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**Running title:** A novel GVHD mouse model

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**Abstract**

Acute graft-versus-host disease (aGVHD) is an important complication of allogeneic hematopoietic cell transplantation (HCT). The majority of aGVHD mouse models are based on radiation conditioning and bone marrow as graft, despite that most allo-HCTs performed now in clinic are based on chemotherapy conditioning and G-CSF mobilized graft. Aiming for a higher translational value, we constructed an MHC major mismatched [C57BL/6 (H-2 Kb) to BALB/c (H-2Kd)] aGVHD mouse model based on busulfan/cyclophosphomide (BU-CY) conditioning and human G-CSF mobilized splenocytes as graft. Allogeneic transplanted mice showed quick and profound donor engraftment. Moreover, there were quick onset (day +7) of typical clinical and histopathological signs of aGVHD, which gradually developed to extensive aGVHD. In addition, CD8+ T cells were the main aGVHD contributing T cell subtype. No toxicity or GVHD signs were observed in the syngeneic setting. This clinical relevant model offers a promising platform for future studies on aGVHD.

## Introduction

Acute graft-versus-host disease (aGVHD) is an important inflammatory complication of allogeneic hematopoietic cell transplantation (HCT) [1, 2]. Poor translational value remains the limitation for many aGVHD mouse models. The majority of aGVHD mouse models are based on total body irradiation (TBI) as conditioning and bone marrow (BM) as graft, despite that most allo-HCTs performed now in clinic are based on chemotherapy conditioning and G-CSF mobilized grafts [3]. van Leeuwen et al constructed the classical MHC major mismatched C57BL/6 (H-2 Kb) to BALB/c (H-2Kd) model based on TBI and bone marrow graft [4]. Subsequently, Sadeghi et al. constructed the first major histocompatibility complex (MHC) major mismatched [C57BL/6 (H-2Kb) to BALB/c (H-2Kd)] model based on busulfan/cyclophosphamide (BU-CY) conditioning [5]. Afterwards, some other chemo-based aGVHD mouse models were also developed to investigate the different contexts of haploidentical [6] and miHA mismatched [7] allo-HCTs. Moreover, aGVHD models based on TBI and G-CSF mobilized grafts has been described [8]. However, none of them have combined these two clinically relevant factors in one model.

Aiming for a higher translational value, we developed a MHC major mismatched aGVHD model using C57BL/6 (H-2 Kb, female) as donor and BALB/c (H-2Kd, female) as recipient. This model manifested typical clinical and histological signs of aGVHD, and may play as a promising clinical-relevant model for future studies.

## Materials and Methods

### Mice

Female C57BL/6j mice (H2kb) and female BALB/c (H2kd) mice were purchased from Janvier (Genest-St-Isle, France). All mice were used at 11-13 weeks of age. Mice were maintained under pathogen free controlled conditions and 12hr light/darkness. Animals had access to food and water *ad libitum*. All protocols were performed according to approval of the "Service Vétérinaires de la Santé et de la Protection Animal" delivered by the Ministry of Agriculture of France.

### HCT procedures

BU (Pierre Fabre Médicament, Idron, UK) and CY (Baxter, Saint-Quentin-en-Yvelines, France) were used for conditioning. BU (4mg/ml) was diluted with PBS, and CY (20mg/ml) was injected without dilution. Female BALB/c mice received IP doses of BU (20 or 25 mg/kg/d) for 4 days (day -7 to day -4), followed by CY (100mg/kg/d) for 2 days (day -3 to day -2). After 1 day rest, the recipient mice were injected IV with 10 x $10^6$ splenic cells from female C57BL/6 mice by tail vein injection. PBS was administrated as a vehicle control. For graft preparation, donors were previously treated by subcutaneous injection of 10 µg per animal of recombinant human G-CSF (Hospira, Hurley, UK) once daily from day -5 to day -1. On the day of transplant, the donors were sacrificed by cervical dislocation. Donor spleens were disrupted in RPMI-1640 culture medium (Thermo Fisher Scientific, Villebon-sur-Yvette, France) and erythrocytes were then lysed with RBC Lysis Buffer (Multi-species) (Thermo Fisher Scientific, Villebon-sur-Yvette, France), washed twice with RPMI-1640 before passing

through a 70 mm strainer. Cells were then resuspended in PBS for injection. Cell viability (>90%) was confirmed with trypan blue. Each experiment included 5 to 6 mice from each group. The Chemo group (BALB/c) received chemotherapy alone without infusion of splenocytes, the Allo group (BALB/c) received chemotherapy followed by infusion of allogeneic splenocytes (C57BL/6), and the Syn group (BALB/c) received chemotherapy followed by infusion of syngeneic splenocytes (BALB/c). The mice were randomly assigned to Chemo, Allo or Syn groups in each experiment. No mice were excluded from analysis. No statistical methods were used to predetermine sample size. The investigators were not blinded to allocation during experiments and outcome assessment.

**Clinical and histological assessment of GVHD**

Mice were monitored for survival and individually scored every two days for five clinical parameters (posture, activity, fur, skin and weight loss) from conditioning initiation until the appropriate sampling day on a scale from 0 to 2 as previously described [9]. Mice survival was the primary endpoint for GVHD evaluation. Clinical GVHD score was assessed by summation of these parameters. Animals with severe aGVHD (scores >6) were sacrificed according to ethical guidelines. For histological analysis, the skin and colon were removed immediately after sacrifice on day +7, +14 and +21 after transplantation, and then fixed in 4% formalin for 24h, transferred to 70% ethanol, dehydrated and embedded in paraffin. Paraffin sections were then stained with hematoxylin and eosin. The histological grade of skin and intestine aGVHD was assessed according to the grading system previously described [10]. Scores were determined in a blinded fashion by two pathologists.

