

## Alantolactone ameliorates graft versus host disease in mice

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### ARTICLE INFO

#### Keywords:

Alantolactone  
 Graft versus host disease  
 Bone marrow transplantation  
 Allogeneic transplantation  
 Autoimmunity

### ABSTRACT

The anti-inflammatory and immunosuppressive drugs which are used in the treatment of Graft-versus-Host Disease (GVHD) have limited effects in controlling the severity of the disease. In this study, we aimed to investigate the prophylactic effect of Alantolactone (ALT) in a murine model of experimental GVHD.

The study included 4 BALB/c groups as hosts: Naïve (n = 7), Control GVHD (n = 16), ALT-GVHD (n = 16), and Syngeneic transplantation (n = 10). Busulfan (20 mg/kg/day) for 4 days followed by cyclophosphamide (100 mg/kg/day) were administered for conditioning. Allogeneic transplantation was performed with cells collected from mismatched female C57BL/6, and GVHD development was monitored by histological and flow cytometric assays. Additionally, liver biopsies were taken from GVHD patient volunteers between ages 2–18 (n = 4) and non-GVHD patients between ages 2–50 (n = 5) and cultured *ex vivo* with ALT, and the supernatants were used for ELISA.

ALT significantly ameliorated histopathological scores of the GVHD and improved GVHD clinical scores. CD8<sup>+</sup> T cells were shown to be reduced after ALT treatment. More importantly, ALT treatment skewed T cells to a more naïve phenotype (CD62L<sup>+</sup> CD44<sup>-</sup>). ALT did not alter Treg cell number or frequency. ALT treatment appears to suppress myeloid cell lineage (CD11c<sup>+</sup>). Consistent with reduced myeloid lineage, liver and small intestine levels of GM-CSF were reduced in ALT-treated mice. IL-6 gene expression was significantly reduced in the intestinal tissue. *Ex vivo* ALT-treated liver biopsy samples from GVHD patients showed a trend of decrease in pro-inflammatory cytokines but there was no statistical significance.

Collectively, the data indicated that ALT may have immunomodulatory actions in a preclinical murine GVHD model.

### 1. Introduction

Graft versus host disease (GVHD) is a life-threatening side effect of

hematopoietic stem cell transplantation (HSCT) which results due to alloreactivity between the donor and the recipient. Donor T cells recognize recipient tissue antigens as foreign and develop an immune

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<https://doi.org/10.1016/j.intimp.2024.111560>

Received 17 October 2023; Received in revised form 17 December 2023; Accepted 15 January 2024

Available online 20 January 2024

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response against these antigens [1]. GVHD could be observed in 40 to 60 % of the patients receiving HSCT [2]. The 5-year survival rates of GVHD patients were 46 % for siblings and 33 % for unrelated donor transplants [3]. Generally, GVHD is classified as acute if appears within 100 days of the transplantation, or chronic if occurs beyond 100 days leading to organ damage in the liver, intestine, and skin [4].

Acute GVHD develops in three consecutive steps: activation of antigen-presenting cells, donor T cell activation-differentiation- migration, and effector phase [5]. Antigen-presenting cell activation takes place in the host due to the underlying diseases, infections, or conditioning regimens (Total Body Irradiation (TBI) and chemotherapy) prior to transplantation which results in the release of endogenous or exogenous damage- or pathogen-associated molecular patterns called DAMPs and PAMPs [6]. These molecules are recognized by various receptors, subsequently, leading to inflammatory changes, including the secretion of proinflammatory chemokines and cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 1 (IL-1), and interleukin 6 (IL-6). Following the activation and expansion, large numbers of alloreactive donor T cells infiltrate the inflamed tissues. Regulatory T cells and suppressive cytokines IL-10 and TGF- $\beta$  have been shown to counter the inflammatory immune responses by inhibiting proliferation and stimulation of alloreactive donor T cells [7 8]. In the third phase of GVHD, cytotoxic T lymphocytes, NK cells, and inflammatory molecules (TNF $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ ) work in synergy to perpetuate the inflammation that damages the target tissues [9].

Although most mouse models of GVHD use the TBI conditioning regimen and bone marrow-derived graft, new models have been developed which employ the chemotherapy conditioning regimen and the G-CSF mobilized graft with remarkable clinical similarity. Evaluation of the GVHD clinical score with respect to the weight loss, posture, activity, fur condition, and skin integrity after transplantation into mice was used in the clinical diagnosis of GVHD [10]. Pathological damage in the liver, skin, kidney, small intestine, and colon tissue samples has been shown in such GVHD models [11–14].

Immunosuppressive drugs such as tacrolimus, sirolimus, and methotrexate take an important place in GVHD treatment [15]. In addition to cyclophosphamide, Janus Kinase 2 (JAK2) Inhibitors, proteasome, and histone deacetylase inhibitors or inhibitors of B cell development could present opportunities for the treatment of GVHD [1]. However, these treatment methods also suppress the immune response to viral and bacterial infections and have side effects. Additionally, the therapeutic agents cannot efficiently treat GVHD because GVHD has already developed by the time treatment started [16].

Sesquiterpene lactone family, which are characterized by presence of a lactone ring in their structure, has been widely used in traditional Chinese medicine in the context of inflammatory diseases and cancer [17]. The malaria drug Artemisinin, for which the 2015 Nobel prize was awarded, is also a sesquiterpene lactone. Parthenolide (PN) and its soluble analog dimethylaminoparthenolide (DMAPT) have been tested in cancer therapy [18,19]. Sesquiterpene lactones have been studied in the context of inflammation by many investigators [20]. *Arctium lappa* L. extract enriched in the germacranolide onopordopicrin was reported to decrease colitis-associated histological damage in rats and prevent mucin layer loss in a model of colitis in rats [21]. Similarly, Alantolactone (ALT) has been demonstrated to be effective in ameliorating Dextran sulfate sodium (DSS) -induced colitis [22]. ALT is a type of sesquiterpene lactone extracted from *Inula helenium* L. plants and has anti-inflammatory, antiproliferative, and antimicrobial biological activities [23,24]. ALT has recently been shown to suppress cigarette smoke extract-induced inflammation, apoptosis, and oxidative stress by modulating the nuclear factor- $\kappa$ B (NF- $\kappa$ B) and nuclear factor erythroid 2-related factor 2 (NRF2) / Heme oxygenase-1 (HO-1) axis [25]. The therapeutic potential of ALT or other sesquiterpene lactone family members in GVHD has not yet been tested to this date.

