Tyrosine Supplement Ameliorates Murine aGVHD by Modulation of Gut Microbiome and Metabolome

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Research

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Abstract

Background

The microbial communities and their metabolic components in gut are essential for immune homeostasis and profoundly influence the host susceptibility to many immune-mediated diseases including acute graft-versus-host disease (aGVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, the functional connections between microbiome and metabolites in aGVHD are poorly understood because of the complexity of gastrointestinal environment. Thus, we initially performed 16S ribosomal DNA gene sequencing and ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS)-based metabolomics to unleash the gut microbiota and fecal metabolic phenotype in aGVHD murine model.

Results

A *Lachnospiraceae_unclassified* was significantly downregulated while the relative abundance of *Clostridium XI*, *Clostridium XIVa* and *Enterococcus* were increased in aGVHD happened group. Meanwhile, a lower content of tyrosine was observed in the gut of aGVHD mice. The correlation analysis revealed that tyrosine-related metabolites inversely correlated with *Clostridium XIVa*, *Blautia* and *Enterococcus* in aGVHD condition. In addition to explore the importance and function of tyrosine, we supplied different tyrosine content diets to mice during transplantation. Additional tyrosine supplements could improve overall survival, ameliorate symptoms at the early stage of aGVHD and changed the structure and composition of gut microbiota and fecal metabolic phenotype, while mice with aGVHD deprived from tyrosine displayed worse manifestations than vehicle.

Conclusions

Overall, a better understanding of the roles of gut microbiota-metabolomes interconnectedness in aGVHD could help identify disease biomarkers and offer better targets for diagnosis and treatment.

1. Introduction

Acute Graft-versus-host disease (aGVHD) is one of the fatal and refractory complications after allogeneic hematopoietic stem cell transplantation (allo-HSCT). The gastrointestinal tract, as the initial damaged organ, is particularly affected by not only activated donor T cells but also the pretransplant conditioning with chemotherapy and irradiation after allo-HSCT. [1, 2] Current treatments concentrate on how to ameliorate the activation of various immune cells especially T cells and inhibit its graft immunology attack. However, the gut microbiota and their metabolites become the primary systemic exposure to epithelial cells, which are essential for mediating gastrointestinal immune responses and regarded as the potential future manipulation for controlling morbidity and mortality after allo-HSCT. [3]

People have recognized gut bacteria acting as an essential modulator fine-tuning homeostasis in human health decades ago. [4] Several disease processes have been verified to associate with the dysbiosis of gut microbiota, such as obesity, insulin resistance, chronic inflammatory and even cancer. [5, 6] People also intended to reveal the relationship between gut microbiota and aGVHD. The importance of microbiome diversity has been first recognized in patients after allo-HSCT and murine aGVHD model. [7–10] Some of the bacteria have been defined as the negative factors by a vary of experiments including *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Escherichia*. [11, 12] Consistent with the results in mice model, the expansion of *Lactobacilliales* and *Enterobacteriales* related to worse outcome in aGVHD patients. Besides, the benefit of increased amounts of *Blautia* associating with reduced GVHD lethality was found in clinical patients but not in mouse models [13]. In aGVHD patients, it is hard to reach a broad consensus on the standards because of the geographical and temporal variations, dietary differences and administrations of antibiotics. Still, researchers focused on modulating gut homeostasis by administration of beneficial microbes via direct supplementation and fecal microbial transplantation (FMT).[14, 15] For refractory and steroid resistant/dependent aGVHD, FMT was performed to attenuate diarrhea and led to sustained changes in the fecal microbiota in aGVHD patients. [16]

In addition to host-derived metabolites, the variations of gut microbiota-derived metabolites were identified at the onset of aGVHD. [17] Gut microbiota affects the bioavailability of metabolites in the gastrointestinal tract, which is proven by the gut metabolic alteration of mice in germ-free compared to conventional environment. [18] The gut microbiota participates in the regulation of multiple metabolic pathways in the host, giving rise to interactions of host-microbiota metabolic, signaling, and immune-inflammatory. [5] Short chain fatty acids (SCFAs) are one of the most widely studied metabolites. Restoration butyrate in intestinal tract or enhance SCFAs sensor could improve junctional integrity of intestinal epithelial cells and upregulate regulatory T cells to mitigate murine aGVHD. [19, 20] Similar ideal results can be obtained by replenishment of butyrate-producing bacteria *Clostridia*, which highlight the mutual influence of gut microbiota and metabolism. [19, 21] Concurrently, acting as precursors for the synthesis of SCFAs, amino acid is another important metabolite in intestine. [22] Numerous researches aim to reveal the pivotal roles of amino acids in allo-HSCT. For example, in mouse models and clinical studies, glutamine supplementation promoted intestinal healing and reduced the severity of mucositis and GVHD. [23] Together, these studies indicate intestinal microbial flora and related metabolites constitute a close correlation with the occurrence and development of diseases. Due to diarrhea, hypermetabolism and inadequate food intake, aGVHD patients experience nutritional and metabolic derangements, and thus, nutritional support can be a novel therapeutic direction. [24]

Tyrosine, a large neutral amino acid normally present in protein food, take part in building essential proteins and providing energy. [25] Tyrosine constitute the precursors of catecholamine neurotransmitters dopamine, norepinephrine, and epinephrine neurotransmitters that can help people effectively response to acute stress and maintenance of homeostasis. [26] As researches recently reported that dietary lactose depletion could attenuate *Enterococcus* outgrowth and reduce the severity of aGVHD in mice, [27] we wonder whether similar roles exist in tyrosine modulating gut microbiome and metabolome under aGVHD status. In our study, we found that the gut abundance of tyrosine markedly decreased in aGVHD mice by

16S rDNA gene sequencing and UHPLC-MS-based metabolomics, which was closely correlated with gut microbiota changes, such as an unclassified Lachnospiraceae, Clostridium XI, Clostridium XIVa and Enterococci. While 2% tyrosine diet improved overall survival and ameliorated aGVHD symptoms at the early stage, the addition of tyrosine shifted the gut microbiota structure and led to fecal metabolome variations, which exhibited a strong correlation between them. Therefore, we suspect that, gut microbiota interacting with metabolites are not only potential therapeutic targets, but also the biomarkers for aGVHD diagnosis and therapeutic response in the future.