## FACS analysis

On day +7, +14, +21 and +28 after transplantation, one random recipient from each group was sacrificed, and splenocytes and BM cells were collected. BM and spleen cellularity were determined by trypan blue staining and microscopic count. Afterwards erythrocytes were lysed with RBC Lysis Buffer and cells were then passed through a 40μm cell strainer, washed twice and stained for 20 minutes at 4 ℃ in PBS/0.5mM EDTA/0.5% BSA with the following mouse mAbs: FITC-CD8a (SONY, Weybridge, UK), PE-CD4 (SONY), PerCPCy5.5-CD11c (SONY), PC7-H2Db (Biolegend, Ozyme, Saint-Quentin-Fallavier, France), A647-H2Dd (Biolegend), APCCY7-CD3 (SONY) and PACIFIC BLUE-CD45 (Biolegend). Analyses were performed with CytoFLEX Flow Cytometer (Beckman Coulter) and Kaluza Flow Cytometry Analysis Software version 1.5a (Beckman Coulter).

## Statistical analysis

Survival data were analyzed using the Kaplan-Meier method and Mantel-Cox log-rank test. For all other data, the two-sided unpaired t-test was used. Normality tests and the F test confirmed Gaussian distribution and equality of variance between different groups. Values were presented as mean±standard error of the mean (SEM). A value of $P < 0.05$ was considered statistically significant in all experiments. Data was computed using GraphPad Prism 5.0 (GraphPad Software).

## Results

For dose optimization purpose, two different doses of BU were evaluated (80 and 100 mg/kg

total dose) in combination with a fixed dose of CY (100 mg/Kg). While dose of BU has no impact on mice survival in the syngeneic setting (groups 3 and 4, Figure 1a), higher dose of BU (100 mg/kg) was associated with very early death, within 10 days, both in the Chemo and the Allo group (groups 6 and 2, figure 1a). On the contrary, with lower dose of BU (80 mg/kg) early deaths were slightly delayed in the Allo group comparing to the Chemo group (groups 1 and 5, figure 1a). Overall GVHD onset and kinetic with the lower dose of BU (80 mg/kg) was more adapted and BU 80 mg/kg in combination with CY 200 mg/kg which was retained has the optimal conditioning regimen for further experiments.

The protocol for transplantation and G-CSF mobilization is shown in Figure 1b. Appropriately conditioned and syngeneic transplanted mice (Syn group) survived over +60 days of transplant, with no signs of aGVHD. Mice who received conditioning but no transplantation (Chemo group) started to die from day +2 and 64.7% (11 out of 17 mice) died within 15 days. Appropriately conditioned and allogeneic transplanted mice (Allo group) started to die from day +4, with 76.5% (13 out of 17 mice) died within 20 days, and all died within 40 days (median survival=12 days) (Figure 1c). In all groups, a weight loss was seen between day -7 and day +5. In the Allo group, weight reached the nadir on day +7 and remained at a low level, until the second phase of weight loss on day +30 (Figure 1d). Meanwhile, features of aGVHD including hunched posture (Figure 2a), hair loss (Figures 2b,c) and ruffled fur (Figure 2c) were observed in the Allo group. Moreover, almost all mice in the Allo group manifested signs of colitis from day +5 until the end of the experiment. Finally, clinical GVHD scores were assessed as previously described [8], and represented the severity of aGVHD (Figure 2d).

With fluorescence activated cell sorting (FACS) analysis, we observed a stable donor chimerism of over 95% in BM, and an increasing donor cell chimerism in the spleen, from 68% from day +7 to full chimerism on day +14 in the Allo group (Figure 3a). T cell engraftment was faster than that of granulocytes in the spleen (Figure 3b). BM and spleen cellularity recovered rapidly in the Syn group. However in the Allo group, the recovery of BM and spleen was slower and at day +28 reached 65% and 71%, respectively when compared with the Syn group (Figure 3c). As previously published [5], percentages of CD3+ T cells were significantly increased in the BM and spleen early after transplant due to chemotherapy conditioning (day +7), and decreased gradually until day +28. However, the percentages of CD3+ T cells were always higher in the Allo group as compared with the Syn group (Figure 4a,c), and the frequencies of CD8+ T cells, but not CD4+ cells, were significantly higher in the Allo group as compared with the Syn group, in both BM and spleen (Figure 4b,d). The ratio of CD4/CD8 was reversed consistently in the Allo group from day +7 until day +28 in both BM and spleen (Figure 4e).

At day +7 the Allo group exhibited significant histopathologic skin changes. Frequent apoptotic figures were observed within the epidermis of the dorsal skin, as well as subepidermal vesical formation, lymphocytic infiltration and slight lifting of the epidermal layer. The Chemo group also manifested epidermis vacuolization probably due to conditioning toxicity, but no immune cell infiltration was observed. The Syn group maintained an essentially intact epidermal and dermal layer (Figure 5a). At day +21, the interfollicular epidermal hyperplasia was extensive and dyskeratotic squamous cells had spread throughout the epidermis. A high density of immune cells had infiltrated the epidermis.

Meanwhile, both the Chemo and Syn groups exhibited normal skin morphology (Figure 5b).

