Heliyon 9 (2023) e13876

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Anti-*E. coli* Immunoglobulin Yolk (IgY): Reduction of pathogen receptors and inflammation factors could be caused by decrease in *E. coli* load

Abdellatif Bouazzaoui [a,b,c,*], Neda M. Bogari [a], Faisal A. Al-Allaf [a], Samar N. Ekram [a], Mohammad Athar [a,b], Anas Dannoun [a], Thomas Schubert [d], Shahzad N. Syed [e], Abdel-Rahman Youssef [f], Mashael Alqahtani [f], Ahmed A.H. Abdellatif [g,h]

[a] *Department of Medical Genetics, Faculty of Medicine, Umm Al-Qura University, 21955, Makkah, Saudi Arabia*
[b] *Science and Technology Unit, Umm Al-Qura University, 21955, Makkah, Saudi Arabia*
[c] *Department of Internal Medicin III (Haematology and Internal Oncology), University Hospital Regensburg, Franz-Josef-Strauß-Allee 11, 93053, Regensburg; Germany*
[d] *Institut für angewandte Pathologie Speyer, Alter Postweg 1, 67346, Speyer, Germany*
[e] *Institute of Biochemistry I, Faculty of Medicine, Goethe-University Frankfurt, 60590 Frankfurt, Germany*
[f] *Department of Basic and Clinical Oral Sciences, Faculty of Dentistry, Umm Al-Qura University, 21955, Makkah, Saudi Arabia*
[g] *Department of Pharmaceutics, College of Pharmacy, Qassim University, 51452, Qassim, Saudi Arabia*
[h] *Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Al-Azhar University, 71524, Assiut, Egypt*

ARTICLE INFO

Keywords:
Stem cell transplantation
Graft-versus-host disease
Hen antibody Immunoglobulin Yolk (IgY)
PRR cytokines And chemokines
Bone marrow transplantation
E. coli

ABSTRACT

Graft versus host disease (GVHD) remains the major cause of morbidity and mortality after allogeneic stem cell transplantation, especially for intestinal GVHD, as steroid resistant GVHD results in high mortality. For this reason, new treatments of GVHD are needed. One approach is the reduction of pathogenic bacteria using anti-*E. coli* Immunoglobulin Yolk (IgY). In a haploidentical murine model, B6D2F1 mice conditioned with total body irradiation (TBI), received bone marrow cells (BM) and splenocytes (SC) from either syngeneic (Syn = B6D2F1) or allogeneic (Allo = C57BL/6) donors. Following this, animals received from day −2 until day +28 chow contained IgY or control chow. Thereafter the incidence and severity of aGVHD, the cytokines, chemokines, IDO1 and different pathogen-recognition receptors (PRR) were analyzed and compared to control animals (received chow without IgY). We found that animals receiving chow with IgY antibody showed reduced GVHD severity compared to control animals. On day28 after alloBMT, IDO, NOD2, TLR2, TLR4 and the inflammatory chemokine CCL3, were reduced in the colon and correlated with a significant decrease in *E. coli* bacteria. In summary chow containing chicken antibodies (IgY) improved GVHD via decrease in bacterial load of E coli conducting to reduction of pathogen receptors (NOD2, TLR2 and 4), IDO, chemokines and cytokines.

* Corresponding author. Department of Medical Genetics, Faculty of Medicine, Umm Al-Qura University, 21955 Makkah, Saudi Arabia.
E-mail addresses: alazzauoi@uqu.edu.sa (A. Bouazzaoui), nmbogari@uqu.edu.sa (N.M. Bogari), faallaf@uqu.edu.sa (F.A. Al-Allaf), snekram@uqu.edu.sa (S.N. Ekram), athar80@gmail.com (M. Athar), anas_dannoun@hotmail.com (A. Dannoun), schubert@pathospeyer.de (T. Schubert), syed@biochem.uni-frankfurt.de (S.N. Syed), amyoussef@uqu.edu.sa (A.-R. Youssef), Msqahtani@uqu.edu.sa (M. Alqahtani), a.abdellatif@qu.edu.sa (A.A.H. Abdellatif).

https://doi.org/10.1016/j.heliyon.2023.e13876
Received 10 May 2022; Received in revised form 14 February 2023; Accepted 14 February 2023
Available online 21 February 2023

1. Introduction

Allogeneic bone marrow transplantation (alloBMT) is a unique curative therapy for diverse hematological and malignant diseases; However, its utility is limited by the development of severe treatment-related complications, most importantly, the occurrence of acute Graft versus host disease (aGVHD) which leads to high morbidity and mortality [1–3]. Acute GVHD occurs within a few days up to 6 months after transplantation and its manifestation depends on the degree of human leukocyte antigen (HLA) differences between recipients and donors, the T cell fraction in the graft, microflora in the gut, patient's age as well as prophylaxis and therapy regimes [1,2,4]. The conditioning regime using total body irradiation caused damages, especially in the gastrointestinal tract [5] resulting in the translocation of pathogen-associated molecular patterns products (PAMP) into the intestinal immune system and sometimes even into the systemic circulation [6]. PAMP interacts with pathogen recognition receptors (PRR) leading to the activation of APC and allor-eactive T cells respectively. It has been understood in early time that the micro-flora in the intestine exert an important role in the activation of donor lymphocytes by recipient antigen-presenting cells (APCs), this activation presents a pivotal step in the induction of alloreactions. This is based on previous researches demonstrated that mice grown under germ-free condition don't get aGVHD, whereas control animal grown under normal condition died early after transplantation [7,8]. Another study from van Bekkum et al. presented evidence for an association between elimination of Lactobacillales before BMT and the GVHD severity [4]. In contrary, reintroduction of probiotic lactobacillus mediated significant protection against GVHD [9,10]. Of further importance is the study from Jenq et al. who showed that GVHD or antibiotics changed the microbiota in a unique pattern [10] and that a loss of diversity and protective Blautia or clostridiales associates with poor outcome and higher treatment related mortality in HSCT patients [11–13].