In the current study, we tested the prophylactic effect of ALT in a murine model of experimental GVHD. The data obtained revealed that

ALT suppressed myeloid lineage and decreased Granulocyte macrophage colony stimulating factor (GM-CSF) production and showed histopathological improvement in skin, liver, kidney small intestine, and colon in a GVHD mouse model. ALT also decreased activated memory T cells. When human tissue biopsies from GVHD patients were treated with ALT ex vivo, especially the liver tissue, decreasing trend in the levels of pro-inflammatory cytokines such as IL-1 $\beta$ , IL-17A, IFN- $\gamma$ , and IL-23 were observed. However, there were no statistically significant changes in the level of these cytokines. Collectively, the data presented herein suggests that ALT may improve GVHD symptoms by reducing GM-CSF production, suppressing myeloid cell frequency, and by reducing T-cell activation.

## 2. Material and methods

Detailed methods are given in [Supplemental Methods](#).

### 2.1. Study approval, mice and reagents

The animal study was approved by the animal ethics committee of Erciyes University (Approval Number 2020/056). The human study was approved by the Institutional Review Board of Erciyes University (Approval Number 2023/612). The Balb/c H2k<sup>d</sup> mice were housed under specific pathogen-free conditions. Four groups of mice were used as hosts: Group 1. Naïve (n = 7), 2. Control GVHD (n = 16), 3. ALT GVHD (n = 16), 4. Syngeneic (n = 10). C57BL/6 H2k<sup>b</sup> mice were used as allogeneic donors. ALT was diluted in Dimethyl sulfo-oxide (DMSO), thus DMSO was given to all other groups as vehicle.

### 2.2. GVHD induction and ALT treatment

The 8 weeks old Balb/c mice were used as host and divided into four groups. 1.Naïve (n = 7), 2.Control GVHD (n = 16), 3.ALT-GVHD (n = 16) and 4.Syngeneic transplantation (n = 10). Allogeneic transplantation was performed with cells collected from MHC mismatched, 6 to 8 weeks old female C57BL/6 mice. Groups and treatments were shown in [Supp. Table 1](#).

For host conditioning, busulfan and cyclophosphamide were given to the control, syngeneic, and ALT groups at 20 mg/kg/day for 4 consecutive days followed by 100 mg/kg/day for 2 additional days, respectively, as described earlier [10]. After busulfan and cyclophosphamide treatment, all mice were rested for one day. Recombinant G-CSF was injected to prepare the donors (both Balbc and C57BL6 mice) at 10  $\mu$ g/ml for 5 days intraperitoneally while the host mice were being conditioned. The donor mice were sacrificed and lymphocytes isolated from the lymph node and spleen were combined and 1x10<sup>7</sup> cells/mice from C57BL6 donors were injected into ALT and control groups *retro-orbitally*. The same number of cells from Balb/c syngeneic donors were injected to the syngeneic group *retro-orbitally*. ALT was given at 15 mg/kg dose twice on day + 5 day and + 7 intraperitoneally. As vehicle 100  $\mu$ l DMSO was injected to control and syngeneic groups [26].

### 2.3. Human samples

Liver biopsies were taken from GVHD patients between ages 2–18 (n = 4) and non-GVHD patients between ages 2–50 (n = 5) at Erciyes University School of Medicine Department of Pediatric Hematology & Oncology and Gastroenterology. After the diagnosis of cGVHD in patient 2, recurrence developed. A second sample from the same patient was included in the study. For pediatric patients, there is no control group. Patient information is shown in [Table 1](#).

**Table 1**  
GVHD information and general characteristics of patients.

GVHD patient	1	2-1	2-2	3	4
Age (y)	14 years 9 months	2 years 3 months	2 years 6 months	6 years 3 months	3 years 2 months
Sex	Male	Female	Female	Male	Male
Diagnosis	cGVHD/ALL	cGVHD/JMML	cGVHD/JMML	aGVHD/ALL	aGVHD/Interferon gamma receptor defect
Stem cell source	HSC	HSC	HSC	HSC	PBSC
GVHD stage	2	2	2	1	2
GVHD prophylaxis	MTX/CSA	MTX/CSA	MTX/CSA	MTX/CSA	MTX/CSA
Match	MSD	MUD	MUD	MUD	MUD
Concomitant drug therapy	Méthylprednisolone and CSA	Méthylprednisolone, tacrolimus, ruxolitinib	Methylprednisolone, ProlastinC/photophoresis	Méthylprednisolone and CSA	Méthylprednisolone, Tacrolimus, Ruxolitinib, MMF, Prolastin C

ALL: Acute lymphoblastic Leukemia, JMML: Juvenile Myelomonocytic Leukemia, aGVHD: acute Graft Versus Host Disease, cGVHD: chronic Graft Versus Host Disease, HSC: Hematopoietic Stem Cell, PBSC: Peripheral Blood Stem Cell, CSA: Cyclosporine A, MTX: Methotrexate, MUD: Matched Unrelated Donor, MSD: Match Sibling Donor, MMF: mycophenolic acid.