At day +7, the Allo group exhibited colon histopathologic changes including crypt cell apoptosis and immune cell infiltration. Crypt damage was also seen in the Chemo group probably due to conditioning toxicity. The Syn group, however, exhibited an essentially normal structure (Figure 5a). At day +21, the crypt structure in the Allo group was severely disturbed with extensive crypt destruction and dense lamina propria lymphocytic infiltration. The Chemo and Syn group however, manifested normal intestinal structure (Figure 5b).

The histopathological scores were assessed as previously described [10]. The scores were higher in the Allo group than in the Syn group for both skin and colon at day +7 (Figure 6a). By day +21, the Allo group showed significantly increased histopathological scores as compared with the Syn group for skin (Allo: $2.5 \pm 0.3$ vs Syn: $0.0 \pm 0.0$; $P<0.0001$) and colon (Allo: $8.7 \pm 0.2$ vs Syn: $3.2 \pm 0.6$; $P<0.0001$). Meanwhile, the scores of the Allo group was also significantly higher than those of the Chemo group for both skin (Allo vs Chemo: $0.0 \pm 0.0$; $P=0.0016$) and colon (Allo vs Chemo: $5.7 \pm 0.3$; $P<0.0001$).

## Discussion

So-far the great majority of aGVHD mouse models have been based on TBI as conditioning and BM as graft [3]. However, the use of lethal TBI without chemotherapy in mouse models is in contrast to the standard procedure in clinical allo-HCT, and profound differences between these two types of conditioning regimens may lead to a distinct GVHD phenotype. Moreover, immune cells derived from different sources (BM or G-CSF mobilized graft)

might have different trafficking capacities and composition, and therefore exert distinct influence on the GVHD phenotype [11].

Compare to the classical MHC major mismatched C57BL/6 (H-2 Kb) to BALB/c (H-2Kd) model based on TBI and bone marrow graft [4], median survival in the Allo group was shorter in our model, being 12 days, versus 18 days in the classical model, suggesting that our model may represent a more typical acute phase of GVHD.

Sadeghi et al. established conditioning with BU-CY in a MHC mismatched model using bone marrow graft [5]. After dose optimization, we use the exact same dose of chemotherapy, BU-CY (80mg/kg-200mg/kg), in our model, with a lethality of 65% in the Chemo group comparable to the study by Sadeghi et al [5]. However, despite BU-CY was not fully myeloablative, chimerism analysis demonstrate that all mice achieved full donor engraftment with either bone marrow[5] or G-CSF mobilized grafts after allo-HCT.

Weight loss was the most well documented manifestation in the GVHD mouse model [3]. In our study, the Allo group manifested weight loss between day -7 and day +7 and remained low afterwards. This weight change indicated a quick onset of GVHD. This early weight loss in the Allo group was comparable to that observed in the model using BU-CY conditioning and bone marrow graft [5]. However, when the follow-up duration was prolonged, we observed a second phase of weight loss which led to death of all mice by day +40, indicating that aGVHD was lethal and progressive in this model.

Concerning immune reconstitution, T cell engraftment in the current model seems to be quicker in the GVHD target organs when comparing to the model using bone marrow as graft.

At day +14, we observed 95.5% of donor T cell chimerism in the recipient spleen, while at the same time point donor chimerism was around 70% in mice receiving bone marrow as reported [5]. This observation is consistent the clinical report that recipients of G-CSF-mobilized grafts from unrelated donors have faster immune reconstitution than BM transplant recipients [14]. Moreover, the T cell engraftment was slower than granulocytes in the spleen, which is consistent with the kinetic of immune reconstitution after Allo-HCT in humans [15].

A reversed CD4/CD8 ratio was observed in the Allo group BM and spleen throughout the process of aGVHD, indicating a disease process largely driven by CD8+ T cells. In Sadeghi et al's model CD8+ T cells also played the major pathogenetic role [5]. In the classic C57BL/6 to BALB/c with TBI and bone marrow graft model a two-phase pathogenesis of GVHD was observed and studies using transgenic mice that have a mutant MHC I or MHC II revealed that CD8+ T cells were the early and leading cause of GVHD mortality (acute phase), while the latter phase was probably mediated by alloreactive CD4+ T cells [11,12]. Overall, our findings are in accordance with previously published data, with early CD8+ T cell drived aGVHD mortality. Further study would be useful to decipher the exact role of T cell subset and in particular to rule out a potential role of CD4+ T cells in latter aGVHD death.

Typical clinical and histopathological signs of aGVHD were observed in our model. Extensive intestinal aGVHD was largely responsible for the weight loss and final death. Moreover, evident pathological alterations including infiltration of inflammatory cells into the skin and large intestine and the presence of high numbers of apoptotic/necrotic cells in all

tested organs were similar to those observed in irradiation-based aGVHD mouse models and human aGVHD [1, 2].

We cannot rule out that chemotherapy toxicity contributed to early death in the Allo group. In fact, lesions induced by conditioning regimen, in particular gut lesions, directly contribute to aGVHD initiation, that once initiated will aggravate the lesion, leading to mice death. However, bone marrow and spleen engraftment with CD8+ T cell infiltration in the Allo group, along with specific impairment in those mice (fur loss, colitis) and increased histopathology score at day 21, confirm that aGVHD was the leading cause of death in those mice.

Overall, this clinically relevant model showed good donor engraftment and typical clinical and histopathological signs of aGVHD, which may be advantageous in reflecting the clinical situation of HCT and aGVHD. There are limitations to this model. Firstly, the one-week long conditioning is a more complicated practice as compared with the 'single shot' TBI. In addition, this is a MHC major mismatched model, while in clinic, minor MHC mismatched and haploidentical HCTs are more routinely practiced. Therefore, haploidentical or minor MHC mismatched models based on chemotherapy conditioning and G-CSF mobilized grafts are expected in the future.

