supernatant using the IL-10 human ELISA kit (Thermofisher) for in vitro studies, and (ii) in plasma using the IL-10 Human ELISA Kit, High Sensitivity (Thermofisher) for in vivo studies, according to the manufacturer's instructions.

2.9 | Gene expression analysis by real-time RT-qPCR

Real-time RT-qPCR was performed as previously described.[17] Primers for the human sequences (Table S1) yielded PCR products of approximately 100–150 bp in size. The abundance of each mRNA was normalized to the β-actin signal.

2.10 | Luciferase biochemical assay

Luciferase assays were performed as previously described.[7] Luciferase activities measured in cell extracts were normalized to protein content (relative light units per mg of protein).

2.11 | Ethics statement

Maintenance of the mice and all experiments were conducted according to the guidelines formulated by the European Community for the use of experimental animals (2010/63/EU). Animal facilities were approved by the French veterinary Department (Agreement D6334515). Animal experiments were approved by the national regulatory committee of the Ministère de l'Enseignement Supérieur, de la Recherche, et de l'Innovation (APAFIS#6545).

2.12 | Animals and experimental diets

Six-week-old NXG males (NOD-*Prkdc*^{scid}-*IL2rg*^{Tm1}/Rj) were purchased from Janvier Labs (SM-NXG-M). Animals were individually housed in plastic cages and subjected to a 12 h light/dark cycle at 22°C, in a pathogen-free environment. For each experiment, five mice per group were used. Animals were excluded from analysis if human T cell expansion in the blood failed at week 3 after injection. Experimental diets were manufactured in our INRAE institute facilities (Unité de Préparation des Aliments Expérimentaux, Jouy-en-Josas, France). The animals had ad libitum access to food and water at all times, unless otherwise indicated. When indicated, the mice were exposed to a control diet or to a leucine-deficient diet for 16 h. Examiners were not blinded with respect to diet administration and treatments. Prior to feeding the mice a leucine-deprived meal, a fasting period of 8 h was required, and newly cleaned cages were provided for the mice during this period. Food intake was monitored in each experiment.

2.13 | Preparation of human cells and injection in mice

Eight days prior to cell injection in mice, primary human AARE-TK-IL-10 T cells were prepared according to "Lentiviral transduction" section. The day of injection in mice, autologous human PBMCs were irradiated at 30 Gy at Pavirma irradiation platform (LPC, UCA Clermont-Ferrand, France). The cells were then pooled and injected into the tail vein of the mice. Every mouse received 10.10^6 AARE-TK-IL-10 T cells and 5.10^6 irradiated PBMCs in 250 µL of sterile PBS.

2.14 | Mice blood collection and analysis

Seven days after cell injection, and then once per week until week 3, 100 µL of blood were collected by submandibular sampling after the 16 h-exposition of mice to control diet or leucine-deficient diet. PBMCs were purified using Cytiva Ficoll-Paque™ PLUS (Fisher) and used freshly for flow cytometry analysis. Plasma (50 µL) was immediately frozen at −20°C.