2. Methods

2.1 Mice

Male BALB/c mice (H-2Kd) and C57BL/6 mice (H-2Kb) (SLAC Laboratory Supplies, Shanghai, China) were used at 8–10 weeks of age. All mice experiments were conducted under specific pathogen free conditions in the laboratory animal center of Zhejiang University.

2.2 Bone marrow transplantation model

Bone marrow transplantation (BMT) recipients received tail vein injections of 5×10^6 T-cell-depleted bone marrow cells (TCD-BM) (Mouse CD3 Positive Selection Kit, Biolegend) with or without 5×10^5 splenic T cells (Pan T cell isolation kit, Miltenyi Biotech) on day 0 after lethal irradiation with 8 Gy (split doses of 2 × 4.0 Gy apart to 4 h) form C57BL/6 → BALB/c at ZJU, China. Clinical scores were assigned following the criteria described by Geoffrey et al. [28] Histopathology scoring for aGVHD was determined according to previously published histopathology scoring systems. [28, 29]

2.3 Dietary Tyrosine dosing of mouse models

According to a pilot study with normal mice comparing the standard feed (0.7% tyrosine) to the same feed supplemented with an additional 2%, 4% or 8% tyrosine, 2% tyrosine established as the highest and safe concentration. [30] Tyrosine intervention groups commenced 0% or 2% tyrosine diet (Hangzhou LiLeng Biotechnology Co., Ltd) a week before BMT and food changed every 2 to 3 days until the end of survival time observation or sacrifice.

2.4 PCR amplification and 16S rDNA gene sequencing

DNA was extracted from around 50–100 mg of fecal utilizing the E.Z.N.A.® Soil DNA Kit (Omega) which was frozen by liquid nitrogen and then stored at -80 °C.The V3-V4 region of the prokaryotic (bacterial and archaeal) small-subunit (16S) rRNA gene was amplified with slightly modified versions of primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'- GGACTACHVGGGTWTCTAAT-3'). The 5' ends of the primers were tagged with specific barcodes per sample and sequencing universal primers. The PCR products were confirmed with 2% agarose gel electrophoresis. The PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA), quantified by Qubit (Invitrogen, USA) and sequenced on an Illumina MiSeq platform. Sequences with similarity over 97% were combined to one

operational taxonomic unit. Differential abundance analysis was analysis and performed on levels of alteration in the gut microbiota using R (3.2.1).

2.5 LC-MS analysis

All samples were acquired by the LC-MS system and all chromatographic separations were performed using an ultra-performance liquid chromatography (UPLC) system (SCIEX, UK). An ACQUITY UPLC T3 column (100mm*2.1 mm, 1.8 μm, Waters, UK) was used for the reversed phase separation, which maintained at 35 °C. Gradient elution parameters and the conditions were set as recommend. [31]

A high-resolution tandem mass spectrometer TripleTOF5600plus (SCIEX, UK) was used to detect metabolites which was operated in 5 kV positive and − 4.5 kV negative modes respectively. The mass spectrometry data was acquired in IDA mode and the TOF mass range was from 60 ~ 1200 Da. The survey scans were acquired in 150 ms and as many as 12 product ion scans were collected if exceeding a threshold of 100 counts per second and with a 1 + charge-state. Total cycle time was fixed to 0.56 s. Four time bins were summed for each scan at a pulser frequency value of 11 kHz through monitoring of the 40 GHz multichannel TDC detector with four-anode/channel detection. Dynamic exclusion was set for 4 s. [32] The mass accuracy was calibrated every 20 samples and a quality control sample was acquired every 10 samples. The metabolites were identified according to accurate molecular weight and fragment pattern by MS/MS and by comparison to online databases such as the Human Metabolome Database, Biofluid Metabolites Database, etc.

2.6 Correlation network analysis and pathway analysis

The correlations of fecal microbiome and metabolomics were calculated as networks based on Spearman correlation analysis. And the correlation network was constructed by Cytoscape.[33] For metabolites Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, selected metabolites were uploaded to MetaboAnalyst database.

2.7 Statistical analysis

For 16S rDNA gene sequencing, we chose top 20 microbiomes in genus level. For metabolites analysis, only the metabolites ratio ≥ 2 or ≤ 1/2, VIP (Multivariate statistical analysis with PLS-DA to obtain Variable Important for the Projection, VIP) ≥ 1, q value ≤ 0.05 and annotated at MS2 level were taken into account. Differences in animal survival (Kaplan-Meier survival curves) were analyzed by log-rank test. For statistical analysis, an unpaired Student's t-test (2-sided) was applied. If the data did not meet the criteria of normality, the Mann-Whitney U test was applied. Data presented as mean ± SEM or SD if indicated. Differences were considered significant when the $P < 0.05$.

3. Result

3.1 Gut microbiome structure shifted in aGVHD murine model

To determine whether aGVHD affects the parameters of gut environment, we initially used a well-established C57BL/6 (H-2Kb) into a BALB/c (H-2Kd) major histocompability complex (MHC) mismatch model (Fig. 1A). [34] TCD-BM + T cells group showed severe aGVHD symptoms including shortened survival time, rapid weight loss, severe diarrhea and injury in multiple organs including liver, intestine and skin (Fig. 1B-E and Supplementary Fig.S1). We wondered if the changes of bacterial flora diversity were associated with occurrence and development of aGVHD. Fecal microbiome was detected on day 14 and 28 after allo-HSCT. Although it is hard to obtain conspicuous differences on phylum levels, we did observe some changes between TCD-BM and TCD-BM + T cells recipients from the fecal microbial 16S rDNA gene sequencing data. *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia* and *Proteobacteria* were the top four dominant components of the fecal microbes (Fig. 1G, H). *Firmicutes*, accounting for the largest proportion in the microbiota compositions, was decreased in TCD-BM + T cells group, while the other three phylum levels were upregulated. These variation trends remained constant across the whole progression of aGVHD.