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## Conflict of Interest

The authors state no conflict of interest.

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311 **Figure Legends**

312 **Figure 1. HCT protocol and survival**

313 (a) Survival curve of mice underwent different treatments. Group 1: Female BALB/c
314 conditioned with BU－CY (80－200 mg/kg) and transplanted with female C57BL/6; Group
315 2: Female BALB/c conditioned with BU－CY (100－200 mg/kg) and transplanted with
316 female C57BL/6; Group 3: Female BALB/c conditioned with BU－CY (80－200 mg/kg) and
317 transplanted with female BALB/c; Group 4: Female BALB/c conditioned with BU－CY
318 (100－200 mg/kg) and transplanted with female BALB/c; Group 5: Female BALB/c
319 conditioned with BU－CY (80－200 mg/kg) and not transplanted; Group 6: Female BALB/c
320 conditioned with BU－CY (100－200 mg/kg) and not transplanted. All transplanted mice
321 received $10x10^6$ splenic cells from donor origin. **(b)** Female BALB/c received IP doses of
322 BU-CY (80mg/kg-200mg/kg) per mouse beginning at day -7 before HCT. For transplantation,
323 $10x10^6$ splenic cells from G-CSF mobilized C57BL/6 mice were given by tail vein injection
324 on day 0. **(c)** and **(d)** show data from 17 mice per group from 3 independent experiments. **(c)**
325 Survival curve, ***$p<0.001$ by Mantel-Cox log-rank test. **(d)** Mean±SEM of mouse weight
326 in aGVHD phase, ***$p<0.001$ by t-test.

327 **Figure 2. GVHD Clinical manifestations**

328 The Allo group exhibited features of aGVHD including hunched posture **(a)**, hair loss **(b, c)**,
329 and ruffled fur **(c)**. **(d)** Mean±SEM of clinical GVHD score from 3 independent experiments,
330 ***$p<0.001$ by t-test. Clinical GVHD score was assessed by summation of five parameters
331 (posture, activity, fur, skin and weight loss).

**Figure 3. Donor engraftment**

**(a)** Donor chimerism (%) in BM and spleen in Allo group. **(b)** T cell and granulocyte (GN) engraftment in the spleen in Allo group. **(c)** BM and spleen cellularity during GVHD, as determined by trypan blue staining and microscopic count. Each time point represents Mean ±SEM of data from at least 3 independent mice.

**Figure 4. Lymphoid subpopulations**

**(a)** T cell (%) in BM. **(b)** CD8+ and CD4+ T cell (%) in BM. **(c)** T cell (%) in spleen. **(d)** CD8+ and CD4+ T cell (%) in spleen. **(e)** CD4/CD8 ratio in BM and spleen. Each time point represents Mean±SEM of data from at least 3 independent mice. **p<0.01 and ***p<0.001 by t-test.