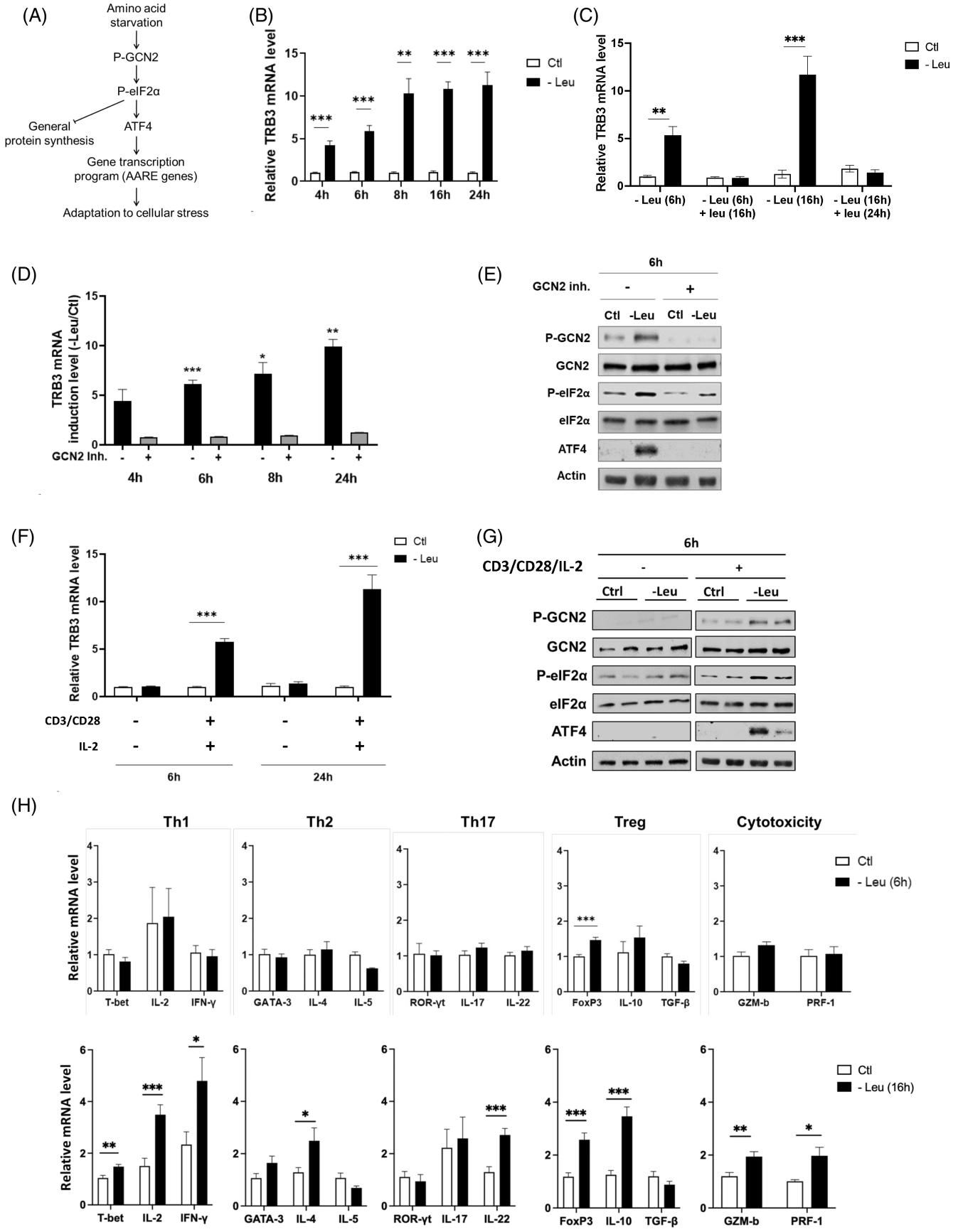
2.15 | Statistics

All statistical analyses were generated using GraphPad Prism 9 (GraphPad Software) and all data are expressed as means ± SEM. For the comparison between two or more experimental groups, statistical significance was assessed via Student's *t* test or two-way ANOVA with an alpha level of 0.05. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. The significance of the time-dependent variation of human IL-10 concentration in mouse plasma was assessed by simple linear regression and the correlation coefficient with a threshold level of 0.05.

3 | RESULTS

3.1 | Essential amino acid deprivation induces the GCN2-ATF4 pathway in human primary activated T cells

Since the NUTRIREG technology exploits the GCN2-ATF4 pathway to induce a transgene expression, the first essential step was to validate that this pathway is inducible by an EAA deprivation in human T cells (Figure 1A). Data from the literature previously highlighted the activation of this signaling pathway mainly in mice T cells in response to IDO (Indoleamine 2.3-Dioxygenase), halofuginone or asparaginase,[18–21] but only a few data were available on GCN2-ATF4 pathway activation in response to AA deficiency in human T cells. We first analyzed the induction of the GCN2-ATF4 pathway in response to leucine starvation in this cell type. Primary activated human T cells were incubated in leucine-free or control media and the mRNA level for TRB3, a known target gene of the GCN2-ATF4 pathway,[13] was measured. Kinetic analysis revealed that TRB3 mRNA level was increased at 4 h of leucine deprivation and reached a plateau from 8 h to at least 24 h (Figure 1B). Similar results were obtained with CHOP and ASNS mRNA levels, two other known ATF4-target genes[22,23] (Figure S1A). As expected, the induction of TRB3 mRNA level by leucine deprivation in T cells was completely lost after the addition of leucine in the culture medium demonstrating that the amino acid induction of the GCN2-ATF4 pathway can be rapidly and fully reversible in T cells, which is an important safety point for NUTRIREG use[7] (Figure 1C). We used a pharmacological