Notably, we obtained some significant changes in genus level. The altered floras predominantly belonged to *Firmicutes* phylum. One of the most abundant genus, *Lachnospiraceae_unclassified*, maintained a high proportion in TCD-BM group but decreased in TCD-BM + T cells group (the mean of TCD-BM and TCD-BM + T cells groups, day14: 54.96% and 9.45%, p□0.05; day 28: 38.28% and 11.90%, p□0.05). Additionally, the consistent and significant differences in fold change on day 14 (p□0.05) and 28 (p□0.05) made the tendency even more convincing (Fig. 1I, J, Supplementary Table 1). The other microbiota belonging to *Firmicutes* were upregulated in TCD-BM + T cells group, such as *Blautia*, *Clostridium XI*, *Clostridium XIVa* (p□0.05 on day 14) and *Enterococcus* (p□0.05 on day 28). Other altered genera such as *Proteus*, belonging to *Proteobacteria*, and high-occupied *Bacteroides*, belonging to *Bacteroidetes*, were also upregulated in TCD-BM + T cells group. Another *Bacteroidetes* component *Alistipes* (p□0.05 on day 28) was downregulated in TCD-BM + T cells recipients. All the tendencies were consistent, which suggested that the gut microbiome composition exhibited remarkable changes along with the occurrence and development of aGVHD.

3.2 Gut metabolome revealed dramatic reduction of tyrosine and related metabolites in aGVHD mice

As microbiota alteration in aGVHD has been extensively explored, some studies stated that the microbiome structure strongly influences the metabolite profiles in multiple disease. [35, 36] We then followed the differences in the fecal metabolome after BMT. To confirm the implication of these metabolites at aGVHD onset, principal component analysis (PCA) was used to confirm whether aGVHD could be discriminated in multivariate analysis (Supplementary Fig.S2A, B). In the partial least squares-discriminant analysis (PLS-DA), the PLS-DA score plots indicated the discrepancies between TCD-BM and TCD-BM + T cells groups were reliable and suggested the rejection reactions leading to significant biochemical changes (Supplementary Fig.S2A,B). Based on pairwise comparisons of different metabolites between two groups and different aGVHD development stages, both of the two groups' fecal metabolic profiles displayed depression at the early stage on day 14. However, the infertile situation was

improved on day 28, which was corresponded to the reconstruction of bone marrow after the irradiation (Supplementary Fig.S2C).

Metabolites with putative biological relevance were identified by comparison of the amount of each metabolite between TCD-BM and TCD-BM + T cells groups on day 14 (Fig. 2A) and 28 (Fig. 2B). Compared with transfusing TCD-BM only, TCD-BM + T cells groups were mainly characterized by a significant decrease in the amounts of lipid and lipid-like molecules, especially physalin P, acylcarnitine and 8-isoprostaglandin E2. Organic acids and derivatives also showed a declined trend in TCD-BM + T cells group, especially dihydrocaffeic acid 3-sulfate and tyrosine (Fig. 2C, Supplementary Table 2). Later, KEGG analysis was performed to analyze the pathways with all the significant different metabolites. According to the KEEG results, several amino acid related pathways attracted our attention. The pathways of phenylalanine, tyrosine and tryptophan biosynthesis ranked on the top (Fig. 2D). As recent discoveries have underscored that changes in the microbiota modulate the host immune system by modulating tryptophan metabolism, [37] we wandered whether another metabolite bears resemblance to it in the same pathway. Strikingly, the following several pathways, such as ubiquinone and other terpenoid-quinone biosynthesis and aminoacyl-tRNA biosynthesis, shared the same metabolite, tyrosine. Thus, we found that not only tyrosine but also the metabolites interacting with tyrosine demonstrating an overall downtrend in TCD-BM + T cells group. While tyrosine was remarkably diminished on day 14 ($p < 0.01$) and 28($p < 0.01$) (Fig. 2E; Supplementary Table 3), L-Aspartic acid, L-Glutamic acid and S-Adenosylhomocysteine became significant ($p < 0.01$) on day 28 as the heatmap showed (Fig. 2F; Supplementary Table 3).

To study the correlations of the host microbiota-metabolomes system, we performed a spearman correlation analysis of tyrosine related metabolites and altered microbiota to explore the internal connections. In general, tyrosine located in the central of correlation network in both comparisons, and bacteria belonging to *Firmicutes* were closest related to selected metabolites (Fig. 2G, H). The components of the network in TCD-BM + T cells and TCD-BM groups were smaller and more scattered on day 14 than day 28. Still, we were able to detect the tyrosine-related metabolites correlated inversely with *Clostridium XlVa*, *Blautia* and *Enterococcus* on day 14 and 28. Interestingly, we found that all of genera in the network belonged to *Firmicutes* on day 28, which implied that this phylum might play a prominent role in aGVHD development. Collectively, these results indicate that the low levels of tyrosine in gut likely correlated with aGVHD occurrence and development.

3.3 Tyrosine supplement partially ameliorated aGVHD phenotype

Our results demonstrated that tyrosine declined in the gut accompanying with microbiota alteration in aGVHD. Subsequently, we reckoned whether tyrosine replenishment could have a positive effect on alleviation for aGVHD and further affect the microbiome structure and metabolite profiles. To test this, we supplied TCD-BM and TCD-BM + T cells group with 2% tyrosine diet that was higher than normal but within safety content and 0% tyrosine diet as the deprivation group respectively. Administration of

tyrosine resulted in extension of total survival time, significant reduction of weight loss and decrease in aGVHD clinical scores in the early period of aGVHD (Fig. 3A-C), especially slighter diarrhea, and better physical status (Supplementary Fig.S3D). Notably, the TCD-BM + T cells + 2% tyrosine diet group displayed lower intestinal and skin pathology scores with improved intestinal epithelial structure, kept microvilli and tight junctions intact and more complete skin structure on day 14 after BMT (Fig. 3D, E). However, these advantages became weaker at the later stage of aGVHD, as shown that the weight variances and clinical scores of TCD-BM + T cells + 2% tyrosine diet group approached to TCD-BM + T cells + vehicle diet groups near the end of day 40. When mice with aGVHD were deprived from tyrosine by given 0% tyrosine diet, they displayed even worse survival rate and clinical scores than TCD-BM + T cells + vehicle diet groups (day 30, p□0.01) .