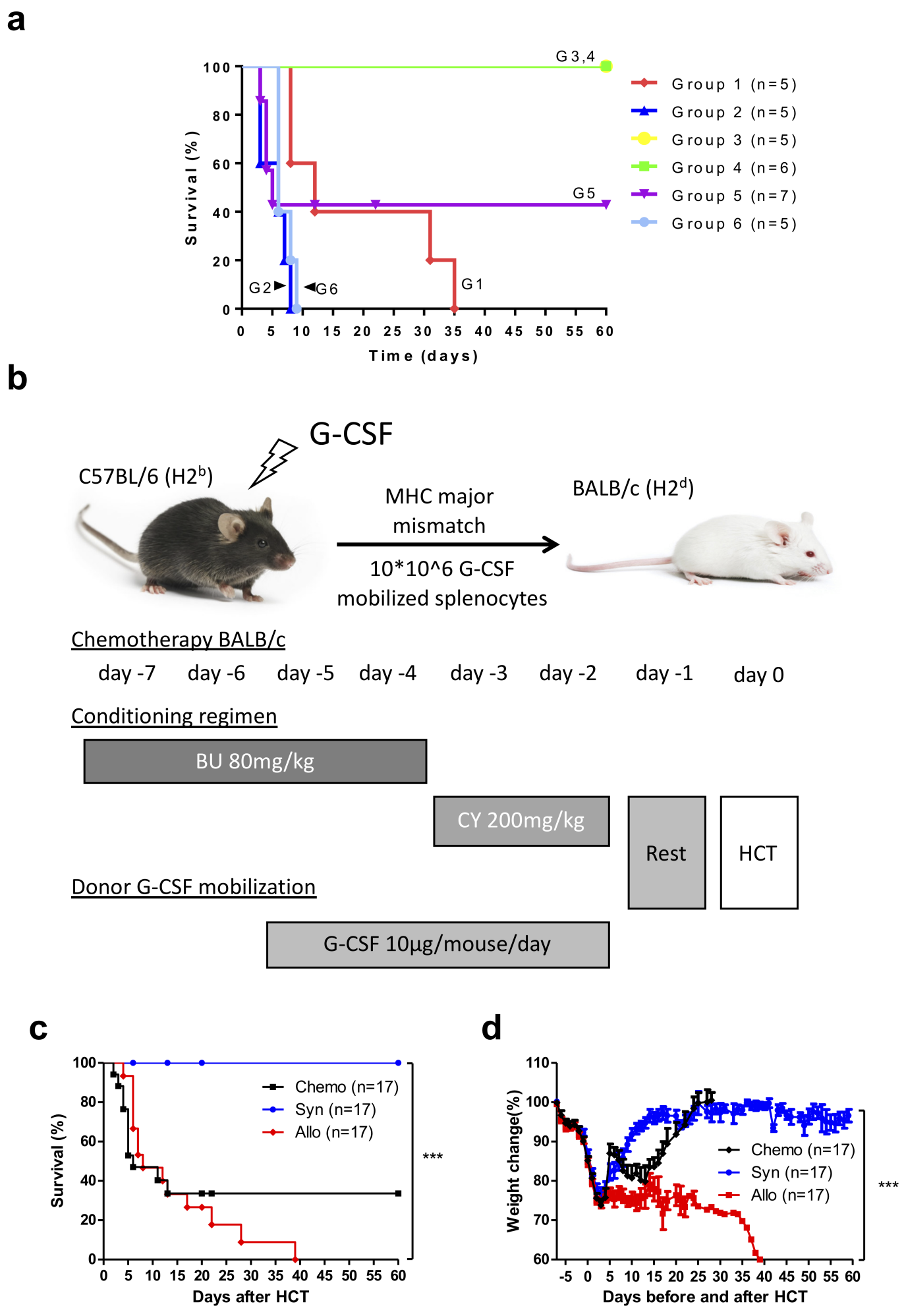
**Figure 5. GVHD pathophysiology**

At day +7 **(a)** and day +14 **(b)** after HCT, mice in the Syn and Allo group were sacrificed for histological examination. Representative H&E-stained sections of dorsal skin and colon from each group are shown. Original magnification was × 200.

**Figure 6. GVHD histopathological score**

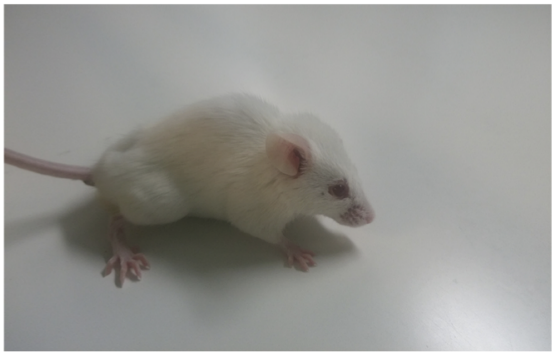
Skin and intestinal aGVHD histopathological scores at day +7 **(a)** and day +21 **(b)** after HCT. Scores of skin and intestinal GVHD were significantly higher in the Allo group than in the Syn and the Chemo group at d+21 after HCT, respectively. Data are shown as Mean±SEM, ***p<0.001 by t-test.

# Figure 1


a
Survival (%)
Time (days)
G3,4
G5
G1
G2
G6
Group 1 (n=5)
Group 2 (n=5)
Group 3 (n=5)
Group 4 (n=6)
Group 5 (n=7)
Group 6 (n=5)
b
G-CSF
C57BL/6 (H2b)
MHC major mismatch
10*10^6 G-CSF mobilized splenocytes
BALB/c (H2d)
Chemotherapy BALB/c
day -7
day -6
day -5
day -4
day -3
day -2
day -1
day 0
Conditioning regimen
BU 80mg/kg
CY 200mg/kg
Rest
HCT
Donor G-CSF mobilization
G-CSF 10µg/mouse/day
c
Survival (%)
Days after HCT
Chemo (n=17)
Syn (n=17)
Allo (n=17)
***
d
Weight change(%)
Days before and after HCT
Chemo (n=17)
Syn (n=17)
Allo (n=17)
***