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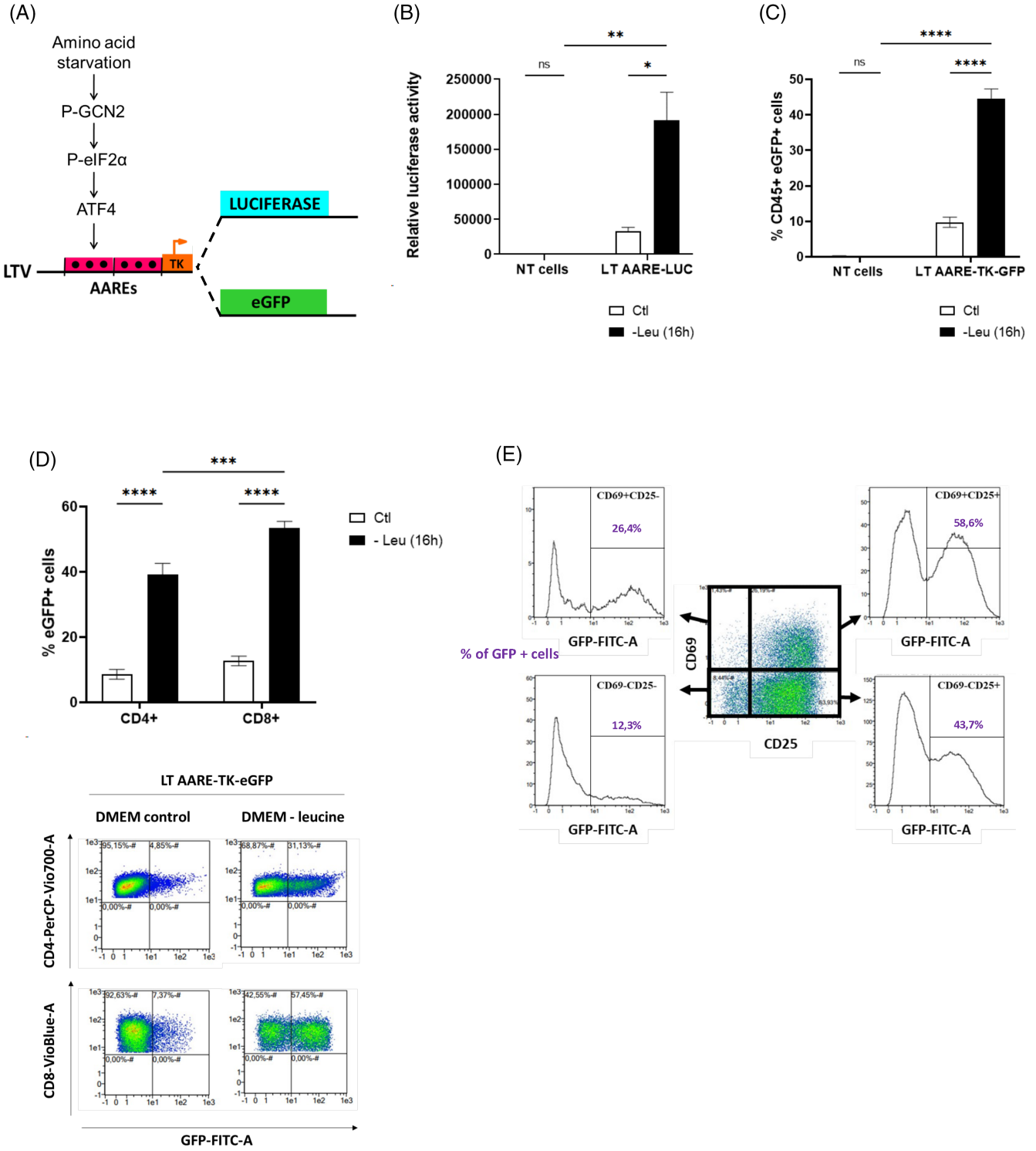
inhibitor of GCN2 to demonstrate that the upregulation of TRB3 expression following 4–24 h-leucine starvation was fully dependent on this kinase, and therefore independent of the other three kinases that can phosphorylate eIF2 (PERK, PKR and HRI)[24] (Figure 1D). Immunoblot analysis of protein expression in human T cells confirmed the role of GCN2 in eIF2α phosphorylation increase and ATF4 protein accumulation in response to leucine starvation (Figure 1E). Then, other EAA starvations were tested for their abilities to activate the GCN2-ATF4 pathway in T cells. Upregulation of TRB3, ASNS and CHOP mRNA expression occurred upon deprivation of each of the nine EAAs, to a different extent depending on the missing EAA (Figure S1B). Interestingly, absence of T cell activation resulted in a loss of TRB3 mRNA inducibility following a 6–24 h-leucine starvation (Figure 1F). These results were confirmed by immunoblot analysis of phospho-GCN2, phospho-eIF2α and ATF4 expression in human T cells. The absence of T cell activation led to losses of (i) GCN2 and eIF2α phosphorylation and of (ii) ATF4 protein increase in response to leucine starvation (Figure 1G). The need for T cell activation to enable GCN2 kinase activation has been described in mouse T cells exposed to either a tryptophan-deficient medium or treatment with IDO,[18,19] and also in human T cells starved for all of EAAs during a few days.[25] To our knowledge, no information was available for human T lymphocytes after short starvation of one EAA. Taken together, these data demonstrate that the GCN2-ATF4 pathway can be upregulated in primary human T cells following EAA starvation, and that activation of T cells is mandatory for the induction of the GCN2-ATF4 pathway in response to leucine starvation.

We finally assessed whether leucine starvation could impact the functionality of T cells through analysis of mRNA expression profile of transcription factors or cytokines implicated in polarization of CD4+ T cells or in cytotoxicity. Leucine deprivation for 6 h had little impact, as only FoxP3 mRNA expression increased significantly (Figure 1H). A longer period of leucine deprivation (16 h) had a higher impact as the mRNA expression of many transcription factors or cytokines increased significantly. However, it appears that no CD4+ polarization subtype was favored. Importantly, short-time leucine starvation appears to have no impact on T cell function, which validates the perspectives of short-time EAA starvations in human.