The Link between bacteria and the immune system is exhibited by the interaction between pathogen-associated molecular patterns (PAMP) on the invasive microorganism and pathogen recognition receptors (PRR) on the human cells. Shortly after discovering the "Toll" receptor in Drosophila as the first PRR [14], Medzhidov et al. group found the Toll-like receptor (TLR) as homologue of "Toll" in human and named it later as TLR4 [15]. From this point, research in the innate immunity increased considerably and to date hundreds of PRRs have been discovered. The TLR are expressed on hematopoietic cells (dendritic cells, T cells and B cells), endothelial cells and epithelial cells [16] and are able to recognize and interact with different PAMPs including lipopolysaccharide (TLR4), bacterial li-poproteins (TLR2), RNA (TLR3+7), cytosine-phosphorothioate-guanine (TLR9) and flagellin (TLR5) [17]. Beside the TLR, the PAMPs are also recognized by NOD-like receptors (NLRs) including proteins such as NALPs (NACHT-, LRR- and PYD-containing protein), NOD1 (nucleotide-binding oligomerization domain) and NOD2 [18–20]. The NOD2 is intracytoplasmatic, highly conserved and can

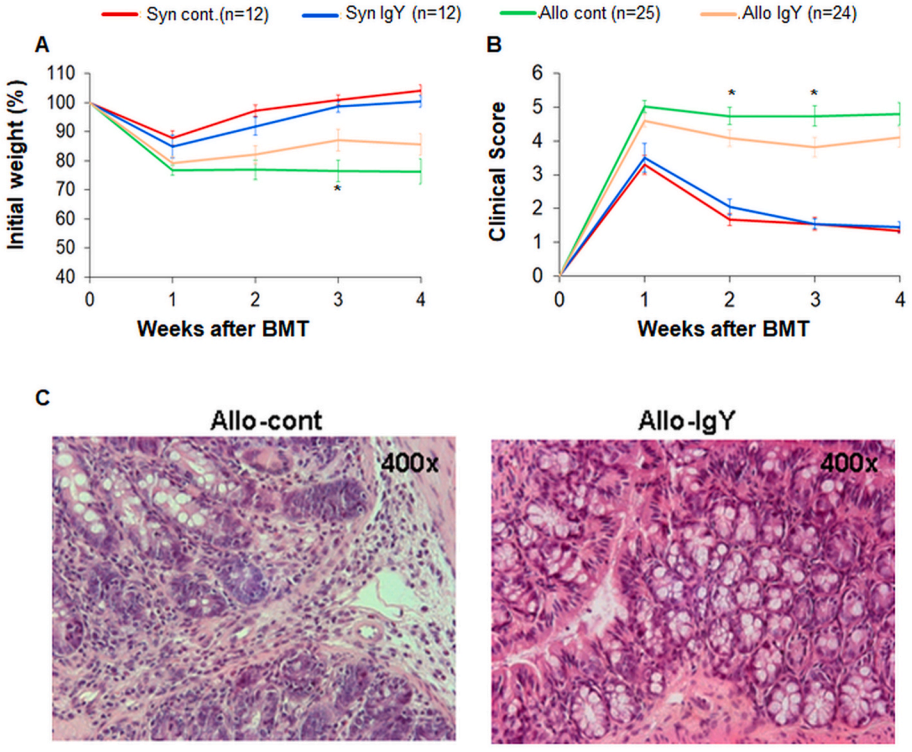

Fig. 1. Weight changes and clinical score after alloBMT. Lethally irradiated B6D2F1 mice received 2 x 10^6 bone marrow cells supplemented with 4 x 10^6 splenocytes from either syngeneic (B6D2F1) or allogeneic (C57BL/6) donors. Thereafter animals received chow with IgY or control chow from day -2 until day 28. A) Weight changes and B) clinical scores after alloBMT are showed for (Syn cont (n=12), Syn IgY (n=11), Allo cont (n=25), Allo IgY (n=24)). C) showed a reprenstative histopathology picture after HE staining for the colon at day 28 after transplantation for Allo cont (on the left) and Allo IgY (on the right). The results are presented as mean ± SEM; *p < 0.05, **p < 0.01.