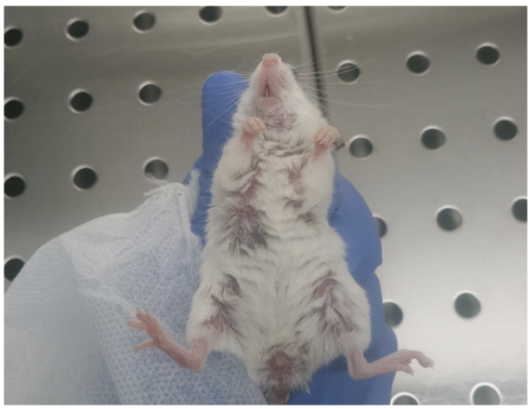
# Figure 2

**a**

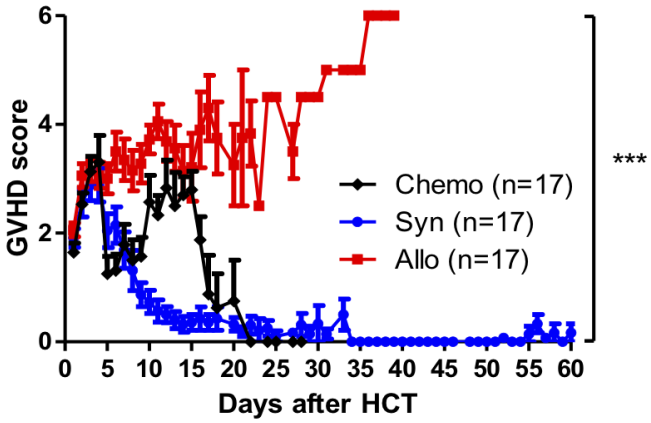


**b**

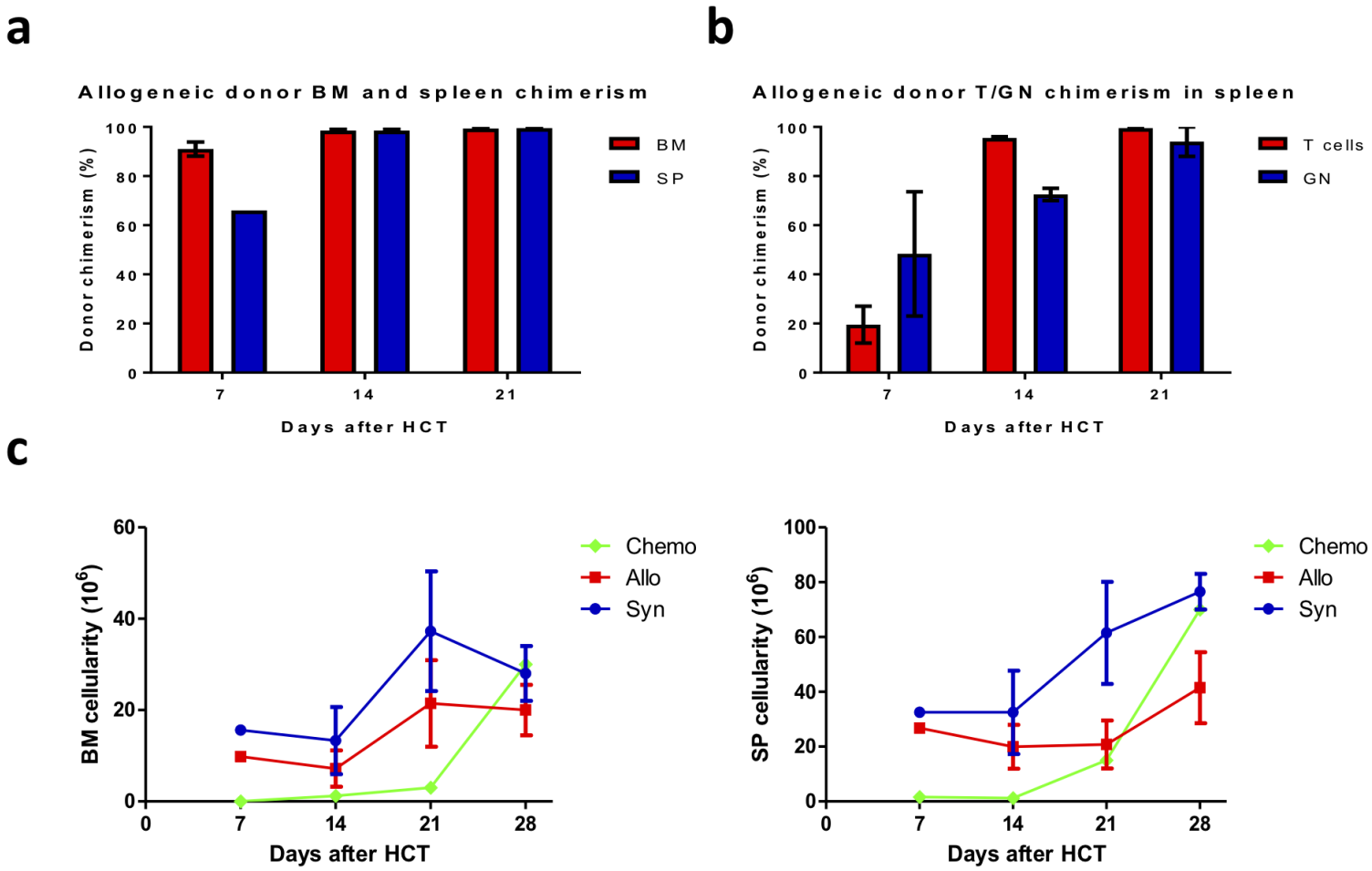
**c**



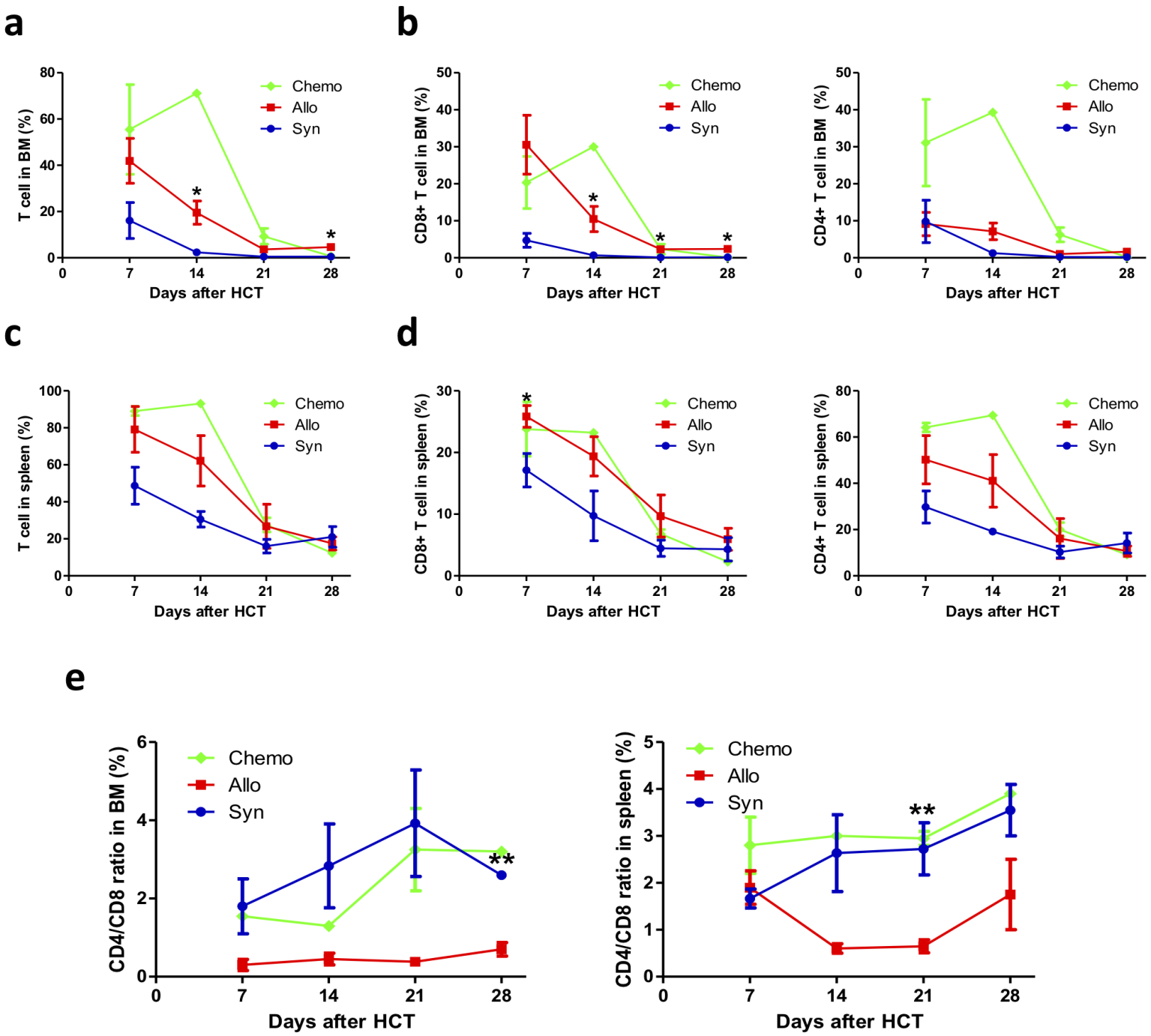
**d**



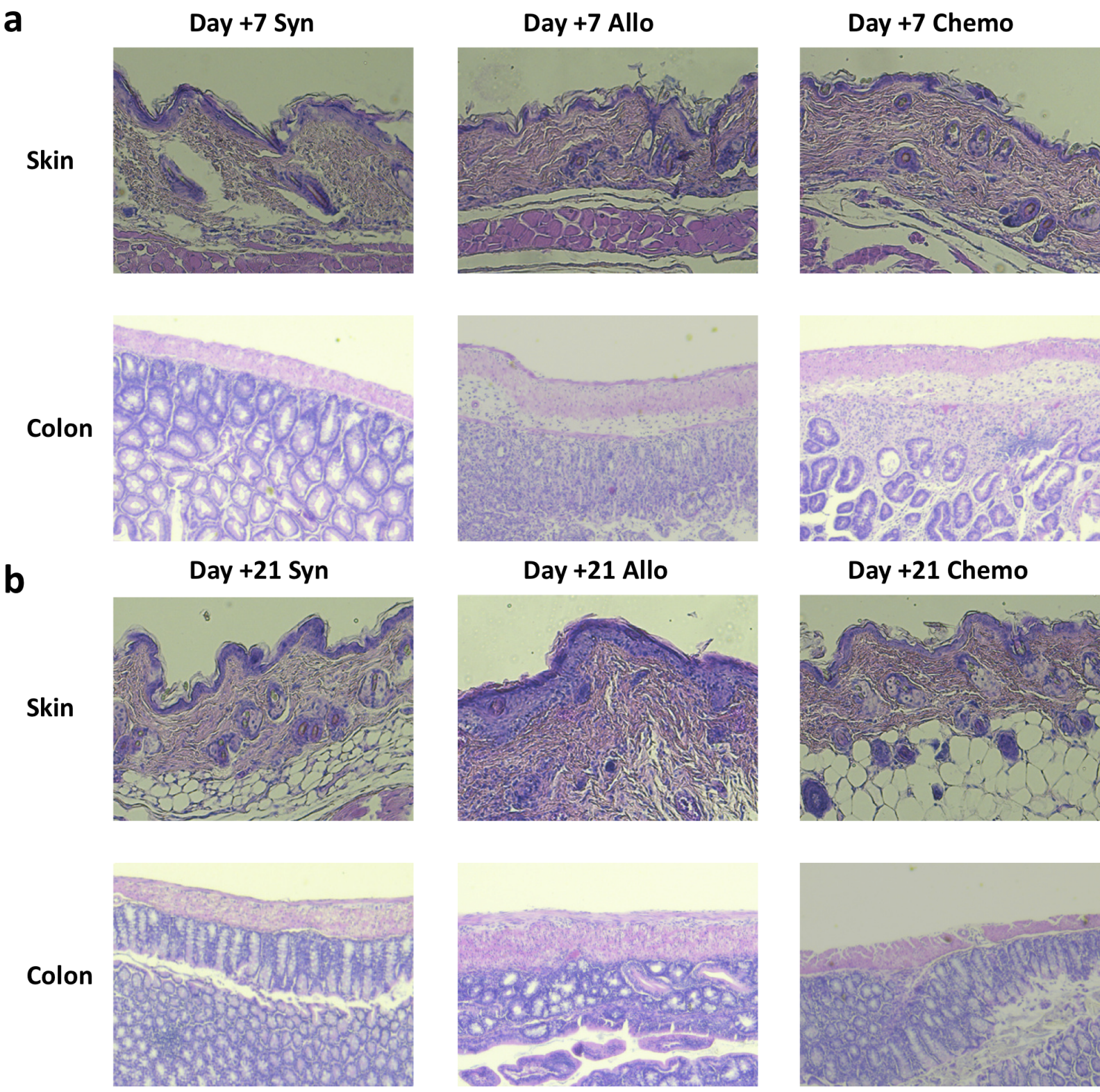
# Figure 3


a
Allogeneic donor BM and spleen chimerism
Donor chimerism (%)
100
80
60
40
20
0
7
14
21
Days after HCT
BM
SP
b
Allogeneic donor T/GN chimerism in spleen
Donor chimerism (%)
100
80
60
40
20
0
7
14
21
Days after HCT
T cells
GN
c
BM cellularity (10^6)
60