FIGURE 1 Short-time EAA deprivation induces the GCN2-ATF4 pathway in primary activated human T cells and does not shape their profile. (A) Scheme of the eIF2α-ATF4 pathway. GCN2 is induced by EAA starvation. This kinase is active in its phosphorylated form (P-GCN2), and phosphorylates eIF2α leading to upregulation of the translation of ATF4 mRNA. The transcription factor ATF4 enhances transcription of many AARE-dependent genes that play a critical role in the adaptation to cellular stress. (B) Kinetics of GCN2-ATF4 pathway induction by leucine deprivation. Activated primary human T cells were incubated for 4–24 h either in control (Ctl) or leucine-free (−Leu) media and harvested after the indicated incubation times. TRB3 mRNA level was assayed by RT-qPCR and normalized on β-actin mRNA level (Student's *t* test −Leu vs. Ctl: $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 6$ per condition; error bars, SEM). (C) Reversibility of the leucine deprivation-induced activation of GCN2-ATF4 pathway by switching to the control level of leucine. Activated primary human T cells were incubated in control (Ctl) or in leucine-free (−Leu) media for 6 or 16 h, then leucine-starved T cells were incubated with leucine (+ Leu) at its control level and harvested after additional 16 or 24 h of incubation. TRB3 mRNA level was assayed by RT-qPCR and normalized on β-actin mRNA level (Student's *t* test −Leu vs. Ctl: $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 6$ per condition; error bars, SEM). (D) GCN2 kinase dependency for induction of TRB3 expression during leucine deprivation. Activated primary human T cells were incubated in control or in leucine-free media that were supplemented with DMSO (vehicle, GCN2 Inh. −) or pharmacological GCN2-inhibitor (2.5uM, GCN2 Inh. +) and harvested after 4–24 h of incubation. TRB3 mRNA level was assayed by RT-qPCR and normalized on β-actin mRNA level. Results are expressed as a mRNA fold change between leucine-free and control media (Student's *t* test –Leu vs. Ctl: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 6$ per condition; error bars, SEM). (E) GCN2-dependent activation of the eIF2α-ATF4 pathway by leucine deprivation in T cells. Activated primary human T cells were incubated in control (Ctl) or in leucine-free (−Leu) media supplemented with DMSO (vehicle, GCN2 Inh. −) or pharmacological GCN2-inhibitor (2.5uM, GCN2 Inh. +) and harvested after 6 h of incubation. Western blot analysis of phospho-GCN2, total GCN2, phospho-eIF2α, total eIF2α, ATF4 and β-actin was performed as described in the "Material and Methods" section. (F) Impact of T cell activation on TRB3 mRNA expression induced by leucine deprivation. Primary human T cells were incubated in control (Ctl) or leucine-free (−Leu) media in the absence (CD3/CD28−) or in the presence (CD3/CD28+) of anti-biotin bead particles loaded with CD3 and CD28 antibodies and in the absence (IL-2−) or in the presence (IL-2 +) of IL-2 supplementation, and harvested after 6 or 24 h of incubation. TRB3 mRNA expression level was assayed by RT-qPCR and normalized on β-actin mRNA level (Student's *t* test –Leu vs. Ctl: $^{***}\ p < 0.001$; $n = 4$–5 per condition; error bars, SEM). (G) Impact of T cell activation on GCN2-ATF4 pathway induction by leucine deprivation. Primary human T cells were incubated for 6 h in control (Ctl) or leucine-free (−Leu) media, in the absence (CD3/CD28/IL-2 −) or in the presence (CD3/CD28/IL-2 +) of anti-biotin bead particles loaded with CD3 and CD28 antibodies and IL-2 supplementation, and harvested after 6 h of incubation. Immunoblot analysis of phospho-GCN2, total GCN2, phospho-eIF2α, total eIF2α, ATF4 and β-actin was performed. (H) Impact of leucine deprivation on T cell. Activated primary human T cells were incubated in control (Ctl) or leucine-free (−Leu) media and harvested after 6 h (upper panels) or 16 h (lower panels) of incubation. Transcription factors or cytokines mRNA expression levels were assayed by RT-qPCR and normalized on β-actin mRNA level (Student's *t* test –Leu vs. Ctl: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 3$–24 per condition; error bars, SEM).

3.2 | NUTRIREG technology is functional in activated primary human T cells

Based on the functionality of the GCN2-ATF4 pathway in human T cells, we then demonstrated the functionality of the NUTRIREG system, allowing flexible and reversible regulation of a transgene, under the control of an EEA deficiency. We established the first proof of concept with the luciferase reporter gene. The luciferase coding sequence was inserted downstream of two copies of the *TRB3* AARE sequences associated with the TK minimal