induce the activation of the inflammasome via interaction with muramyl dipeptide (MDP) and muramyl tripeptide (MTP). To characterize the roles of PRR in the inflammation process, early publications showed varied results. On one hand Heimesaat et al. supported the boosting effect of bacterial products on the inflammation via TLR2 and TLR4 and directing the recruitment of inflammatory cells to the inflammation site [21]. Blazar et al. found in animal models, that the administration of cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpGODNs) significantly accelerated the aGVHD in treated animals [22]. On the other hand, the group of Vijay-Kumar et al. and Lee et al. demonstrated in knock-out mice model that TLR5 and TLR9 induced anti-inflammatory effects [23, 24]. Furthermore, other groups showed that the administration of synthetic TLR agonists in low dose can induce tolerance and inhibit inflammation [25–27]. Another factor which can affect the tolerance and modulate the inflammation is the Indoleamine 2,3-dioxygenase-1 (IDO), this enzyme catalyzes the first step of tryptophan (Trp) catabolism and is induced in different cells in response to bacterial components, such as Toll-like receptor (TLR) ligands during infection [28]. In a recent study from Comim et al. the author found that treatment with the IDO inhibitor prevented long-term cognitive impairment triggered by sepsis [29], other early works showed favorable effects of IDO inhibition in human immunodeficiency virus (HIV)- patients [30] and in trauma patients [31]. Furthermore the IDO inhibitor 1-methyl-D,L tryptophan (1-MT) could increase the anti-HIV immunity [32]. However, blocking of IDO protected mice against lipopolysaccharide (LPS)-induced endotoxin shock via modulation of IL-10 production in DCs [33].

In the present study, a haploidentical murine model was used, B6D2F1 mice conditioned with total body irradiation (TBI), received bone marrow cells (BM) and splenocytes (SC) from either syngeneic (Syn = B6D2F1) or allogeneic (Allo = C57BL/6) donors. Following this, the mice received from day −2 until day +28 chow contained hen antibodies (IgY = Immunoglobulin Yolk). Thereafter the effects on the incidence and severity of aGVHD, the PRR expression (TLR2-6, NOD2) and the level of IDO, CCL3, CXCL9 were analyzed.

2. Results

2.1. Treatment with chicken antibodies improve the aGVHD outcome compared to control

After transplantation syngeneic recipients demonstrated minor changes related to conditioning toxicity (Fig. 1A and B), but continuously recovered and all animals survived until end of the experiment. In contrary, allogeneic recipients developed severe GVHD, in allogeneic control animals 44% died, whereas in the IgY group less than 27% mice died [34]. Furthermore, we found significant weight reduction and high clinical score within the first week after transplantation (Fig. 1A and B). During the second and third week, treatment with IgY antibody in allogeneic recipients decreased clinical GVHD scores and reduced the weight loss when compared to allogeneic control animals and reached a significant low value at week 3. Interestingly, when we compare the colon pathology score, the difference between allogeneic control mice versus animals received IgY is very clear as the pictures in Fig. 1C

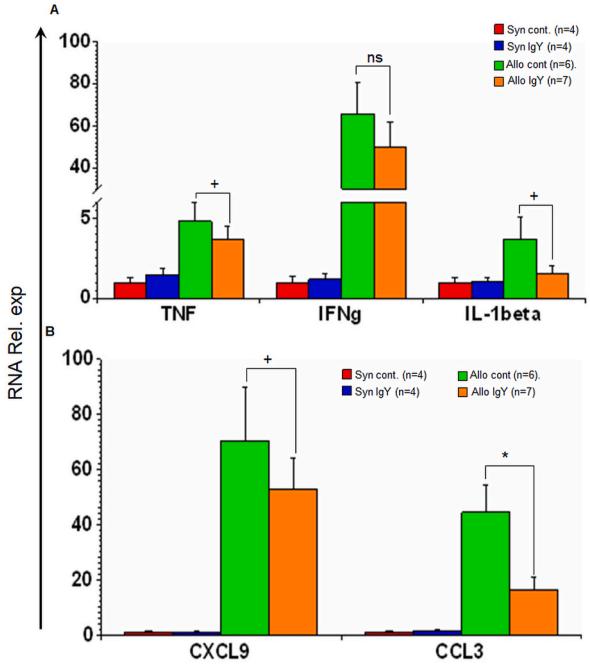

Fig. 2. Expression of cytokines, chemokines after allo-BMT. Lethally irradiated B6D2F1 mice received 2 x 10^6 bone marrow cells supplemented with 4 x 10^6 splenocytes from either syngeneic (B6D2F1) or allogeneic (C57BL/6) donors. Thereafter animals received chow with IgY or control chow from day -2 until day 28. The figure shows the relative expression on the RNA level for cytokines (A) and chemokines (B) in the colon 28 days after transplantation for ■ Syn cont (n=4), ■ Syn IgY (n=4), ■ Allo cont (n=6), ■ Allo IgY (n=7). The data are presented as mean ± SEM; *p < 0.05, **p < 0.01, + tendency to significance.

shows. In the allogeneic control animals, the colon presents high T cell infiltration compared to IgY treated animals.

2.2. Expression of cytokines and chemokines after allo-BMT

After treatment with IgY in pellet chow, in the next step, and based on early work [35], we studied the expression of selected chemokines and cytokines. Twenty-eight days after transplantation, samples from the colon of animals were taken and the expression of genes on mRNA level have been assessed. Analysis of the cytokines expression in the gut showed reduced level of TNF and IL-1beta with tendency to significance (Fig. 2A). The level for TNF reached (4.83 ± 1.19relexp) in control versus (3.70 ± 0.83relexp) in IgY animals and for IL-1beta (3.71 ± 1.42relexp) in control versus (1.56 ± 0.50relexp) in IgY animals. The chemokines CCL3 also showed a significant difference ($p = 0.026$) in the expression between control and IgY animals (Fig. 2B). The expression reached (44.36 ± 9.91 relexp) in the control versus (16.41 ± 4.31 relexp) in the mice received IgY chow.