As mice that had received TCD-BM only did not present any signs of aGVHD, deprived or extra tyrosine-diet had no impact on their survival rate until the end (Fig. 3A). In comparison, TCD-BM + 0% tyrosine diet group exhibited delayed weight regain and slight aGVHD symptoms at initial stage after BMT. The histopathologic analysis also displayed mild lesions on intestine and skin on day 14 (Fig. 3D, E). Above all, these results implied additional tyrosine diets only played a role in aGVHD status and influenced the early stage after allo-HSCT.

3.4 Tyrosine supplement amended gut microbiome and metabolome

Since we already observed the roles of tyrosine supplement in ameliorating aGVHD, we further verified its effects on gut environment. From the fecal microbiome analysis, we found that, after the tyrosine intervention, it is hardly to summarize the regularity of phylum variances. When phylum did not differ between the groups on day 14, *Firmicutes* persisted at low level in TCD-BM + T cells + 2% tyrosine diet group ($p < 0.05$) on day 28, while *Bacteroidetes* ($p < 0.05$) and *Verrucomicrobia* showed higher levels. (Fig. 4A, B). However, through the analysis of genera level, we supposed that tyrosine treatment could not only ameliorate aGVHD symptoms, but also restore the gut microflora to aGVHD-free condition. After 2% tyrosine supplement, the abundance of *Lachnospiraceae_unclassified* increased ($p < 0.05$, on day 28) and the fold changes of *Bacteroides* ($p < 0.05$, on day 14), *Clostridium XI* ($p < 0.01$, on day 28), *Clostridium XIVa* ($p < 0.05$, on day 14) and *Enterococcus* decreased (Fig. 4C, D; Supplementary Table 4). Notably, some microbiota that was insignificant in the comparison between TCD-BM and TCD-BM + T cells groups became striking after tyrosine intervention. For instance, not only the proportion ($p < 0.05$) but also the fold change ($p < 0.01$) of *Barnesiella* remarkably increased in TCD-BM + T cells + 2% tyrosine diet group on day 14. Although *Escherichia* only possessed a small fraction in all aGVHD groups, it reduced after 2% tyrosine intervention on day 14 ($p < 0.01$), whereas the opposite trend was observed on day 28 ($p < 0.05$). Besides, there was a sudden amplification of *Lactobacillus* on day 28 (TCD-BM + T cells + vehicle and TCD-BM + T cells + 2% tyrosine diet group: 37.48% and 15.34%, $p < 0.01$), which significant downregulated in 2% tyrosine diet group ($p < 0.05$).

The PCA and PLS-DA revealed grander different metabolites between TCD-BM + T cells with vehicle diet and 2% tyrosine diet groups (Supplementary Fig.S3A, B; Supplementary Table 5). From comparison statistics, we found the number of differential metabolites on day 14 was larger than that of day 28 (Supplementary Fig.S3C). Still, most of the different metabolites belong to the superclass of lipids and lipid-like molecules following by organheterocyclic compounds and organic acids and derivatives (Supplementary Fig.S4A-C; Supplementary Table 5). The fecal metabolomic profile showed the TCD-BM + T cells with 2% tyrosine diet group maintained high levels of tyrosine on day 14 ($p < 0.01$), moreover, its related metabolites including L-Glutamic acid, oxalacetic acid, S-Adenosylhomocysteine and homogentisic acid also showed upward trends (Fig. 4E; Supplementary Table 6). Yet, these differences disappeared on day 28 in accordance with the unsatisfied clinical and microbiota changes (Fig. 4F; Supplementary Table 6). Together, these data exemplified that the replenishment of tyrosine could ameliorate aGVHD at the early stage, at the same time, change the structure of the gut microbiota and the fecal metabolic phenotype.

The larger network components displayed in the comparison of TCD-BM + T cells with vehicle diet and 2% tyrosine diet groups. On day 14, L-Aspartic acid was positively connected with *Escherichia* and *Akkermansia*. Another attractive network node, tyramine, a monoamine compound and trace amine derived from tyrosine, was positively correlated with *Bacteroides*, *Escherichia* and *Clostridium_sensu_stricto*. (Fig. 4G). At the same time, *Bacteroides* negatively connected with tyrosine and S-Adenosylhomocysteine. The oxalacetic acid also displayed a positive correlation with *Mucispirillum*, but which only occupied a small proportion. Just as the treatment of tyrosine did not meet the expectation on day 28, the correlation network showed that tyrosine faded out of the center at the later stage of aGVHD. As there was only negative correlation between tyrosine and *Firmicutes* microbiota on day 28, S-Adenosylhomocysteine became the central node of five microbiota including correlated positively with *Lactobacillus*, *Clostridium_XI* and inversely with *Blautia*. (Fig. 4H). In all, we still believed that there was a close correlation between the gut microbiota and tyrosine in aGVHD models.

4. Discussion

In this study, an integrated 16S rDNA gene sequencing and LC-MS-based metabolomics approach was utilized to explore the impact of tyrosine supplement in murine aGVHD after BMT. Our results indicated for the first time that tyrosine-replenished diet affected the gut microbiota composition in stool samples as well as the dynamic changes in gut metabolomics in aGVHD.