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promoter in a lentiviral vector (Figure 2A). We confirmed the functionality of NUTRIREG in human T cells with significant induction of luciferase activity following 16 h of leucine starvation (Figure 2B). The induction of luciferase activity in response to leucine starvation was detectable from 3 h of leucine deficiency (Figure S2A). In order to be able to analyze more finely the expression of the transgene, we used eGFP as the second reporter gene (Figure 2A). Measurement of eGFP fluorescence by flow cytometry demonstrated that a 16 h leucine deprivation markedly induced the expression of eGFP transgene in T cells (Figure 2D). Our experiments also showed that induction of eGFP expression was higher in CD8+ T cells than in CD4+ T cells (Figure 2D). In addition, flow cytometry analyses of T cells co-expression of activation markers (CD69 and CD25) and of eGFP protein enabled us to confirm the key necessity of T cell activation to induce the expression of the transgene under the control of NUTRIREG system. Indeed, non-activated cells ($CD25^-$ $CD69^-$) did not express eGFP, whereas moderately activated cells ($CD69^-$ $CD25^+$ or $CD69^+$ $CD25^-$) expressed it weakly, and strongly activated cells ($CD69^+$ $CD25^+$) expressed the highest level of eGFP protein (Figure 2E). Induction of eGFP expression via NUTRIREG was possible in the case of each one of the nine EAAs deprivation, with different magnitudes according to the missing EAA, leucine deprivation being one of the most effective inducers (Figure S2B). Then, we used a pharmacological inhibitor of GCN2 to demonstrate that induction of eGFP expression in response to leucine starvation was fully dependent on the GCN2 kinase (Figure S2C). Collectively, these results demonstrate the functionality of the NUTRIREG technology in activated primary human T cells in vitro. The essential activation of T cells provides a really reassuring advantage for the use of NUTRIREG in the field of cellular immunotherapy. Indeed, the transgene will be expressed only in activated T cells as the GCN2-ATF4 pathway cannot be induced in quiescent T cells. It thus represents a major physiological safety control.

3.3 | NUTRIREG technology can be used in human T cells to control the expression of a gene of interest

As an example, we propose here a preventive or curative control of GvHD with a NUTRIREG-T cells system comprising an IL-10 expression transgene promoting the expansion of human Tr1 lymphocytes, to slow down the cytotoxic response responsible for GvHD. T cell transduction with an IL-10 expressing lentivirus has indeed been described as able to prevent GvHD in mouse models.[26,27] To transiently express IL-10 through the NUTRIREG technology, we inserted in a lentiviral vector IL-10 cDNA downstream of (i) two copies of the AARE sequences (2xAARE) and (ii) the Thymidine-Kinase (TK) minimal promoter (Figure 3A). We demonstrated that a 16 h-leucine deprivation significantly induced mRNA (Figure 3B) and protein (Figure 3C,D) expression of the IL-10 transgene, in comparison to the transduced T cells cultured in control medium and the non-transduced T cells starved for leucine. Furthermore, we showed that induction of IL-10 mRNA expression was completely lost 24 h after leucine addition to the culture medium (Figure 3B). IL-10 protein levels decreased from 48 h after the addition of leucine, demonstrating that the induction of IL-10 transgene via NUTRIREG can be deactivated in T cells (Figure 3C). The delay between the disappearance of mRNA and protein is likely to be due to

FIGURE 2 NUTRIREG system is functional in activated primary human T cells and allows conditional expression of transgenes. (A) Schematic representation of 2xAARE-TK-LUC and 2xAARE-TK-eGFP constructs. Constructs were transduced through a lentiviral vector 24 h after activation of T cells. (B) Functionality of LUC-NUTRIREG in T cells after leucine deprivation. Activated primary human non-transduced T cells (NT cells) or AARE-TK-LUC transduced T cells (LT AARE-TK-LUC) were incubated in control (Ctl) or in leucine-free (−Leu) media and harvested after 16 h of incubation. Expression of luciferase was measured and normalized on protein amount (Two-way [leucine deprivation, transduction] ANOVA: ns, non-significant; *$p < 0.05$; **$p < 0.01$; $n = 5$ per condition; error bars, SEM). (C) Functionality of eGFP-NUTRIREG in CD45+ cells after leucine deprivation. Activated non-transduced T cells (NT cells) or AARE-TK-eGFP transduced T cells (LT AARE-TK-eGFP) were incubated in control (Ctl) or in leucine-free (−Leu) media and harvested after 16 h of incubation. Expression of eGFP was analyzed by flow-cytometry. Results are expressed as percentage of eGFP+ cells among CD45+ cells (Two-way [leucine deprivation, transduction] ANOVA: ns, non-significant; ****$p < 0.0001$; $n = 2$–9 per condition; error bars, SEM). Dot plots are extracted from a representative experiment. (D) Functionality of eGFP-NUTRIREG in CD4+ and CD8+ cells after leucine deprivation. Activated primary AARE-TK-eGFP transduced human T cells (LT AARE-TK-eGFP) were incubated in control (Ctl) or in leucine-free (−Leu) media and harvested after 16 h of incubation. Expression of eGFP was analyzed by flow cytometry. Results are expressed in percentage of eGFP+ cells among CD4+ or CD8+ cells (Two-way [leucine deprivation, phenotype] ANOVA: ***$p < 0.001$; ****$p < 0.0001$; $n = 9$ per condition; error bars, SEM). Dot plots are extracted from a representative experiment. (E) Impact of T cell activation on eGFP-NUTRIREG expression. Activated primary AARE-TK-eGFP transduced human T cells (LT AARE-TK-eGFP) were incubated in control (Ctl) or in leucine-free (−Leu) media and harvested after 16 h of incubation. Expression of CD69 and CD25 activation markers and eGFP was analyzed by flow cytometry. Dot plots and histograms are extracted from a representative experiment.