40
20
0
0
7
14
21
28
Days after HCT
Chemo
Allo
Syn
SP cellularity (10^6)
100
80
60
40
20
0
0
7
14
21
28
Days after HCT
Chemo
Allo
Syn

# Figure 4

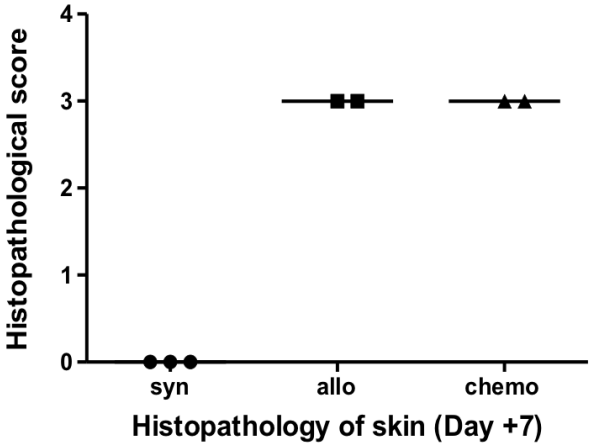

a
b
c
d
e
T cell in BM (%)
CD8+ T cell in BM (%)
CD4+ T cell in BM (%)
T cell in spleen (%)
CD8+ T cell in spleen (%)
CD4+ T cell in spleen (%)
CD4/CD8 ratio in BM (%)
CD4/CD8 ratio in spleen (%)
Days after HCT
Chemo
Allo
Syn
*
**

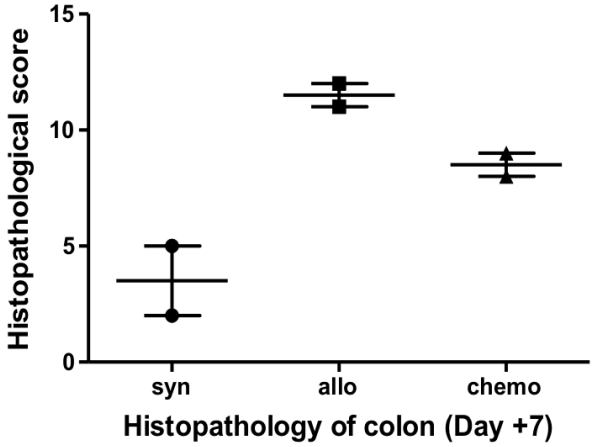
# Figure 5


a
Day +7 Syn
Day +7 Allo
Day +7 Chemo
Skin