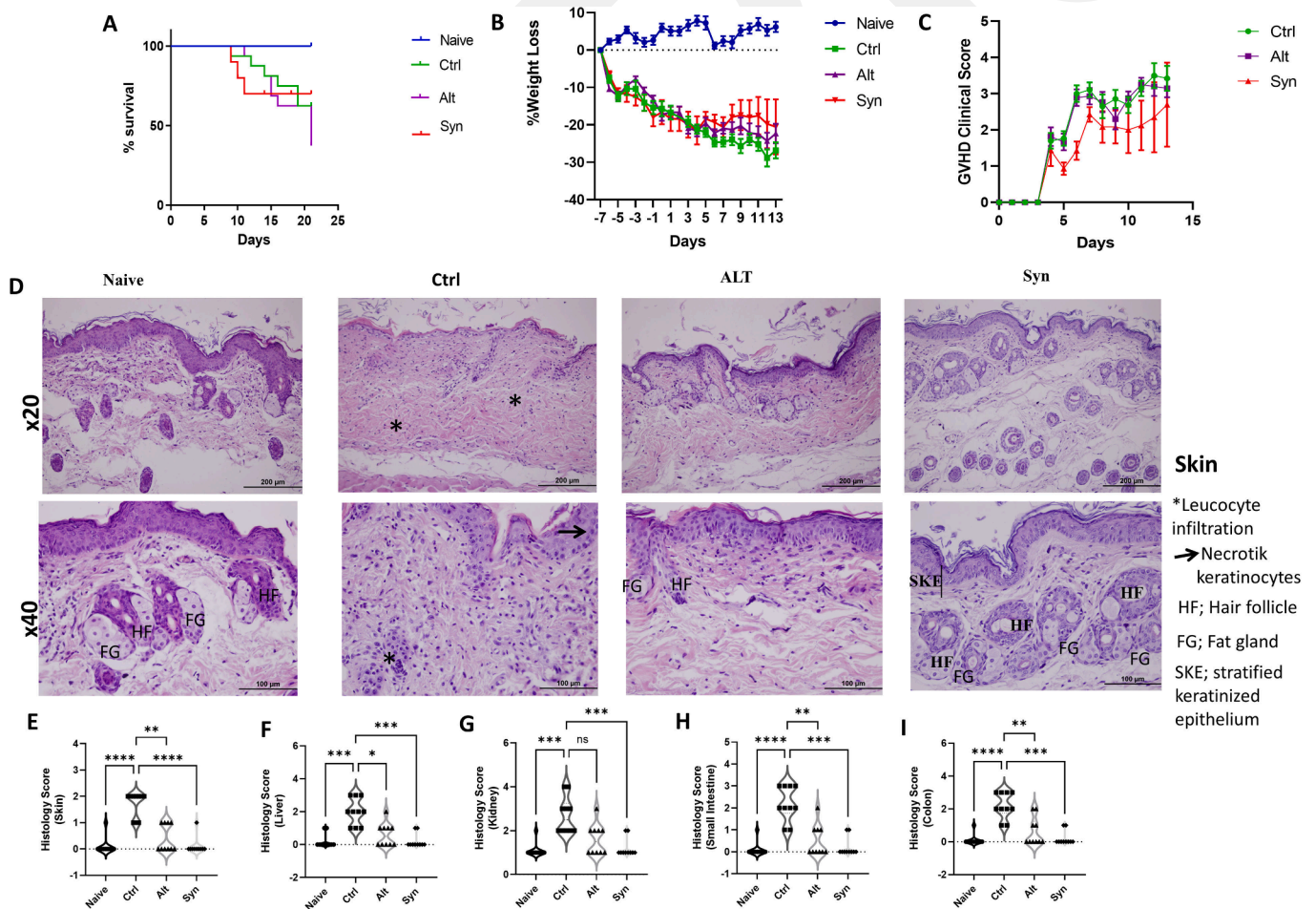
### 3. Results

#### 3.1. ALT does not affect survival or weight loss in GVHD mice but ameliorates GVHD clinical and histopathological scores

The mismatch of MHC I alleles between Balb/c and C57BL/6 in our

facility was verified by H2K<sup>b</sup> staining. Host mice both in the naïve and syngeneic transfer groups (both having Balb/c derived hematopoietic cells) stained negative for H2K<sup>b</sup> whereas most mice in both control and ALT groups (which received C57BL/6 derived lymphocytes) stained positive for H2K<sup>b</sup> (Supp. Fig. 1A-B).