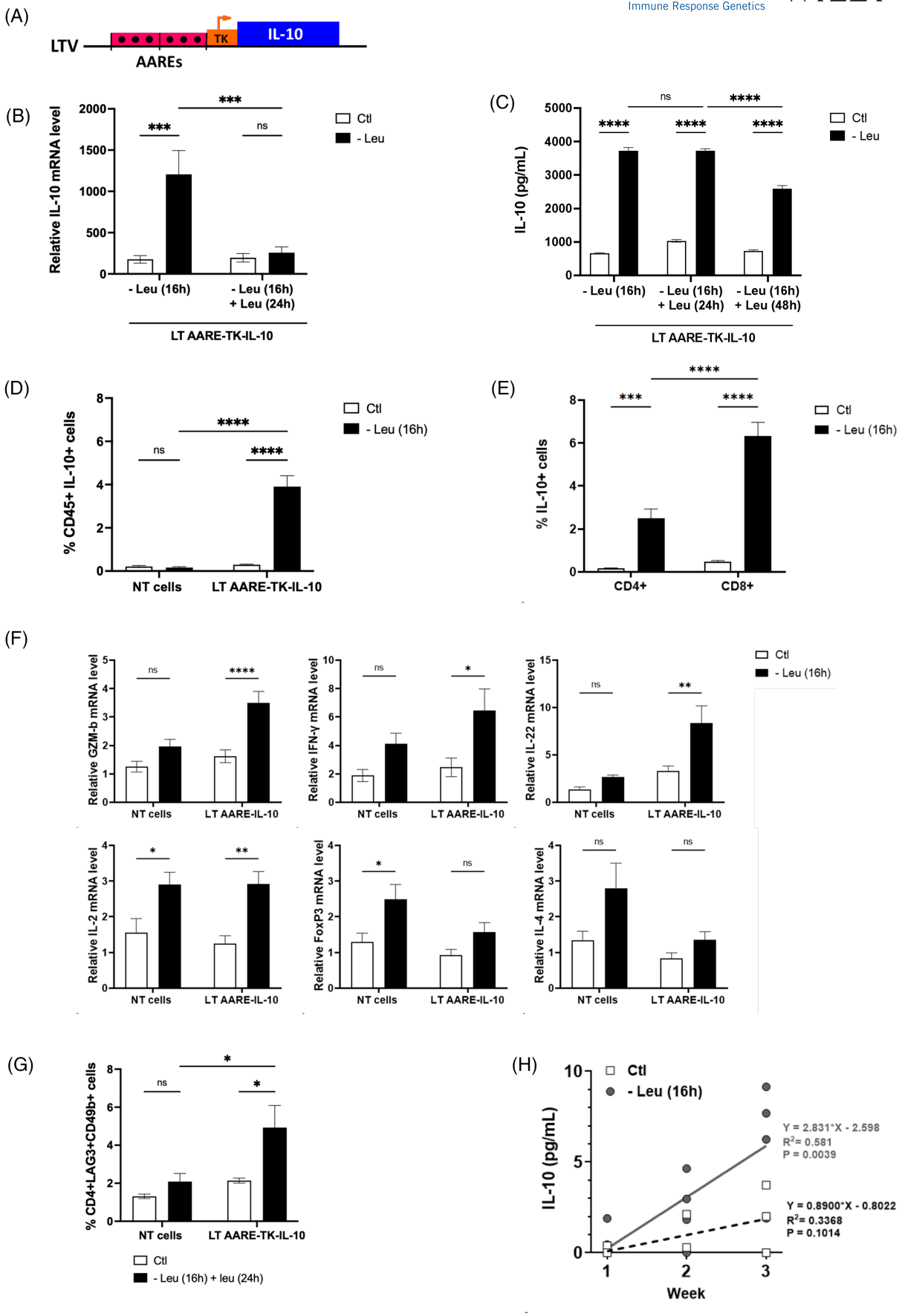

FIGURE 3 Legend on next page.

the stability of IL-10 protein. As shown above with the eGFP reporter gene, IL-10 expression was higher in CD8+ T cells than in CD4+ T cells (Figure 3E).

Type 1 regulatory T cells (Tr1) have a specific cytokine profile since they strongly express IL-10, and to a lesser extent TGF-β, GZMb, IFN-γ and IL-22 while they do not express IL-2, IL-4 and IL-17, nor the FoxP3 transcription factor.[28] Our mRNA expression analysis showed that transient expression of IL-10 transgene via NUTRIREG led to a Tr1 type transcription profile IL-10^{++} $GZMb^{+}$ IL-22^{+} IFN-γ^{+} $FoxP3^{-}$ and IL-4^{-} (Figure 3F). We also analyzed surface co-expression of the two markers CD49b and LAG-3 considered as specific to Tr1 cells.[29] To maximize induction of marker expression without having the adverse effects of a long-term leucine deficiency, T cells were cultured in a leucine-deficient medium for 16 h, and then returned for 24 h to a medium containing this amino acid. Under these conditions, in which IL-10 is still detected (see above), we showed that CD49b/LAG-3 co-expression was significantly induced in CD4+ T cells following leucine starvation (Figure 3G). Altogether, these results demonstrate that transient expression of IL-10 via NUTRIREG in human T cells directs the immune response toward a Tr1 type response.