Colon
b
Day +21 Syn
Day +21 Allo
Day +21 Chemo
Skin
Colon

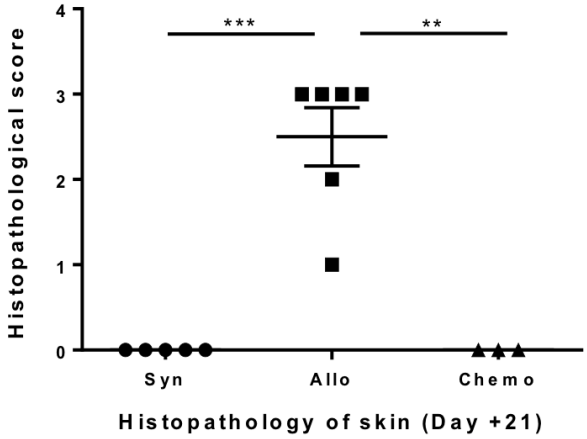
# Figure 6

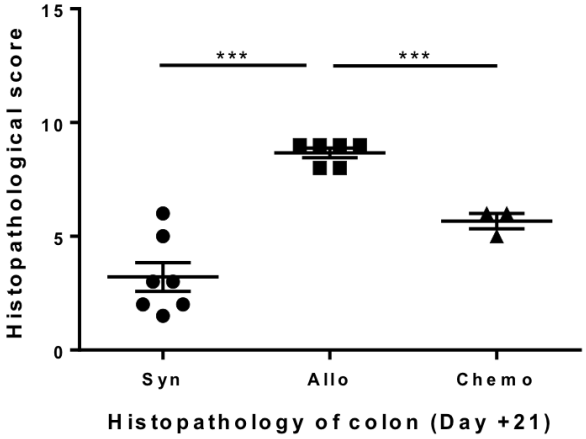
**a**


Histopathological score
4
3
2
1
0
syn
allo
chemo
Histopathology of skin (Day +7)


Histopathological score
15
10
5
0
syn
allo
chemo
Histopathology of colon (Day +7)

**b**


Histopathological score
***
**
4
3
2
1
0
Syn
Allo
Chemo
Histopathology of skin (Day +21)


Histopathological score
***
***
15
10
5
0
Syn
Allo
Chemo
Histopathology of colon (Day +21)