Using 16S rDNA gene sequencing, we revealed the robust linkage between dietary intake and gut microbial community structure in murine aGVHD model. As researchers emphasized the importance of *Firmicutes* in patients after allo-HSCT, we agreed that *Firmicutes* held the balance on gut microbiota because of its largest proportion in each group. Both TCD-BM group and TCD-BM + T cells group contained a larger percentage of *Firmicutes* but they displayed opposite prognosis, which indicated that we could not judge it properly or hardly obtain the overall trend on phylum levels because they harbored dense and diverse microbial communities with both advantage and disadvantage components. What's

more, individual difference and limitation of case number were potential contributing factors that might explain differences in responding. Hence, lower levels such as family and genus were worth discussing. Many results in our study were consistent with the previous publications. Many researchers described a relative shift towards *Enterococcus* in gastrointestinal aGVHD after allo-HSCT, and the degree of this *Enterococcus*-dominant dysbiosis was correlated with the severity of aGVHD. [27, 38] In our results, groups with severe aGVHD accumulated large proportion of *Enterococcus*. Jenq RR et al considered high proportion of *Blautia* was associated with reduced death rate in aGVHD patients, while we found it was increased in aGVHD groups.[13] It may be due to the complexity in clinical practice situation, such as the use of antibiotics and conventional nutritional supplement in the post-BMT period. What's more, the eating habits with salient features of localism and the different dietary structures between human and animal models also play a decisive role. [39] Recently, new hypotheses were proposed, such that increased abundance of *Clostridiales* was associated with reduced lethal GVHD and improved overall survival following allo-HSCT. [40] Likewise, we brought out some original findings in our research. The groups with higher proportion of *Lachnospiraceae_unclassified*, one of the major microbiota in our data, showed less aGVHD. Besides, *Porphyromonadaceae_unclassified* processed large ratio in groups after tyrosine intervention on day 14. Although deep sequencing technology has made it possible to characterize the composition of mixed bacterial samples and distinguish thousands of bacteria, there are still many limitations in identification and functional verification of all the species due to the complexity and multiformity.[41] Consequently, further studies are warranted to explore the unknown but exceptional species mainly by separation, purification and study of their genome.

After allo-HSCT, extra nutrients are needed not only for repairing damaged tissue from chemotherapy or radiation in the initial period of transplantation, but also for bone marrow reconstitution, as well as to face the challenge on the body such as infection, wound healing and hypermetabolism after transplantation.[42] Meanwhile, the measures to avoid loss of beneficial microbiota mainly through antibiotic selection or probiotics administration. Therefore, nutritional supplementation appeared to be a more suitable treatment. Some researchers illustrated that the presence of the gut microbiota resulted in increased levels of tyrosine, glutamate, alanine and aspartate in the small intestine, and of creatine, glutamine and aspartate in the colon compared to germ-free counterparts.[43] Previous works usually focused on studying the impacts of microbiota on the metabolites, such like indole metabolites produced by the intestinal microbiota could limit gut epithelial damage and reduce subsequent GVHD pathology via type I interferons to. [44] In our study, we set a certain metabolite tyrosine as an affecting factor for the first time. We found that a comparatively high content of tyrosine could attenuate aGVHD clinical manifestations and improve the overall metabolites belonging to tyrosine biosynthesis pathway. So far, although we could not differentiate the source of tyrosine form self-synthesis or external uptake, it was certain that a comparatively high-level tyrosine played an active role in preventing damages during aGVHD. Even though the content of tyrosine is our unique intervention, the alterations of certain metabolites could influence the whole metabolic network through interactions with other elements leading to the change of the metabolic homeostasis of the host organism.[45] Unexpectedly, we did not observe the sustained effectiveness of tyrosine supplemented diet, possibly because that the dose of the

tyrosine was incapable to remedy at a later stage aGVHD or the compensatory mechanism of the organism under the stimulation of pathological stress. Therefore, further studies are warranted to verify whether there is a dose-dependent relationship between tyrosine and curative property or whether combination therapy of complex amino acids are needed to control aGVHD progression.

Correlation network analysis is an emerging field that captures the relations between different parts of a complex biological system, such as molecules, processes, organs or even microbes and metabolites.[46] We charted the relations between changes in the microbiota members and metabolites, and identified specific groups of correlations relevant to aGVHD development. Specifically, we observed many changes in the abundance of *Firmicutes* and *Bacteroidetes* correlated to tyrosine and its biosynthesis in the host. Zhang et al. suggested that the levels of tyrosine and SCFAs is negatively associated with *Enterococcus* [47] that was in accordance with our result. Moreover, we also found tyrosine related metabolites such as L-Glutamic acid and L-Aspartic acid were inversely correlated with *Clostridium XIVa* and *Enterococcus*. In addition, *Bacteroides* displayed opposite correlations with different selected metabolites during aGVHD. That could be explained that tyrosine metabolism in the gut microbiota and the underlying metabolic interactions with the host may be important in the regulation of immune homeostasis. Just as researchers demonstrated an interleukin-17-sensitive gut microbiota controlled susceptibility to aGVHD mediating a protective effect to intestinal damage, [48] accordingly, we intended to find a tyrosine-sensitive gut microbiota which might act as an important regulator of gut microenvironment and influence the severity and penetrance of aGVHD. Collectively, these results provide us with a broader view of the effects of tyrosine on gut microbiome and metabolome, and further studies are essential to uncover the underlying mechanisms and the potential biological significances.

5. Conclusion

Overall, our data indicated that the additional supply of tyrosine in diet could shifted the gut microbiota structure leading to fecal metabolome variations, which could improve overall survival and ameliorated clinical symptoms at the early stage of aGVHD after BMT. These findings exhibited a strong correlation between gut microbiota and metabolomes, in turns, affected both pathophysiology and clinical manifestations of disease.

Declarations

Ethics approval and consent to participate: All experiments were approved by the Institutional Animal Care and used Committee of Zhejiang University.

Consent for publication: Not applicable.

Availability of data and material: The datasets generated during the current study have been deposited in Sequence Read Archive (SRA). The SRA accession is PRJNA637751. The Submission ID is SUB7556797. The links is https://submit.ncbi.nlm.nih.gov/subs/bioproject/SUB7556797/overview.

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Authors' contributions: Xiaoqing Li and Yu Lin designed the study; Xiaoqing Li and Xiaoxiao Xu and Yanmin Zhao conducted most of the experiments; Xiaoqing Li and Xue Li analyzed the data and created the figures of heatmap and correlation network; Yamin Tan, Lin Xu, Yang Gao and Yixue Li performed some of the experiments and provided the research guidance. Xiaoqing Li and Yu Lin pre-pared the first draft; Pengxu Qian and He Huang provided subsequent revisions and revised the article. All authors commented on the manuscript and approved the final version.