2.3. Expression of PRR, NOD2 and IDO after allo-BMT

The reduction of cytokines and chemokines is in line with aGVHD improvement in IgY animals compared to allogeneic control animals. In addition, the role of PRR (TLR2-6, NOD2) and IDO in detection of bacteria, activation/regulation of the immune system and in the outcome of GVHD is very important [36–38]. For this reason, we also analyzed the expression of these genes. We found a significant decrease of the TLR2, 4 and NOD2 expression in mice which received IgY chow compared to control (Fig. 3) the value reached (0.34 ± 0.04 relexp) versus (0.61 ± 0.09 relexp) for TLR2, (0.13 ± 0.02 relexp) versus (0.33 ± 0.08 relexp) for TLR4 and (0.7 ± 0.14 relexp) versus (1.08 ± 0.14 relexp) for NOD2. Moreover, a very significant decrease in the IDO expression level in mice with IgY versus control was seen and the value reached (22.0 ± 6.94 relexp) in IgY animals and (45.47 ± 9.96 relexp) in controls.

3. Discussion

Since many years, the relationship between the microflora and GVHD has been well established [4]. Based on earlier publications [39,40], we selected *E. coli* as prototypical TLR4 agonists and *C. perfringens* as prototypical TLR2 agonist. We assumed that the resulting IgY antibodies, after oral intake, will have an impact on the overall gastrointestinal (GI) LPS, lipoteichoic acid and glycoprotein load at the GI mucosal lining level. To reach this target, the generated chicken antibodies were used to capture and eliminate bacteria and/or bacterial products in order to improve the aGVHD outcome. As we showed in Fig. 1, allogeneic recipients developed severe clinical symptoms whereas treatment with IgY-antibody resulted in significant improvement of the allogeneic recipients compared to allogeneic control. However, the allogeneic animals with chicken antibody still presented high GVHD score compared to syngeneic mice. Furthermore, 4 weeks after transplantation, the disease severity exacerbated again in allogeneic animals which indicated that aGVHD has not been completely abolished by IgY antibody treatment. Concerning the cytokine production and the importance of the conditioning regimen in aGVHD pathophysiology, a correlation between conditioning intensity, inflammatory cytokines and aGVHD severity was shown in different studies [41,42]. Moreover, in previous study from our group [43], we found that early after transplantation, the organ injury in syngeneic recipients exhibited only minor pathological changes independent of the conditioning regimen, indicating that the inflammation caused by allo reactive T cells is clearly the leading cause of organ injury at early stage.

In our previous work, 15 days after transplantation, we found that the stool samples of allogeneic animals treated with IgY presented high increase of *L. reuteri* and *Firm u. bact1* (21.95 ± 4.18% and 4.18 ± 0.08% respectively) compared to allogeneic control animals (0.85 ± 0.3% and 0.17 ± 0.05% respectively) [34]. In contrary, the stool samples of allo IgY animals showed decreased level of *E. coli* and *Bact. u. bacteria* (0.05 ± 0.02% and 5.35 ± 2.62% respectively) compared to allo control (2.18 ± 0.76% and 35.65 ± 8.13% respectively). But, at day 28 after transplantation the difference between the bacterial levels in allo IgY and control animals was not significant. Interestingly, only *E. coli* showed continuous significant difference between IgY animals at day 15 and day 28 after

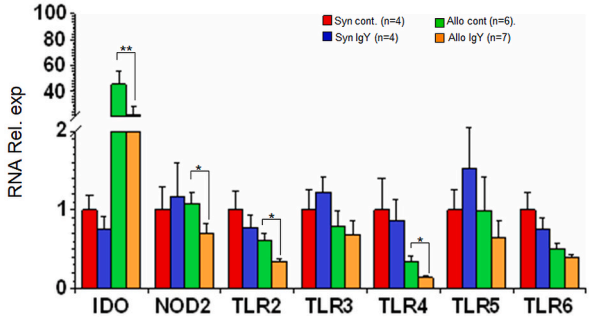

Fig. 3. Expression of PRR and IDO after allo-BMT. Lethally irradiated B6D2F1 mice received 2 x 10^6 bone marrow cells supplemented with 4 x 10^6 splenocytes from either syngeneic (B6D2F1) or allogeneic (C57BL/6) donors. Thereafter animals received chow with IgY or control chow from day -2 until day 28 The figure shows the relative expression on the RNA level for PRR and IDO in the colon 28 days after transplantation for ■ Syn cont (n=4), ■ Syn IgY (n=4), ■ Allo cont (n=6), ■ Allo IgY (n=7). The data are presented as mean ± SEM; *$p < 0.05$, **$p < 0.01$.