We chose to use the GVHD model developed by Ye et al. which

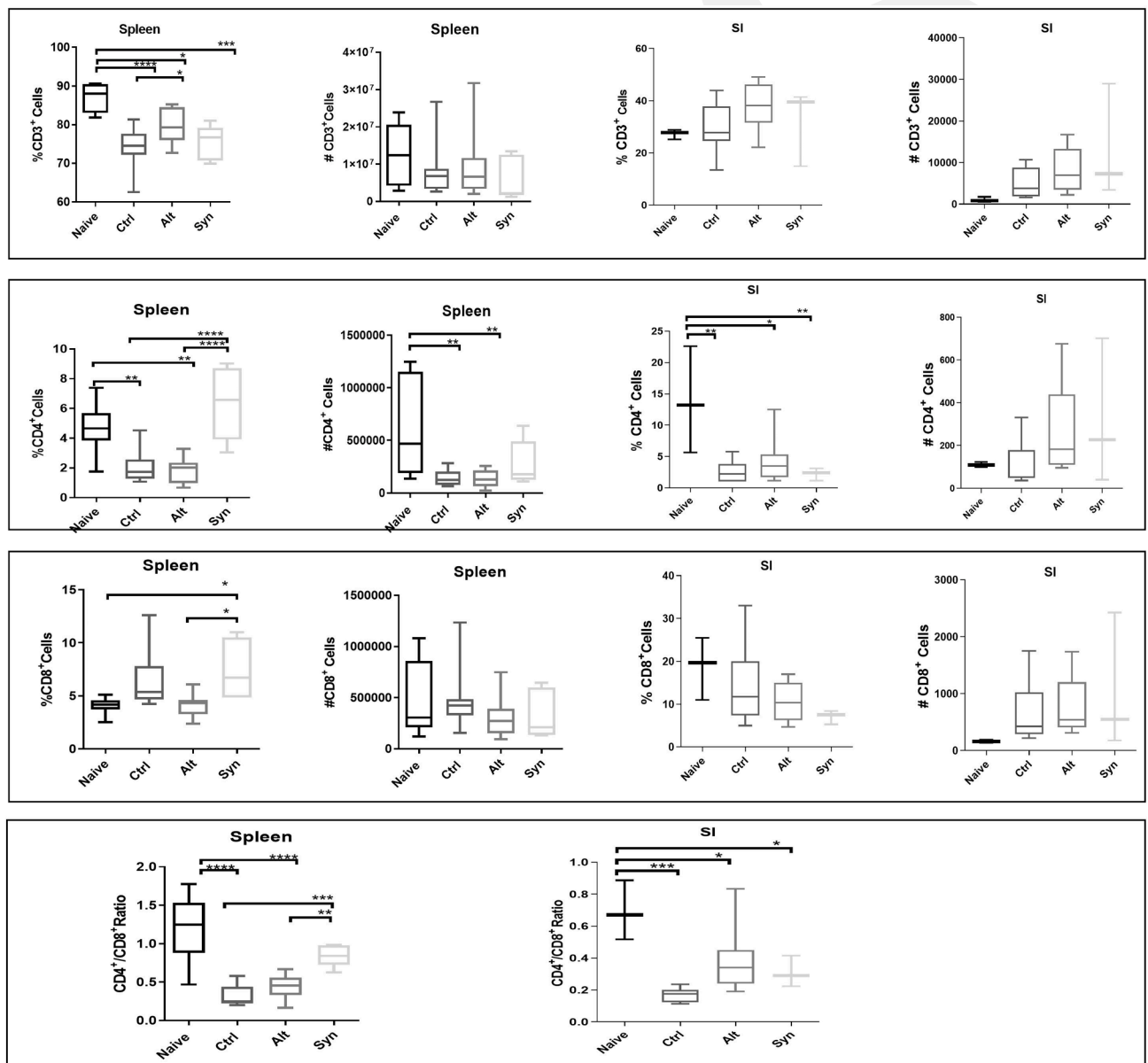


**Fig. 1.** ALT does not affect survival or weight loss in GVHD mice but ameliorates GVHD clinical scores and GVHD histopathological scores. (A) Survival graph of mice groups ( $p = 0.370$ ). (B) Percent weight loss over time in all experimental groups. (C) GVHD clinical scores ( $p > 0.05$ ). (D) Representative histology pictures from the skin of each experimental group. \* Leucocyte infiltration, HF; Hair follicle, FG; Fat gland, SKE; stratified keratinized epithelium, Necrotic keratinocytes. (E-I) ALT reduced histopathological scores in the skin, liver, kidney, small intestine, and colon, respectively.

employed HLA-mismatched *in vivo* G-CSF expanded splenocyte/lymphocyte transfer due to its ease [10]. Our experiment was terminated on the + 13th day of the transplantation protocol (and the 21st day of the initiation of the conditioning) due to weight loss and rapid development of GVHD. None of the mice in the naive group showed GVHD symptoms. While 5 (50 %) of the mice in the syngeneic group died, likely due to conditioning regimen-related complications the remaining (50 %) survived, and the mean survival time was found to be 16.7 days. In the control GVHD group, the survival percentage was 62.5 % and the mean survival time was found to be 18.7 days. ALT-GVHD group had a 62.5 % survival percentage which was higher than the syngeneic group, but identical to the control group. There was no significant difference in survival between the groups ( $p = 0.370$ ) (Fig. 1A). Since the 7th day, weight loss was similar in all groups except the naive group. The experiment was terminated on the + 13th day due to dramatic weight loss in the control GVHD group which reached 30 % (Fig. 1B). Although a trend towards reduction was observed in clinical scores of the ALT

group, it did not reach the statistical significance ( $p > 0.05$ ) (Fig. 1C).

The histopathological effects of ALT were evaluated in all groups based on the previously defined scoring systems of the liver, skin, small intestine, and colon [11,12]. When the skin sections of the naive group were evaluated histologically, the epidermis consisting of stratified squamous keratinized epithelium and the dermis layers consisting of hair follicles and skin appendages consisting of sebaceous glands could be clearly distinguished. When compared to the control-GVHD group, statistically significant improvements were recorded in the histopathological scores of the ALT-treated group skin. The histology of the control GVHD group showed standard pathological features of acute GVHD, including necrotic keratinocytes, mononuclear cell infiltration, atrophy of hair follicles and sebaceous glands, and dermal fibrosis. Significant improvement in skin pathology scores was observed in the ALT-GVHD group (Fig. 1D-E). Significant histopathological improvement was observed also in the liver, kidney, small intestine, and colon samples of the ALT-GVHD group compared to the control GVHD group (Fig. 1F-I).



**Fig. 2.** ALT treatment increased CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio. (A) Percentage and absolute number of CD3<sup>+</sup> cells in the spleen or small intestine (SI) lamina propria across GVHD mice groups. (B) Percentage and absolute number of CD4<sup>+</sup> T cells in the spleen or small intestine (SI) lamina propria across GVHD mice groups (C) Percentage and absolute number of CD8<sup>+</sup> T cells in the spleen or small intestine (SI) lamina propria across GVHD mice groups. (D) CD4<sup>+</sup>/CD8<sup>+</sup> cell ratio in spleen and small intestine (SI). (\*) indicates  $p < 0.05$ , (\*\*) indicates  $p < 0.01$ , (\*\*\*) indicates  $p < 0.001$ , (\*\*\*\*) indicates  $p < 0.0001$ .

Collectively the data support a beneficial role for ALT in GVHD in mice.