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Figures

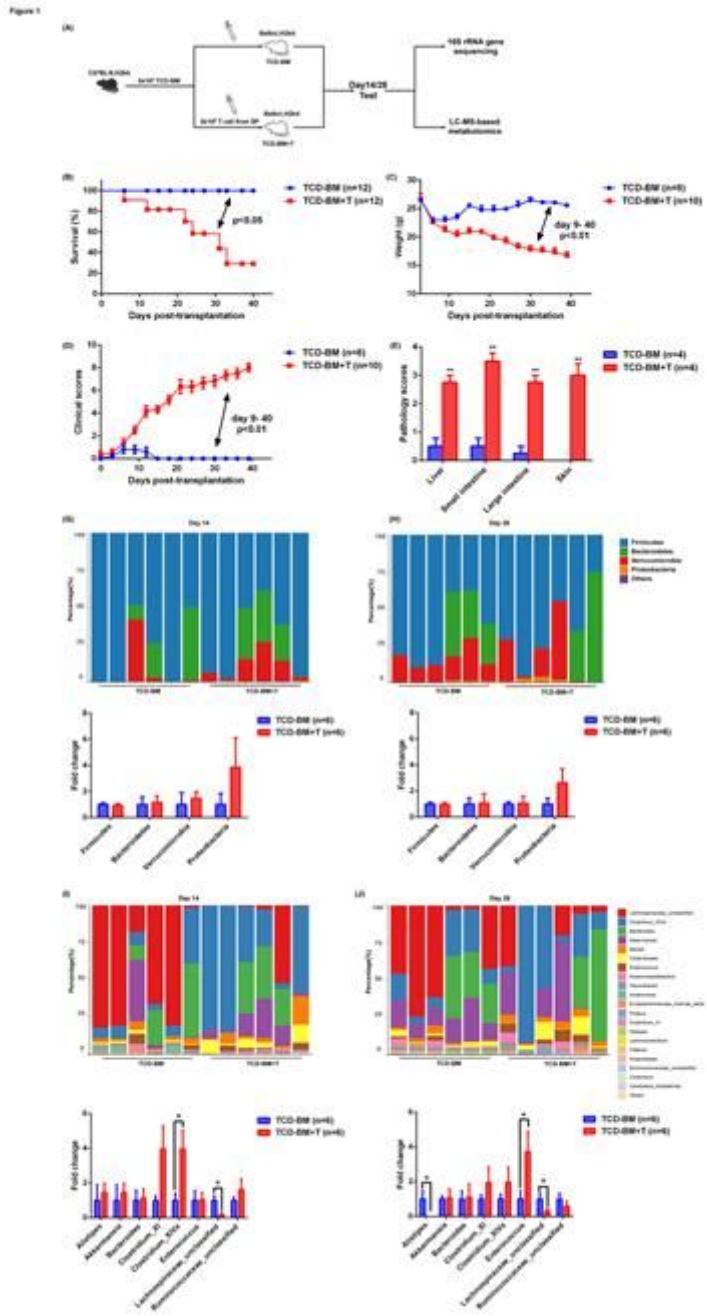

Figure 1

Relative gut microbiota abundance at the phylum and genus levels. (A) BALB/c host mice transplanted with C57BL/6 TCD-BM (n=12) or TCD-BM+T cells (n=12) to established aGVHD murine model. (B-E) The significant changes of (B) survival time (p□0.05), (C) weight changes(p□0.01), (D) clinical scores (p□0.01)

and (E) pathology scores on day14 of each group were displayed. (G-H) 16S rDNA gene sequencing of fecal microbiota from TCD-BM (n=6) and TCD-BM+T cells groups (n=6), the relative gut microbiota abundance with the histogram of the fold change (TCD-BM+T/TCD-BM) at the phylum level on day 14 (G) and 28 (H). (I-J) The top 20 relative abundance of genus were displayed with the histogram of the fold change (TCD-BM+T/TCD-BM) on day 14 (I) and 28 (J). All data reflect mean ± SEM from 3-4 independent experiments.