transplantation [34]. This result is in line with a previous study from Heimsaat et al. [21] who showed in a colitis murine model, a correlation between high organ damage and a significant population shifts towards *E. coli*. In addition, the adherent-invasive *E coli* strains, have been isolated from ileal crohn's disease (iCD) biopsy specimens and the abundance of this strain was very high in patients with CD compared with healthy person [44,45]. Taken altogether, we assumed that the reduced level of *E. coli* in allogeneic IgY mice at an early stage after transplantation maybe important in aGVHD development and explains, why the treatment with IgY could not abolish aGVHD but only slowed it down. In this project, beside *E. coli* we also targeted *C. perfringens* in the phylum Firmicutes, which comprise between 80 and 90% of the whole bacterial load and contains several hundreds of species [34]. *C. perfringens* was not detectable in the analyzed stool samples however, other species were still present and a significant increase of *L. reuteri* and *Firm u. bact1* (in the phylum Firmicutes) was seen in IgY animals versus controls. This result support early publication from Jenq et al. who showed that the development of GVHD or treatment with antibiotics induced unique microbial shifts [10] and that the elimination of Lactobacillales from the flora of mice before BMT aggravated GVHD, whereas reintroducing the predominant species of Lactobacillus mediated significant protection against GVHD. In the same context, a study from Gerbitz et al. [9] showed significant GVHD improvement by prophylactic oral administration of Lactobacillus rhamnosus GG. Interestingly, early publication showed that *L. reuteri* can produce antimicrobial products [46,47] which can inhibit the grow of *E. coli* [47].

Pathogen-associated molecular patterns (PAMPs) activate the innate immune system via pathogen recognition receptors (PRR). Prototypical representatives of PAMPs are lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid and glycoprotein. Lipopolysaccharide (LPS) is a component of the outer membrane of almost all Gram-negative bacteria. LPS is recognized by Toll-like receptor 4 (TLR4) on host immune cells and can induce high production of pro-inflammatory cytokines via activation of the transcription factor NF-κB, which leads to short term inflammatory responses [39]. Whereas lipoteichoic acid and peptidoglycan are typically found in the cell wall of gram positive bacteria and are potent activators of TLR2 [40].

We found in our study a significant decrease in the expression of TLR2 and TLR4 in mice receiving IgY chow compared to controls. This is in line with previous results which demonstrated the role of TLR2 and TLR4 in inducing pro-inflammatory intestinal bacteria in a colitis model [21]. For NOD2 the quantification of the expression showed a reduced level in animals with IgY chow versus control and confirms the importance of NOD2 in elimination of bacteria in the gut. This is supported by several studies which demonstrated this effect of NOD2 on the chemokine and cytokines expression via MDP and NF-κB pathway [48–52].

In our study, we found also a significant reduction in CCL3 expression between control and IgY animals ($p = 0.026$). The expression value reached (44.36 ± 9.91 relexp) in the control versus (16.41 ± 4.31 relexp) in the mice received IgY chow. It is known that TLR4 is the receptor of LPS, after activation, the TLR4 leads to the synthesis of pro-inflammatory chemokines and cytokines [53,54]. Duo to the fact, that the treatment with IgY reduced the *E. coli* as well as the total bacterial load [34] which subsequently conducted to the reduction of LPS. We can conclude that this reduction resulted in decreases of the CCL3 chemokine in animals treated with IgY chow.

Another reason for the reduction of inflammation chemokine CCL3 could be the reduction of IDO. In our study, we found a significant difference in IDO expression between IgY treated animals and controls. It is known, that IDO modulates the inflammation, in a recent publication, the author founds that the inhibition of IDO prevents long-term cognitive impairment triggered by sepsis [29] and the blockade of IDO protects mice against endotoxin shock via modulation of IL-10 production in DCs [33]. Moreover, previous study showed that the IDO, TLR and NOD2 expression is affected by inflammatory mediators, such as Toll-like receptor ligands [55]. Taken all together, we could assume that the reduction in IDO, and other PRR in IgY treated animals, could results in reduction of the CCL3 chemokine. The reduction of IDO and other PRR could be driven by the reduced load of *E. coli* and other microbiota via reduction of LPS.

4. Conclusions

In summary we found that the aGVHD improvement of animals treated with IgY has been mediated by reduction of the bacterial load and decrease in the pathogenic bacteria like *E. coli*. This microbial change may result in reduction of TLR2, 4 NOD2 and IDO expression via LPS reduction which can cause also a subsequent decrease in chemokine and cytokine expression and finally improves the inflammation.

5. Materials and methods

5.1. Transplantation procedure

Female C57BL/6 (H-2^b) and B6D2F1 (H-2^{bxd}) mice were purchased from Charles River (Charles River, Sulzbach, Germany) and acclimatized in the animal facility for at least 7 days before starting the experiments. All animal experiments were approved by the animal committee of the University of Regensburg (54-2532.1-19/12) and were in accordance with German animal protection laws. Transplanted mice were between 11 and 14 weeks old at the time of BMT and were housed in micro-isolator cages with autoclaved bedding and received sterilized chow and water.

The transplantation was done as described previously [34]; in summary on the day of the transplantation (day 0) B6D2F1 recipient received a TBI dose of 9Gy delivered in one fraction using a linear accelerator (150 cGy/min). Thereafter animals were infused with cell mixtures of 2×10^6 BM cells supplemented with 4×10^6 SC from either syngeneic (B6D2F1 = syn) or allogeneic (C57BL/6 = allo) donors. After transplantation, the mice received pellet chow with antibodies from hens immunized with heat inactivated *E. coli* (E.C), *C. perfringes* (C.P). and *S. typhimorium* (S.T) from the company *IgNova GmbH (IgNova GmbH, Oberursel, Germany*) or control chow (without IgY) from day −2 until day 28 after transplantation. After that, survival was monitored daily, the weight and the clinical

GVHD scores were assessed weekly. Furthermore, the colon pathology score, the expressions of chemokines, cytokines, TLR2-6, IDO and NOD2 were measured in the colon 28 days after transplantation using real-time PCR.