### 3.2. ALT does not affect CD3<sup>+</sup> T cell number and percentages but alters CD4/CD8 ratio in GVHD mice

In order to test whether ALT had an impact on the expansion of T cell subsets, we examined CD3<sup>+</sup> T cells (CD4<sup>+</sup> and CD8<sup>+</sup>) in the spleen and small intestine in all mice groups. The gating strategy of T cell subsets in spleen and small intestine is shown in [supplement Fig. 2](#). The percentage of CD3<sup>+</sup> cells was significantly lower in the control GVHD, ALT and syngeneic groups in the spleen, although the absolute numbers remained comparable across all groups. Additionally, CD3<sup>+</sup> cell percentage was significantly higher in the ALT-GVHD group compared with the control-GVHD group ([Fig. 2A](#)). Small Intestine CD3<sup>+</sup> T cell numbers and frequency were comparable across mice groups. The percentage and absolute number of CD4<sup>+</sup> cells were significantly reduced in the control-GVHD and ALT-GVHD groups in the spleen compared with naïve mice groups ([Fig. 2B](#)). This reduction in CD3<sup>+</sup> cell frequency was also seen in the small intestine. CD8<sup>+</sup> T cell percentage was slightly elevated in the control GVHD group compared with the naïve and ALT-GVHD group. Similarly, in the syngeneic group, CD8<sup>+</sup> T cell frequency was significantly higher compared with ALT-GVHD and the naïve group in the spleen, although the absolute numbers were similar statistically across all groups ([Fig. 2C](#)). In addition, the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> cells was decreased in control GVHD, ALT-GVHD and syngeneic groups compared to the naïve group in the spleen and small intestine ([Fig. 2D](#)). However, the CD4/CD8 ratio was higher in the ALT-GVHD group compared with the control GVHD (although did not reach significance) reflective of a decrease in CD8<sup>+</sup> T cells in the ALT-GVHD group. ([Fig. 2C](#)). Collectively, these data indicate that ALT may affect the CD4/CD8 ratio in favor of the CD4<sup>+</sup> T cells and that this happens through ALT's negative action on CD8<sup>+</sup> rather than CD4<sup>+</sup> T cells.

### 3.3. ALT treatment decreased effector memory T cells in GVHD mice

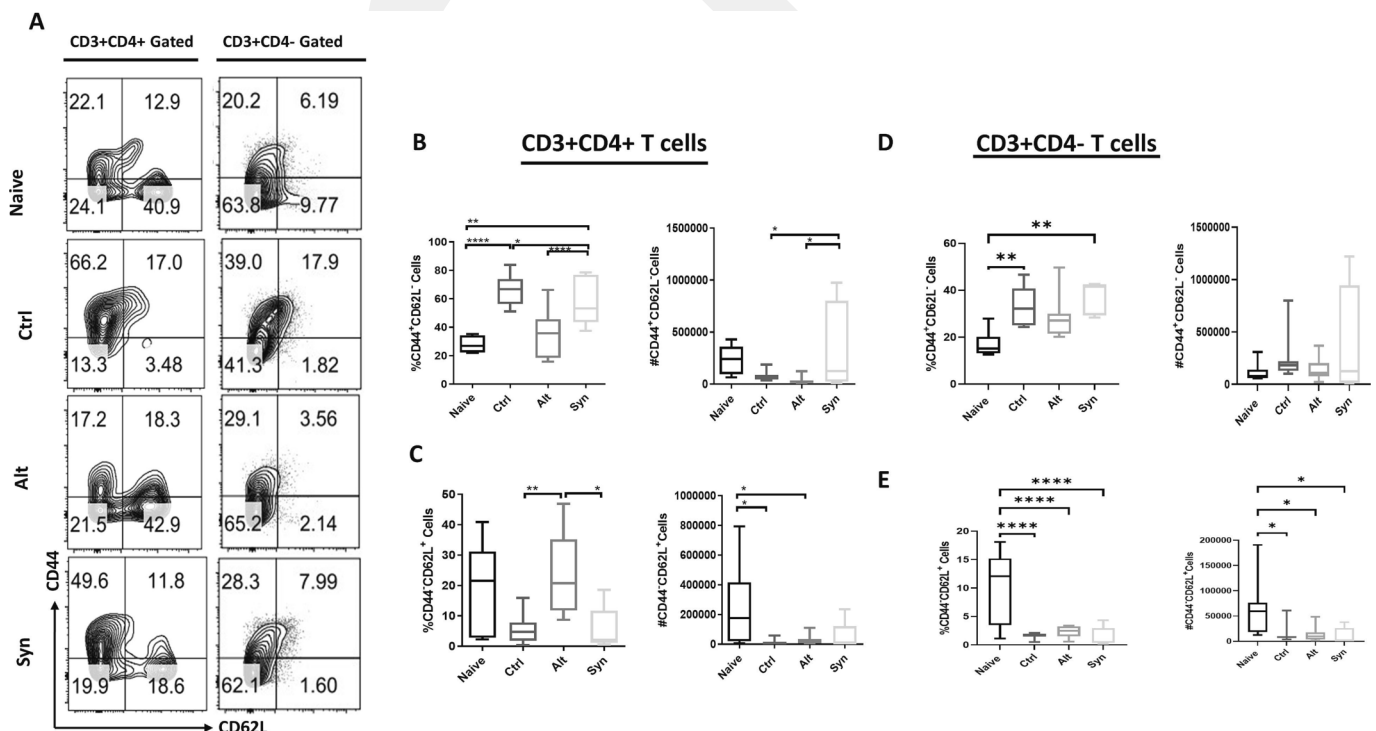
We also investigated naïve and memory T cell profiles in all groups by staining for CD44 and CD62L markers. A decrease in the ratio of activated T lymphocytes (CD44<sup>+</sup> CD62L<sup>-</sup>) in the spleen was observed in the ALT-GVHD group compared to the control GVHD ([Fig. 3A-B](#)). While CD44<sup>+</sup> effector memory cells were decreased in the ALT-GVHD group, CD44<sup>+</sup>CD62L<sup>+</sup> naïve T cells were significantly higher compared to the control GVHD group ([Fig. 3B-C](#)). Also we analyzed CD44<sup>+</sup> CD62L<sup>-</sup> and CD44<sup>+</sup>CD62L<sup>+</sup> T cells gated from CD3<sup>+</sup> CD4<sup>+</sup> cells (majority of which will be CD8<sup>+</sup> T cells) and observed the same trend in the cell population ([Fig. 3A-D-E](#)). In the small intestine, no statistically significant changes were observed ([Supp. Fig. 3A-B](#)). These data suggest that ALT may partly suppress T cell activation in this murine GVHD model.