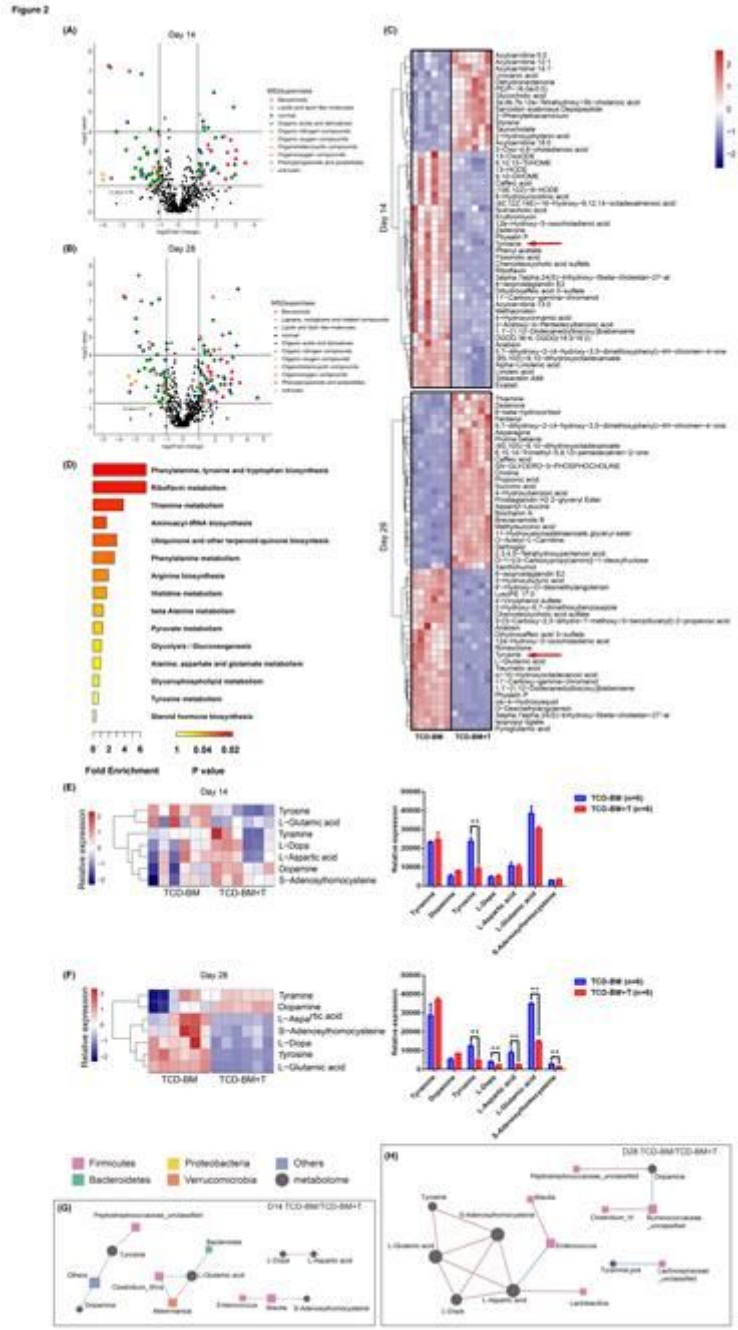

Figure 2

Metabolomics changes between TCD-BM and TCD-BM+T cells groups. (A, B) The detected metabolites were compared with a paired Student test followed by a Bonferroni correction. The volcano plot represents the variation of metabolites amount between TCD-BM (n=6) and TCD-BM+T cells (n=6) groups

according to the −log (q value) and log2 (fold change) on day 14 (A) and 28 (B). (C) Heatmap representation of the most significant 50 metabolites after Student's test and hierarchical clustering of samples. (D) KEGG-annotated metabolic pathways between TCD-BM and TCD-BM+T cells groups. (E) On day 14, the heatmap summarized the altered fecal metabolites related to tyrosine between TCD-BM and TCD-BM+T cells groups and only tyrosine decreased in TCD-BM+T cells group ($p<0.01$). (F) On day 28, tyrosine, L-dopa, L-aspartic acid, L-glutamic acid and S-adenosylhomocysteine showed a significant decrease in TCD-BM+T cells group ($p<0.01$). The corresponding reten-tion times, m/z and VIP values of different metabolites are shown in Supplementary Table 3. (G, H) The correlation network between altered metabolites (circular) and differentially abundant microbiota (square). A connection indicates that the microbe has a correlation with the metabolite, a red line indicates a positive correlation, a blue line indicates a negative correlation, and the volume size indicates the strength of the correlation. The networks between TCD-BM and TCD-BM+T cells groups were shown on day 14 (G) and 28 (H).