We lastly investigated the capacity of a diet deficient in leucine to upregulate the AARE-driven IL-10 expression in human T cells injected into mice. Indeed, the lack of any of the EAAs in the diet of mammals represents a potential inducer of the AARE-transgene expression.[14] As activation of T cells is mandatory for the EAA-induction of the GCN2-ATF4 pathway, we set up a mouse model in which we injected transduced human T cells in association with autologous irradiated human PBMCs. We assumed that transduced human T cells would be transiently activated by irradiated PBMCs and that this activation would be sufficient for the GCN2-ATF4 pathway to be inducible by an EAA nutritional deficiency. Briefly, immunodeficient NXG mice were injected into the tail vein with (i) human T cells transduced with lentiviruses carrying the 2xAARE-TK-IL-10 construct and (ii) human irradiated PBMCs, and were then fed for 16 h, once a week until week 3, with either a control diet or a leucine-devoid diet. Weekly flow cytometry analysis of PBMCs enabled us to follow the correct expansion of human CD45+ cells, with no significant impact of leucine-deficient diet on cells expansion nor on CD4+ or CD8+ phenotype (Figure S3). Human IL-10 increased linearly with time in the leucine-deficient

FIGURE 3 NUTRIREG technology allows the control of IL-10 expression in vitro and in vivo in human T cells, to shape the response toward a Tr1 profile. (A) Schematic representation of 2xAARE-TK-IL-10 construct. (B) Functionality and reversibility of IL-10-NUTRIREG in T cells after leucine deprivation-induced activation of IL-10 transgene mRNA expression. Activated primary AARE-TK-IL-10 transduced human T cells (LT AARE-TK-IL-10) were incubated in control (Ctl) or in leucine-free (−Leu) media during 16 h, then leucine-starved T cells were supplemented with leucine and harvested after additional 24 or 48 h of incubation. IL-10 mRNA expression level was assayed by RT-qPCR and normalized on β-actin mRNA level (Two-way [leucine deprivation, leucine supplementation] ANOVA: ns, non-significant; ***$p < 0.001$; $n = 6$ per condition; error bars, SEM). (C) Functionality and reversibility of leucine deprivation-induced activation of IL-10 protein expression. Activated primary AARE-TK-IL-10 transduced human T cells (LT AARE-TK-IL-10) were incubated in control (Ctl) or in leucine-free (−Leu) media during 16 h, then leucine-starved T cells were supplemented with leucine and harvested after additional 24 or 48 h of incubation. IL-10 concentration was assessed by ELISA (Two-way [leucine deprivation, leucine supplementation] ANOVA: ns, non-significant; ****$p < 0.0001$; $n = 3$–6 per condition; error bars, SEM). (D, E) Functionality of IL-10-NUTRIREG in CD45+, CD4+ and CD8+ cells after leucine deprivation. Activated primary human non-transduced T cells (NT cells) or AARE-TK-IL-10 transduced T cells (LT AARE-TK-IL-10) were incubated in control (Ctl) or in leucine-free (−Leu) media and harvested after 16 h of incubation. Expression of intra-cellular IL-10 was analyzed by flow cytometry. Results are expressed as a percentage of IL-10+ cells among CD45+ cells (left) or CD4/CD8+ cells (right) (Two-way [leucine deprivation, transduction] ANOVA: ns, non-significant; ***$p < 0.001$; ****$p < 0.0001$; $n = 7$–12 per condition; error bars, SEM). (F, G) Induction of Tr1 cell profile by transient expression of IL-10. (F) Activated primary human non-transduced T cells (NT cells) or AARE-TK-IL-10 transduced T cells (LT AARE-TK-IL-10) were incubated in control (Ctl) or in leucine-free (−Leu) media and harvested after 16 h of incubation. Transcription factors or cytokines mRNA expression level was assayed by RT-qPCR and normalized on β-actin mRNA level (Two-way [leucine deprivation, transduction] ANOVA: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; $n = 3$–6 per condition; error bars, SEM). (G) Activated primary human non-transduced T cells (NT cells) or AARE-TK-IL-10 transduced T cells (LT AARE-TK-IL-10) were incubated in control (Ctl) or in leucine-free (−Leu) media during 16 h, then leucine-starved T cells were supplemented with leucine and harvested after additional 24 h of incubation. Co-expression of CD49b and LAG-3 was analyzed by flow-cytometry. Results are expressed in percentage of cells with co-expression of CD49b and LAG-3 among CD4+ cells (Two-way [leucine deprivation then supplementation, transduction] ANOVA: ns, non-significant; **$p < 0.01$; ***$p < 0.001$; $n = 5$–6 per condition; error bars, SEM). (H) Monitoring of human IL-10 in mouse plasma. NXG immunodeficient mice received 10.10^{6} human T cells transduced with a viral vector containing AARE-TK-IL-10 construct and 5.10^{6} human irradiated PBMC by tail vein injection. Seven days later, and then once a week until week 3, mice were fed with a control diet or leucine-deficient diet for 16 h, prior to blood sampling for flow cytometry analysis and IL-10 measurement. Plasmatic human IL-10 concentration was assayed by ELISA according to Methods section, every week from week 1 to week 3, just after mice consumption of control diet or leucine-deficient diet for 16 h ($n = 3$–4 per condition; linear regression analysis).

group ($R^2 = 0.581$; $p = 0.004$) and was not significantly changed over time in control group (Figure 3H). Collectively, these results demonstrate that the NUTRIREG technology is able to regulate, in vitro and in vivo, a therapeutic gene expression, such as IL-10, in activated human T cells.