### 3.4. ALT does not change Treg cell numbers but reduces myeloid lineage in the spleen of GVHD mice

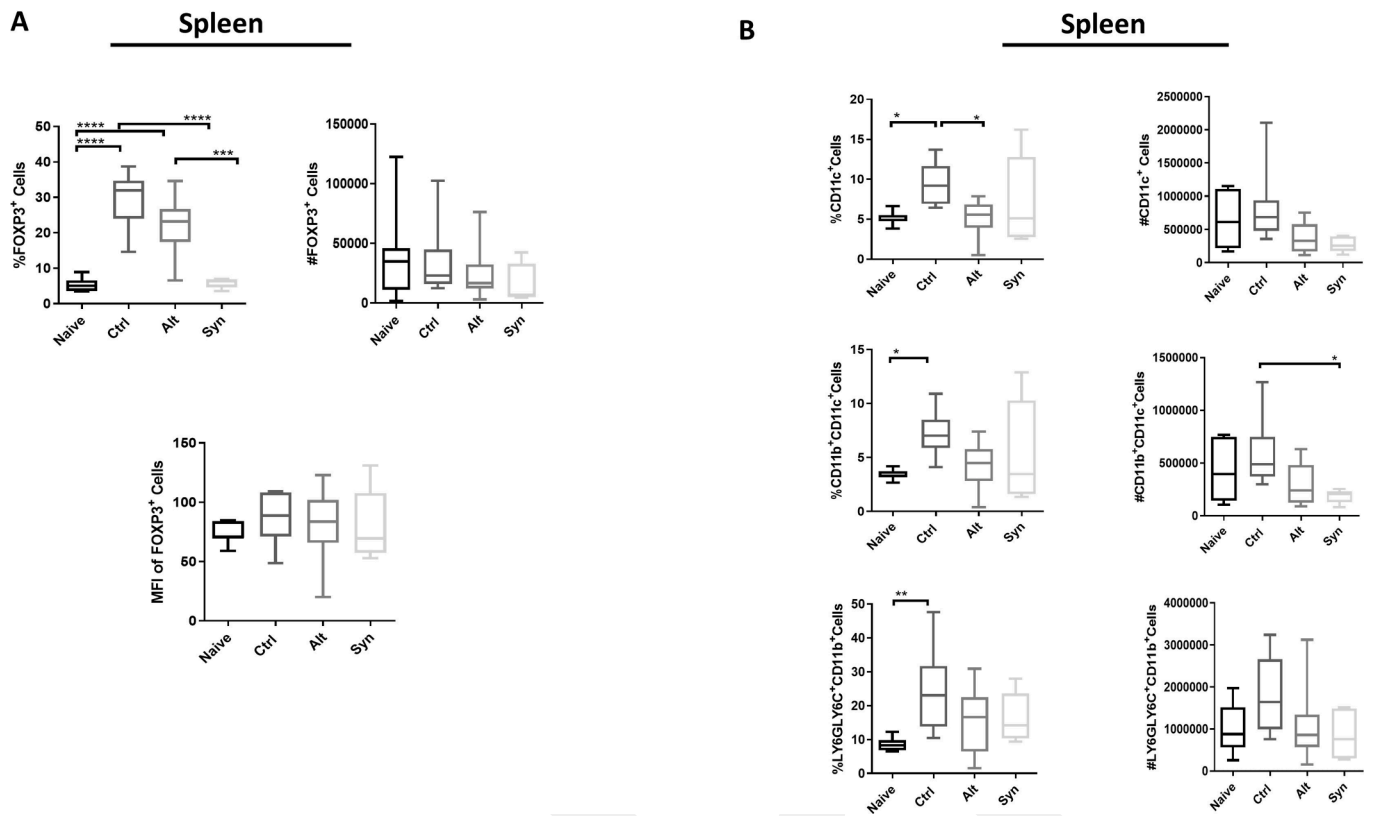
Treg cells are critical in restraining inflammation, thus CD3, CD4, and FOXP3 staining was performed on tissue-derived samples of GVHD mice. Also, we stained the cells with LY6GLY6C, LY6G, CD11b, CD11c, and NK 1.1 in all groups to examine the impact of ALT treatment on the myeloid cells. No increase in Treg cells was observed in the mice group given ALT ([Supp Fig. 4, Fig. 4A](#)). However, lymphocytes obtained from the spleen were stained significantly less with CD11c in the ALT-GVHD group compared to the control GVHD group, ([Fig. 4B](#)), suggesting that ALT may have impact on myeloid lineage, particularly dendritic cells, but not on Treg cells.

### 3.5. ALT reduces GM-CSF and IL-6 expressions and may play a role in myeloid cell generation and activity in GVHD mice