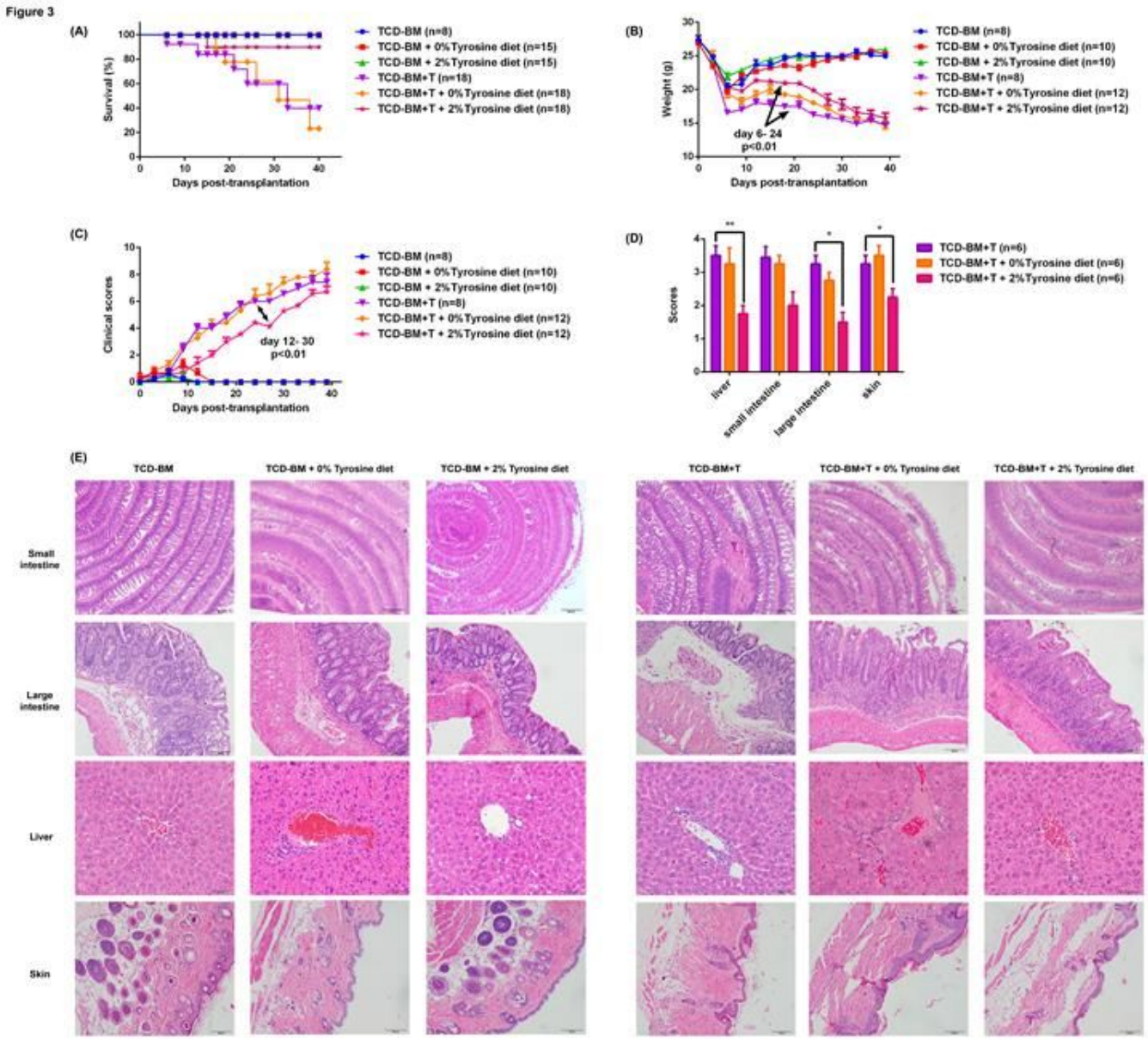

Figure 3

Tyrosine supplement ameliorated aGVHD. 0% or 2% tyrosine diet were served to TCD-BM (n=18) and TCD-BM+T cells (n=18) groups from day -7 of irradiation until the end of the experiments. Comparing with TCD-BM+T cells + vehicle, 2% tyrosine diet could (A) prolonged survival time, (B) lose less weight (day 6 to 24, p□0.01; day 27 to 33, p<0.05), (C) lower clinical scores (day 12 to 30, p□0.01; day 9 and 33, p<0.05) at early stage of aGVHD. The administration of 2% tyrosine could decrease the (E) pathological scores of large in-testine and skin on day 14. (F) 2% tyrosine diet protected intestines and skins from allogenic T-cell attacks as the HE images showed. All data reflect mean ± SEM from 3-4 independent experiments.

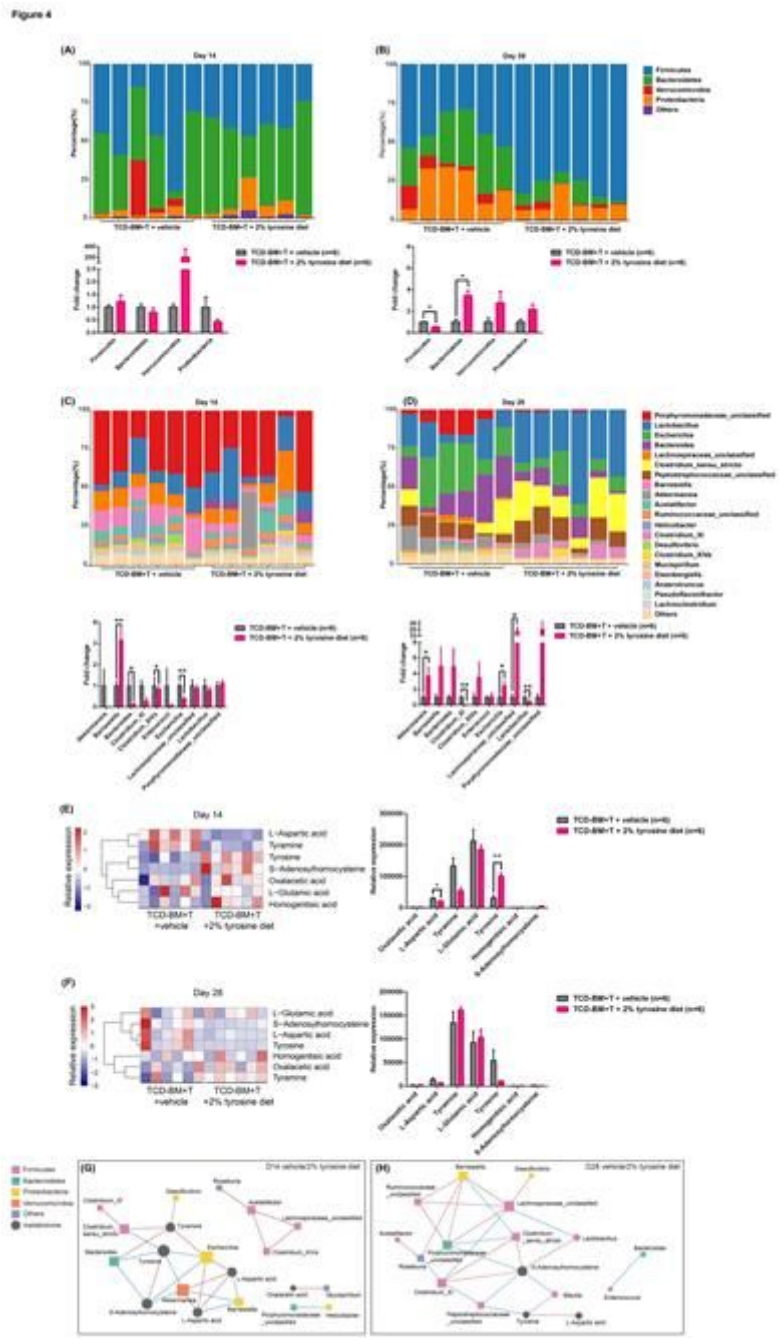

Figure 4

Tyrosine supplement alter microbiome and metabolome. (A-D) The relative gut microbiota abundance between TCD-BM+T cells + vehicle (n=6) and 2% tyrosine diet (n=6) groups and the histogram of the fold

change (TCD-BM+T cells + 2% tyrosine diet groups/ TCD-BM+T cells + vehicle) at the phylum level on day 14 (A) and day 28 (B). The top 20 relative abundance of genus were displayed with the histogram of the fold change (TCD-BM+T cells + 2% tyro-sine diet groups/ TCD-BM+T cells + vehicle) on day 14 (C) and day 28 (D). (E-F) The heatmap summarized the different fecal metabolites related to tyrosine between TCD-BM+T cells + vehicle (n=6) and 2% tyrosine diet (n=6) groups. Tyrosine was increased ($p<0.01$) in 2% tyrosine-diet group on day 14 (E), but there was no signifi-cant difference in tyrosine and other metabolites on day 28 (F). The corresponding retention times, m/z and VIP values of different metabolites are shown in supplemen-tary Table 6. (G-H) The same correlation analysis was used to build a network ac-cording to the figure 2 G-H, where nodes represent microbiota or metabolites between TCD-BM+T cells + vehicle and TCD-BM+T cells + 2% tyrosine-diet groups on day 14 (G) and day 28 (H).

Supplementary Files

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