4 | DISCUSSION AND CONCLUSIONS

These present results open up new avenues for considering number of NUTRIREG applications to control T cell therapy strategies. Hence in allo-HSCT, allogeneic donor T cells restore tumor immunosurveillance in transplanted patients, resulting in elimination of residual leukemic cells, a phenomenon called the Graft versus Leukemia (GvL) effect. Unfortunately, the GvL effect is often accompanied by the destruction of patient's normal tissues, the graft versus host disease (GvHD), which is a major side effect of allo-HSCT. We demonstrated here that the NUTRIREG-T cells system comprising an IL-10 expression transgene promotes the expansion of human Tr1 lymphocytes and could then slow down the cytotoxic response responsible for GvHD, as already shown in case of T cell transduction with an IL-10 expressing lentivirus in mouse models.[26,27] Donor T cells (present in allo-stem cells in the graft, or in secondary Donor Lymphocytes Injection) could genetically be modified ex vivo with a lentiviral vector including a Tr1-inducing IL-10 transgene before reinjection into the patient. This could allow, by a simple consumption of a diet deficient in an EAA for a few hours, (i) to express this Tr1-inducing transgene in activated T cells (which are responsible for GvHD), and (ii) to promote polarization of the T cells response toward a Tr1 regulatory phenotype. The On/Off modulation enabled by the NUTRIREG technology in T cells is furthermore a key asset since the consumption of the missing EAA will stop the expression of the transgene that promotes human T cell differentiation into Tr1 in due time. It could thus preserve the anti-tumoral effect of the graft, which is not possible with traditionally transduced T cells or immunosuppressive drugs used in standard care. Other potential applications of NUTRIREG in cellular immunotherapy may concern the control of genetically modified T cells, such as CAR-T or TCR-T cell therapies. The high level of T cell activation following tumor antigen recognition is a key asset for the use of NUTRIREG due to the need of T cell activation for the induction of GCN2-ATF4 signaling pathway. NUTRIREG could thus be easily used in CAR-T cell or in TCR-T cell either to transiently (i) boost their activity or (ii) break down their toxicity. As an example, NUTRIREG could allow transient expression of a gene of interest in preventing the exhaustion of the T cells, which is one of the main issues for CAR-T or TCR-T cells persistence and efficacy. Exciting results have been described in the literature regarding the overexpression of c-JUN in CAR-T cells, making them resistant to exhaustion.[30] A transient and repeated expression of c-JUN under the dependence of NUTRIREG in the CAR-T cell or in the TCR transgenic T cell could thus make it resistant to exhaustion and promote the maintenance of its efficacy over time.

To conclude, our results represent a solid basis for the promising use of NUTRIREG in numerous preventive or curative cellular immunotherapy strategies.

AUTHOR CONTRIBUTIONS

Aurore Dougé, Alain Bruhat and Paul Rouzaire designed the experiments; Aurore Dougé, Cyrielle Vituret, Valérie Carraro, Laurent Parry and Cécile Coudy-Gandilhon performed the experiments; Aurore Dougé analyzed the data; Alain Bruhat and Paul Rouzaire supervised Aurore Dougé's work; Aurore Dougé, Alain Bruhat, Paul Rouzaire wrote the paper; Cyrielle Vituret, Richard Lemal, Lydie Combaret, Anne-Catherine Maurin, Julien Averous, Céline Jousse, Jacques-Olivier Bay, Pierre Verrelle and Pierre Fafournoux reviewed the paper.

ACKNOWLEDGMENTS

We thank (i) Julien Hermet, Mehdi Djelloul-Mazouz and Yoann Delorme (animal facilities of INRAE institute, Clermont-Ferrand) for animal care and technical assistance, (ii) Silvère Baron, Philippe Mazuel and Sandrine Plantade (animal facilities of UCA, Clermont-Ferrand) for animal care and technical assistance, (iii) Christophe Ferrand and Marina Deschamps (INSERM, Besançon) for technical advices and (iv) Miltenyi Biotec society for the initial loan of MacsQuant. We also thank Isabelle Papet, UNH, INRAE for the critical reading of the manuscript.

FUNDING INFORMATION

This study was supported by a grant from the UCA I-Site Emergence Program, by Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), France, by the University Hospital of Clermont-Ferrand (DRCI) and by the startup NUTRITHERAGENE.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Dougé A, Vituret C, Carraro V, et al. Temporal regulation of transgene expression controlled by amino acid availability in human T cells. *HLA*. 2023;1-12. doi:10.1111/tan.15252