5.2. Clinical GVHD score

For the clinical GVHD score, a scoring system incorporating five clinical parameters: weight loss, posture (hunching), mobility, fur texture, and skin integrity [43,56] was used. Each parameter was graded between 0 and 2. Once an animal reached a cumulative score $\geq$6.5 or a weight loss of >35%, it was sacrificed and counted as death due to transplantation-related mortality.

5.3. Colon pathological score

On day 28 after transplantation, animals were sacrificed. Hematoxylin-eosin-stained organ sections from individual mice were coded without reference to mouse type and prior treatment and detailed analysis of changes in colon was performed in a blinded fashion as described previously [34]. Analysis was performed by light microscopy (Axioskop 2 plus, Carl Zeiss GmbH, Jena, Germany). Photographs of histopathology were acquired by AxioCam HRc (Carl Zeiss GmbH) and processed with AxioVision Release 4.6.3 (Carl Zeiss GmbH, Jena, Germany).

5.4. Determination of mRNA-expression levels using real-time PCR

After transplantation and treatment as described in transplantation procedure, the colon samples were isolated from different animals 28 days after allo-BMT. After that, total cellular RNA was extracted using mammalian total RNA Miniprep Kit (Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer's protocol. First-strand cDNA was synthesized using 1 μg of total RNA (DNase-treated) with Maxima first strand cDNA Syn-Kit (Thermo-scientific, Dreieich, Germany) and stored at −20 °C until use. After synthesis of cDNA, all real-time PCR reactions to measure the selected cytokines and chemokines (Table 1) were performed according to a standard protocol as previously described [35]. The real-time PCR program was conducted under successive cycling conditions of 95 °C for 5 min, then 35 cycles at 95 °C for 1 min, 60 °C for 30 s, and 75 °C for 15 s, after 35 cycles a final elongation step at 72 °C for 10 min was done. The quantification was performed using the LightCycler® 480 from Roche (Roche, Mannheim, Germany) and the fluorescence threshold value was calculated using the SoftwareLightCycler® 480 version 1.5.0. Normalization with the housekeeping gene Beta-actin was applied for data calculations with results as $2^{-\Delta\Delta CT}$ expression. The primers used for real-time PCR are listed in Table 1.

5.5. Statistical methods

Weight, GHVD scores, expression levels of cytokines and bacterial constitution were compared between IgY treated animals and control at individual time points using Mann-Whitney *U* test. For the analysis of the differences for microbial constitution among the treatment groups, first we analyzed the variance by ANOVA. After that, species tested significant were compared using the Mann-Whitney *U* test. Statistical significance for all analyses was established when the value was less than $p < 0.05$. Data are presented as mean ± SEM (standard error of the mean). IBM Statistic 22 program (SPSS, Chicago, USA) was used for analysis.

Author contribution statement

Abdellatif Bouazzaoui: Conceived and designed the experiments; Performed the experiments and analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; drafting the article or critically revising its important intellectual content; Wrote the paper.

Neda M. Bogari: Conceived and designed the experiments; Performed the experiments and analyzed and interpreted the data;

Table 1
Primer used for real time RT-PCR.

	sens	anti.sens
B-act	gctgtccctgtatgcctctg	gtggtggtgaagctgtagcc
TNF	tacgtgctcctcacccacac	agttggtccccctctccag
IFNg	caggccatcagcaacaacat	cgcttcctgaggctggatt
IL-1beta	ctgcagctggagagtgtgga	ggcttgtgctctgcttgtga
IDO	cccagtccgtgagtttgtca	ccacatttgagggctcttcc
NOD2	gggagatgtcggagtggaac	ccaccctcagggacaagaag
TLR2	cgagctcctgaagctgttgc	gtctgactccgaggggttga
TLR3	tgaaccccacctcacagag	cgaggggacagacgctgtat
TLR4	cgtccatcggttgatcttgg	cctgccagagacattgcaga
TLR5	caccgaagactgcgatgaag	gcattctgtgcccattcaaa
TLR6	tgtgctgtctcccacttca	tccaagtacccgcagcttgt
CCL3	tgcctgctgcttctcctaca	tggacccaggtctctttgga
CXCL9	tgggcatcatcttcctggag	ccggatctaggcaggtttga

Contributed reagents, materials, analysis tools or data; drafting the article or critically revising its important intellectual content; Wrote the paper.

Faisal A. Al-Allaf: Performed the experiments and analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; drafting the article or critically revising its important intellectual content.

Samar N. Ekram: Performed the experiments and analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; drafting the article or critically revising its important intellectual content.

Mohammad Athar: Performed the experiments and analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; drafting the article or critically revising its important intellectual content.

Thomas Schubert: Conceived and designed the experiments; Performed the experiments and analyzed and interpreted the data; drafting the article or critically revising its important intellectual content.

Shahzad Nawaz Syed: Conceived and designed the experiments; Performed the experiments and analyzed and interpreted the data; drafting the article or critically revising its important intellectual content.

Ahmed A. H. Abdellatif: Conceived and designed the experiments; Performed the experiments and analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; drafting the article or critically revising its important intellectual content; Wrote the paper.

Abdel-Rahman Youssef: Performed the experiments and analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; drafting the article or critically revising its important intellectual content.

Mashael Alqahtani: Performed the experiments and analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; drafting the article or critically revising its important intellectual content.

Anas Dannoun: Performed the experiments and analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; drafting the article or critically revising its important intellectual content.