To have insight into the impact of ALT on inflammatory cytokine



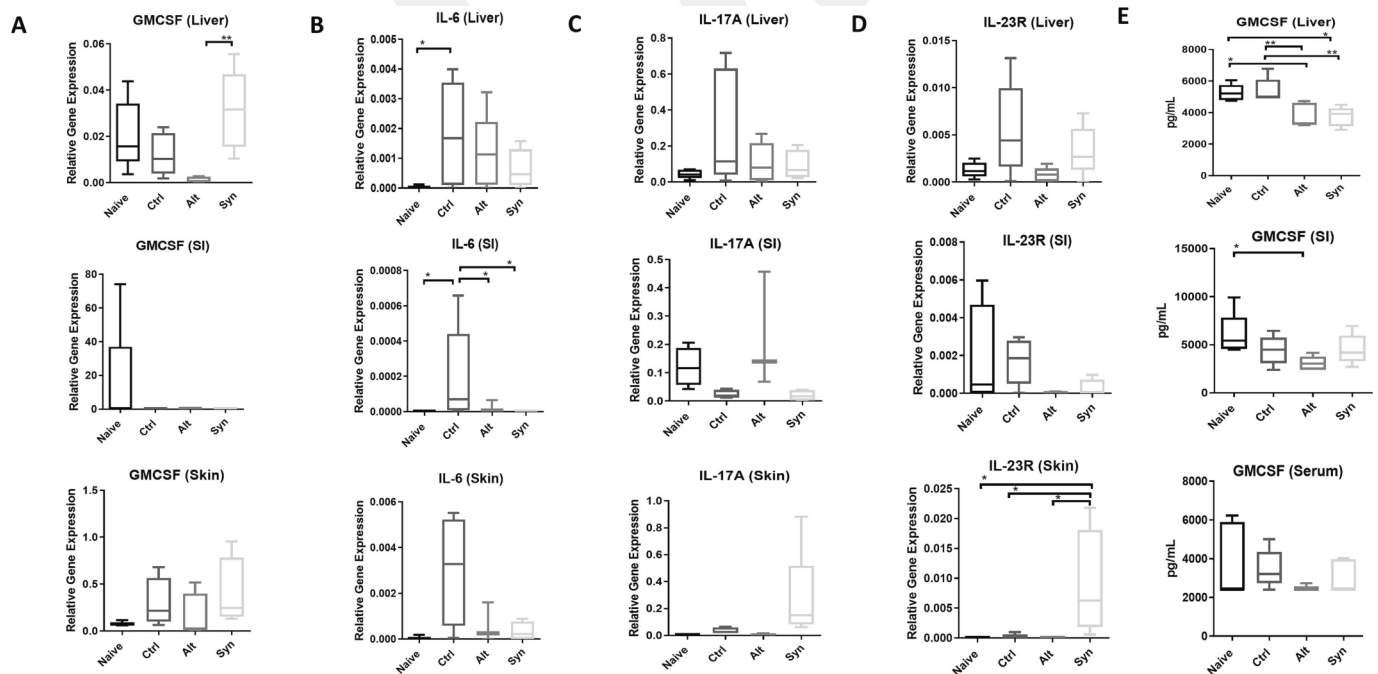
**Fig. 3.** ALT reduces naïve to effector memory phenotype switch *in vivo*. (A) Representative flow plots of CD3<sup>+</sup> CD4<sup>+</sup> or CD3<sup>+</sup> CD4<sup>-</sup> T cells showing CD44/CD62L expression. (B) Frequency and absolute number of CD44<sup>+</sup> CD62L<sup>-</sup> effector memory cells gated from CD3<sup>+</sup> CD4<sup>+</sup> T cells. (C) Frequency and absolute number of CD44<sup>+</sup> CD62L<sup>+</sup> effector memory cells gated from CD3<sup>+</sup> CD4<sup>+</sup> T cells. (D) Frequency and absolute number of CD44<sup>+</sup> CD62L<sup>-</sup> effector memory cells gated from CD3<sup>+</sup> CD4<sup>-</sup> T cells. (E) Frequency and absolute number of CD44<sup>+</sup> CD62L<sup>+</sup> effector memory cells gated from CD3<sup>+</sup> CD4<sup>-</sup> T cells. (\*) indicates  $p < 0.05$ , (\*\*) indicates  $p < 0.01$ , (\*\*\*) indicates  $p < 0.001$ , (\*\*\*\*) indicates  $p < 0.0001$ .



**Fig. 4.** ALT does not change Treg cell numbers but reduces CD11c<sup>+</sup> cells in the spleen of GVHD mice (A) The frequency and absolute number of Treg cells in the spleen and the mean fluorescent intensity (MFI) of FOXP3<sup>+</sup> in gated Treg cells across mice groups (B) Myeloid cell subsets in the spleen across all mice groups, CD11c, CD11b, LY6G, LY6C staining were performed. (\*) indicates  $p < 0.05$ , (\*\*) indicates  $p < 0.01$ .

expression, we analyzed mRNA expression levels with real-time qPCR in the target tissues of GVHD mice (liver, small intestine, and skin) (Fig. 5A-D). The qPCR data revealed that *CSF2* and *IL-6* gene expression

was the most dramatically impacted cytokines in the ALT-GVHD group. (Fig. 5A-B). Additionally, GM-CSF protein levels were measured by ELISA in the liver, small intestine, and the serum. These data also



**Fig. 5.** ALT reduces GM-CSF and IL-6 levels in GVHD mice. (A) *CSF2* mRNA expression in the liver, SI, and skin. (B) *Il6* gene expression in liver, SI, and skin. (C) *Il17A* gene expression in liver, SI, and skin (D) *Il23r* gene expression in liver, SI, and skin. (E) GM-CSF cytokine levels in supernatants of the liver, small intestine (SI), and serum. (\*) indicates  $p < 0.05$ , (\*\*) indicates  $p < 0.01$ .

confirmed that ALT reduced GM-CSF production in GVHD mice significantly (Fig. 5E). In addition, although there was a decreasing trend for *Il17a* (in liver and skin) and *Il23R* (in the liver, small intestine, and skin) they did not reach significance (Fig. 5C-D). Given the role of GM-CSF in dendritic cell maturation and generation, the reduced CD11c<sup>+</sup> cell data are in line with reduced GM-CSF and IL-6 levels, supporting the hypothesis that ALT may alleviate GVHD through its action on GM-CSF-myeloid cell axis.

We also acquired liver biopsy tissues from GVHD patients and exposed the biopsy tissues to ALT *ex vivo* and investigated the effects of ALT on inflammatory cytokines released to the supernatants. In the supernatant of liver biopsies treated with ALT, IL-1 $\beta$ , IL-18, and IL-33 were slightly decreased but they did not reach significance (Supp Fig. 5A-C). Similarly, there were trends towards reduction for IL-17A, IFN- $\gamma$ , TNF- $\alpha$ , IL-12p70, and IL-23 cytokines as well however these did not reach significance either (Supp Fig. 5D-H).

#### 4. Discussion

Acute GVHD develops after allogeneic HSCT in up to 50 % of fully matched recipients and is treated by algorithms that take into account the disease severity, and the affected organs [27,28]. The first line of therapy is often steroids and is effective in almost 50 % of the cases. A second line of therapy is added to steroids in resistant cases. To this day, several treatment options have been developed and used from biologicals to chemical inhibitors, however, there is no standard treatment, and thus novel preventive and therapeutic drugs are needed to effectively prevent or cure GVHD. ALT is a sesquiterpene lactone whose anti-inflammatory properties have been demonstrated [29]. It remains unclear by which mechanisms this molecule suppresses inflammation, though studies implicated NF- $\kappa$ B and Nrf2/HO-1 axis [25]. In the current study, we investigated for the first time the efficacy of ALT treatment in the acute GVHD mouse model. Our study revealed that ALT may ameliorate symptoms of GVHD when given prophylactically, by reducing T cell activation, reducing CD8<sup>+</sup> T cell and dendritic cell/myeloid cell expansion, and GM-CSF production.