Funding statement

Associ. Prof Abdellatif Bouazzaoui was supported by Dean of Scientific Research, at Umm Al-Qura University of the Kingdom of Saudi Arabia [22UQU4350178DSR01].

Data availability statement

Data will be made available on request.

7. Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Additional information

No additional information is available for this paper.

Acknowledgments

The authors would like to thank Dr. Bassim bin Yusuf Muhammad Al-Kazemi (Dean of Scientific Research) and the staff of the Science and Technology Unit and Deanship of Scientific Research at Umm Al-Qura University for their continuous support.

References

[1] J.L. Ferrara, et al., Graft-versus-host disease, Lancet 373 (2009) 1550–1561.
[2] S. Paczesny, et al., Acute graft-versus-host disease: new treatment strategies, Curr. Opin. Hematol. 16 (2009) 427–436.
[3] J.O. Armitage, Bone marrow transplantation, N. Engl. J. Med. 330 (1994) 827–838, https://doi.org/10.1056/NEJM199403243301206.
[4] D.W. van Bekkum, S. Knaan, Role of bacterial microflora in development of intestinal lesions from graft-versus-host reaction, J. Natl. Cancer Inst. 58 (1977) 787–790.
[5] F. Paris, et al., Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice, Science 293 (2001) 293–297.
[6] F.P. Nestel, et al., Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor alpha during graft-versus-host disease, J. Exp. Med. 175 (1992) 405–413.
[7] D.W. Beelen, et al., Evidence that sustained growth suppression of intestinal anaerobic bacteria reduces the risk of acute graft-versus-host disease after sibling marrow transplantation, Blood 80 (1992) 2668–2676.
[8] D.W. van Bekkum, et al., Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora, J. Natl. Cancer Inst. 52 (1974) 401–404.
[9] A. Gerbitz, et al., Probiotic effects on experimental graft-versus-host disease: let them eat yogurt, Blood 103 (2004) 4365–4367.
[10] R.R. Jenq, et al., Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation, J. Exp. Med. 209 (2012) 903–911.
[11] R.R. Jenq, et al., Intestinal Blautia is associated with reduced death from graft-versus-host disease, Biol. Blood Marrow Transplant. 21 (2015) 1373–1383.
[12] Y. Taur, et al., The effects of intestinal tract bacterial diversity on mortality following allogeneic hematopoietic stem cell transplantation, Blood 124 (2014) 1174–1182.