ALT did not improve weight loss and survival rates in our study. However, although not statistically significant, ALT showed a tendency to improve in clinical GVHD scores. The improvement in clinical scores would probably have been more pronounced in the ALT-treated group had we had more mice in the groups and had not recorded high post-transplant mortality rates. Nevertheless, twice ALT treatment significantly improved histopathological scores of GVHD suggesting a beneficial role for ALT in this murine model.

T cells encounter activated antigen-presenting cells (APC) which come from the damaged tissues to the draining lymph nodes, and in the last phase, the effector phase, T lymphocytes which arrive in the tissues initiate the events that lead to the development of GVHD. In the pathogenesis of GVHD, the high HLA-reactive CD8<sup>+</sup> T cell counts in the effector phase are the main source of the pathology in the target organs such as skin, liver, and colon [30]. In this study, we observed a decrease in the amount of CD8<sup>+</sup> T cells and an increase in the ratio of CD4/CD8 in the spleen and intestine samples of the GVHD mice group administered ALT, but there was no statistical significance between these data. Dunn's test is significant only when a paired comparison was made ( $p < 0.005$ ). When post-hoc Dunn's is applied, the p-value is 0.0509. The data suggest that ALT's effect was on CD8<sup>+</sup> T cells rather than CD4<sup>+</sup> since the numbers of CD4<sup>+</sup> T cells were similar in ALT-treated and non-treated GVHD groups. Although others have shown that ALT may reduce CD4<sup>+</sup> T cells' differentiation into Th17 [31], and that ALT may exert cytotoxicity over leukemia cells [32], it is yet unclear how ALT may selectively target CD8<sup>+</sup> T cells. This may perhaps be partly explained by the reduction in CD11c<sup>+</sup> cells which are ultimately the cells that will present donor antigens to CD8<sup>+</sup> T cells and prime them. The data regarding CD44/CD62L staining showed that ALT inhibited T cell activation *in vivo* post-transplant. Thus, the effector phase of GVHD may be

delayed or partly blocked by ALT. These results are in line with the study by Aggarwal et al., which showed reduced severity of GVHD after blockade of CD44 [33].

Our data also showed a significant decrease in the frequency of CD11c<sup>+</sup> cells (a dendritic cell marker) in the spleen tissue samples in the ALT-GVHD group. Our data also confirmed an increase in the frequency of myeloid and Treg cells in the intestine and spleen due to alloreactivity during the development of GVHD [34]. However, ALT did not alter Treg cell frequency or counts, suggesting that the beneficial effects of ALT are probably mediated by mechanisms other than Treg. The decrease in the myeloid, especially CD11c<sup>+</sup> cells is accompanied by a decrease in GM-CSF cytokine, which is implicated in DC development, maturation, and function [35]. This reduction was more evident in the liver at the protein levels and was showing similar trends in other organs, as well as at the mRNA levels. These data suggest that ALT may also control the first two phases of GVHD such as antigen-presenting cell activation and presentation of host antigens to donor T cells. Because our results show that ALT inhibits myeloid lineage, CD11b<sup>+</sup>, CD11c<sup>+</sup> cells which are the antigen presenting cells (APC), the decrease in alloreactive CD8<sup>+</sup> T cell expansion, and also reduction in the inactivation status of T cells could in part be attributed to this reduction in APCs, which prime and activate the T cells to allogeneic antigens in the first place.

Our data also revealed a reduction in IL-6 mRNA levels in the GVHD mice small intestine after ALT treatment which may be also related to improvement in GVHD symptoms in this study (liver and skin data did not reach significance despite a trend toward reduction). Betts et al. reported improvement in GVHD clinical scores, similar to our data, in the GVHD mouse model after treatment with tocilizumab, an IL-6 receptor blocker [36]. Although it has not been tested in this report, ALT may further suppress the JAK2/STAT3 pathway used by IL-6 given that prior research demonstrated its negative impact on STAT3 signaling [31]. We also tested ALT's impact on human liver biopsies from GVHD patients *in vitro*, however, the cytokines tested did not differ significantly from those not treated with ALT. This may be due to the limited number of patient samples, tissue samples taken from patients with cGVHD, and ineffective penetration of ALT into the liver tissue due to the thickness of the biopsies.

In summary, statistically significant improvement was observed in tissue damage scores with respect to lymphocyte infiltration in skin, liver, and intestinal tissue samples, in GVHD when ALT was prophylactically given. The data also suggest that ALT may negatively impact CD11c<sup>+</sup> cells, GM-CSF, and IL-6 levels and naïve to effector transition of CD3<sup>+</sup> T cells. These results indicate that ALT may have potential for GVHD treatment as an immunomodulatory agent. More clinical studies are needed to determine its safety and efficacy in human GVHD.

#### CRedit authorship contribution statement

**Gul Pelin Odabas:** Conceptualization, Methodology, Writing – review & editing, Writing – original draft. **Kubra Aslan:** Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Pinar Alisan Suna:** Methodology. **Perihan Kader Kendirli:** Methodology. **Şerife Erdem:** Methodology. **Mustafa Çakır:** Methodology. **Alper Özcan:** Data curation. **Ebru Yılmaz:** Data curation. **Musa Karakukcu:** Data curation. **Hamiyet Donmez Altuntas:** Conceptualization. **Arzu Hanim Yay:** Methodology. **Kemal Deniz:** Data curation. **Derya Altay:** Data curation. **Duran Arslan:** Data curation. **Halit Canatan:** Conceptualization, Methodology. **Ahmet Eken:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Ekrem Unal:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing.

#### Data availability

The data that has been used is confidential.

## Acknowledgments

The authors thank to patients and their families. Also, we thank to administrative personnel of Genome and Stem Cell Center (GENKOK).

## Funding sources

This work was supported partly by the Erciyes University BAP grant, [TTU-2020-10478] to EU and Grant number: 4313 by TUSEB, TUBA GEBIP 2021, and BAGEP 2022 awards by the Turkish Academy of Sciences (TUBA) and Science Academy (BA) respectively to AE.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2024.111560>.

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