[13] D. Weber, et al., Low urinary indoxyl sulfate levels early after transplantation reflect a disrupted microbiome and are associated with poor outcome, Blood 126 (2015) 1723–1728.
[14] B. Lemaitre, et al., The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults, Cell 86 (1996) 973–983.
[15] R. Medzhitov, et al., A human homologue of the Drosophila Toll protein signals activation of adaptive immunity, Nature 388 (1997) 394–397.
[16] M. Schnare, et al., Toll-like receptors control activation of adaptive immune responses, Nat. Immunol. 2 (2001) 947–950.
[17] D.N. Cook, et al., Toll-like receptors in the pathogenesis of human disease, Nat. Immunol. 5 (2004) 975–979.
[18] J.D. Hansen, et al., Sensing disease and danger: a survey of vertebrate PRRs and their origins, Dev. Comp. Immunol. 35 (2011) 886–897.
[19] N. Inohara, et al., Human Nod1 confers responsiveness to bacterial lipopolysaccharides, J. Biol. Chem. 276 (2001) 2551–2554.
[20] F. Martinon, J. Tschopp, NLRs join TLRs as innate sensors of pathogens, Trends Immunol. 26 (2005) 447–454.
[21] M.M. Heimesaat, et al., Shift towards pro-inflammatory intestinal bacteria aggravates acute murine colitis via Toll-like receptors 2 and 4, PLoS One 2 (2007) e662.
[22] P.A. Taylor, et al., TLR agonists regulate alloresponses and uncover a critical role for donor APCs in allogeneic bone marrow rejection, Blood 112 (2008) 3508–3516.
[23] M. Vijay-Kumar, et al., Deletion of TLR5 results in spontaneous colitis in mice, J. Clin. Invest. 117 (2007) 3909–3921.
[24] J. Lee, et al., Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells, Nat. Cell Biol. 8 (2006) 1327–1336.
[25] D.K. Podolsky, et al., Colitis-associated variant of TLR2 causes impaired mucosal repair because of TFF3 deficiency, Gastroenterology 137 (2009) 209–220.
[26] M. Vijay-Kumar, et al., Activation of toll-like receptor 3 protects against DSS-induced acute colitis, Inflamm. Bowel Dis. 13 (2007) 856–864.
[27] M. Ehlers, J.V. Ravetch, Opposing effects of Toll-like receptor stimulation induce autoimmunity or tolerance, Trends Immunol. 28 (2007) 74–79.
[28] A.L. Mellor, D.H. Munn, Ido expression by dendritic cells: tolerance and tryptophan catabolism, Nat. Rev. Immunol. 4 (2004) 762–774.
[29] C.M. Comim, et al., Inhibition of indoleamine 2,3-dioxygenase 1/2 prevented cognitive impairment and energetic metabolism changes in the hippocampus of adult rats subjected to polymicrobial sepsis, J. Neuroimmunol. 305 (2017) 167–171, https://doi.org/10.1016/j.jneuroim.2017.02.001.
[30] M.F. Murray, Tryptophan depletion and HIV infection: a metabolic link to pathogenesis, Lancet Infect. Dis. 3 (2003) 644–652.
[31] K. Pellegrin, et al., Enhanced enzymatic degradation of tryptophan by indoleamine 2,3-dioxygenase contributes to the tryptophan-deficient state seen after major trauma, Shock (Augusta, Ga.) 23 (2005) 209–215.
[32] A. Boasso, et al., HIV inhibits CD4+ T-cell proliferation by inducing indoleamine 2,3-dioxygenase in plasmacytoid dendritic cells, Blood 109 (2007) 3351–3359, https://doi.org/10.1182/blood-2006-07-034785.
[33] I.D. Jung, et al., Blockade of indoleamine 2,3-dioxygenase protects mice against lipopolysaccharide-induced endotoxin shock, J. Immunol. 182 (2009) 3146–3154, https://doi.org/10.4049/jimmunol.0803104.
[34] A. Bouazzaoui, et al., Reduction of aGVHD using chicken antibodies directed against intestinal pathogens in a murine model, Blood 129 (2017) 1052–1055, https://doi.org/10.1182/blood-2016-06-722538.
[35] A. Bouazzaoui, et al., Chemokine and chemokine receptor expression analysis in target organs of acute graft-versus-host disease, Gene Immun. 10 (2009) 687–701, https://doi.org/10.1038/gene.2009.49.
[36] E. Holler, et al., Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination, Blood 107 (2006) 4189–4193.
[37] E. Holler, et al., Both donor and recipient NOD2/CARD15 mutations associate with transplant-related mortality and GvHD following allogeneic stem cell transplantation, Blood 104 (2004) 889–894.
[38] K. Landfried, et al., Tryptophan catabolism is associated with acute GVHD after human allogeneic stem cell transplantation and indicates activation of indoleamine 2,3-dioxygenase, Blood 118 (2011) 6971–6974.
[39] N. Maeshima, R.C. Fernandez, Recognition of lipid A variants by the TLR4-MD-2 receptor complex, Front. Cell. Infect. Microbiol. 3 (2013) 3, https://doi.org/10.3389/fcimb.2013.00003.
[40] M.J. Aldape, et al., Innate immune recognition of, and response to, Clostridium sordellii, Anaerobe 16 (2010) 125–130, https://doi.org/10.1016/j.anaerobe.2009.06.004.
[41] R. Chakraverty, et al., An inflammatory checkpoint regulates recruitment of graft-versus-host reactive T cells to peripheral tissues, J. Exp. Med. 203 (2006) 2021–2031.
[42] C.Q. Xun, et al., Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice, Blood 83 (1994) 2360–2367.
[43] A. Bouazzaoui, et al., Cytostatic conditioning in experimental allogeneic bone marrow transplantation: busulfan causes less early gastrointestinal toxicity but Treosulfan results in improved immune reconstitution, Immunopharmacol. Immunotoxicol. 36 (2014) 158–164.
[44] A. Darfeuille-Michaud, et al., High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease, Gastroenterology 127 (2004) 412–421.
[45] M. Martinez-Medina, et al., Molecular diversity of Escherichia coli in the human gut: new ecological evidence supporting the role of adherent-invasive E. coli (AIEC) in Crohn's disease, Inflamm. Bowel Dis. 15 (2009) 872–882.
[46] T.L. Talarico, et al., Production and isolation of reuterin, a growth inhibitor produced by Lactobacillus reuteri, Antimicrob. Agents Chemother. 32 (1988) 1854–1858.
[47] M.G. Ganzle, et al., Characterization of reutericyclin produced by Lactobacillus reuteri LTH2584, Appl. Environ. Microbiol. 66 (2000) 4325–4333.
[48] K.S. Kobayashi, et al., Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract, Science 307 (2005) 731–734.
[49] S. Maeda, et al., Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing, Science 307 (2005) 734–738.
[50] Coulombe, F. et al. Muramyl dipeptide induces NOD2-dependent Ly6C(high) monocyte recruitment to the lungs and protects against influenza virus infection. PLoS One 7, e36734.
[51] Kim, Y.G. et al. The Nod2 sensor promotes intestinal pathogen eradication via the chemokine CCL2-dependent recruitment of inflammatory monocytes. Immunity 34, 769-780.
[52] Hausmann, M. et al. MIP-3alpha expression in macrophages is NOD dependent. Digestion 85, 192-201.
[53] S. Janssens, R. Beyaert, Role of toll-like receptors in pathogen recognition, Clin. Microbiol. Rev. 16 (2003) 637–646, https://doi.org/10.1128/CMR.16.4.637-646.2003.
[54] H. Kanzler, et al., Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists, Nat. Med. 13 (2007) 552–559, https://doi.org/10.1038/nm1589.
[55] S. Fujigaki, et al., Lipopolysaccharide induction of indoleamine 2,3-dioxygenase is mediated dominantly by an IFN-gamma-independent mechanism, Eur. J. Immunol. 31 (2001) 2313–2318, https://doi.org/10.1002/1521-4141(200108)31:8<2313::aid-immu2313>3.0.co;2-s.
[56] A. Bouazzaoui, et al., Steroid treatment alters adhesion molecule and chemokine expression in experimental acute graft-vs.-host disease of the intestinal tract, Exp. Hematol. 39 (2011) 238–249 e231.