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Interactions between the gut microbiota, short-chain fatty acids and the immune system in pediatric patients undergoing hematopoietic stem cell transplantation

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Esame finale anno 2015

To my family with extreme gratitude and love

"Philosophy is like being in a dark room and looking for a black cat.
Metaphysics is like being in a dark room and looking for a black cat that isn't there.
Theology is like being in a dark room and looking for a black cat that isn't there, and shouting "I found it!"
Science is like being in a dark room and looking for a switch. The light will reveal a cat... if there is one."

[The Black cat analogy]

Remember to remember.
Remember to believe.
Remember to live.

ABSTRACT

The gut microbiota (GM) is essential for human health and contributes to several diseases; indeed it can be considered an extension of the self and, together with the genetic makeup, determines the physiology of an organism.

In this thesis has been studied the peripheral immune system reconstitution in pediatric patients undergoing allogeneic hematopoietic stem cell transplantation (aHSCT) in the early phase; in parallel, have been also explored the gut microbiota variations as one of the of primary factors in governing the fate of the immunological recovery, predisposing or protecting from complications such as the onset of acute graft-versus-host disease (GvHD).

Has been demonstrated, to our knowledge for the first time, that aHSCT in pediatric patients is associated to a profound modification of the GM ecosystem with a disruption of its mutualistic asset. aGvHD and non-aGvHD subjects showed differences in the process of GM recovery, in members abundance of the phylum Bacteroidetes, and in propionate fecal concentration; the latter are higher in the pre-HSCT composition of non-GvHD subjects than GvHD ones.

Short-chain fatty acids (SCFAs), such as acetate, butyrate and propionate, are end-products of microbial fermentation of macronutrients and distribute systemically from the gut to blood. For this reason, has been studied their effect *in vitro* on human DCs, the key regulators of our immune system and

the main player of aGvHD onset. Has been observed that propionate and, particularly, butyrate show a strong and direct immunomodulatory activity on DCs reducing inflammatory markers such as chemokines and interleukins.

This study, with the needed caution, suggests that the pre-existing GM structure can be protective against aGvHD onset, exerting its protective role through SCFAs. They, indeed, may regulate cell traffic within secondary lymphoid tissues, influence T cell development during antigen recognition, and, thus, directly shape the immune system.

INDEX

1.Gut microbiota

1.1. General overview

Human beings have been recently reconsidered as super-organisms in co-evolution with an immense microbial community living in the gastrointestinal tract (GIT), the human intestinal microbiota (Gill, Pop, Deboy, & al., 2006) (Ley, Hamady, Lozupone, & al, 2008). With a concentration of 10^{12} CFU/g of intestinal content, the human intestinal microbiota probably represent one of the most dense, biodiverse and rapidly evolving bacterial ecosystem on Earth (Ley, Hamady, Lozupone, & al, 2008).

The gut microbiota can be considered an extension of the self and, together with the genetic makeup, determines the physiology of an organism. For example, two supposedly genetically identical organisms, such as same-sex inbred mice, can have widely different metabolic and inflammatory responses depending on the makeup of their microbiota. The intestinal microbiota is derived at least in part from the mother during the birthing process and is modified thereafter by factors such as diet, antibiotic use, host genetics and other environmental factors. Whereas microbes in the gut were once considered only harmful or pathogenic, it is now clear that commensal bacteria accomplish many beneficial functions, such as vitamin synthesis, the digestion of dietary fiber and the regulation of inflammatory responses. Microbes and vertebrates have evolved together over the millennia, so normal functioning of the digestive and immune systems depends on the

presence of nonpathogenic 'beneficial' bacteria (symbionts). As just mentioned, its collective genome provides functional features that humans have not evolved by their own, and several of our metabolic, physiological and immunological features depend on the mutualistic association with our intestinal microbial community (Neish, 2009) (Lee & Marzamian, 2010). In fact, the intestinal microbiota enhances our digestive efficiency by degrading otherwise indigestible polysaccharides and also represents a fundamental barrier against GIT colonization by enteropathogens and their invasion with a higly dynamic modality.

Further, the crosstalk between the immune system (IS) and the GIT microbial community is essential for the development, education and functionality of our immune system (Garrett, Gordon, & Glimcher, 2010) (Hooper & Macpherson, 2010) (Figure 1).

The interaction between intestinal microbiota and human host is dynamic and pivlotal for the ultrastructural development of the gastrointestinal tract (Round & Marzmanian, 2009). This dynamism consists in a predisposition to adaptation, which guarantees rapid variations of microbial community and metabolic functions in response to diet and habits, and, it represents an essential component for the education of the immune system to homeostasis (Candela, Biagi, Maccaferri, & al., 2012).

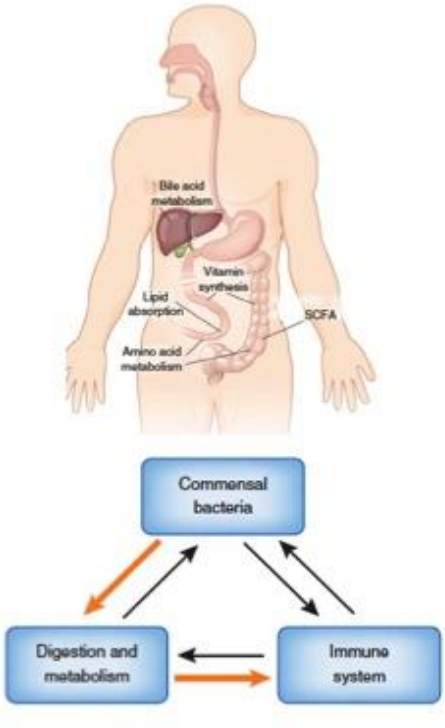

Figure 1: Crosstalk scheme illustrating the interactions between GM and immune system (Modified by Brestoff & Artis, 2013).

1.1.1. GM composition

Gut microbiota is composed by an amongous taxonomic diversity at species level and more than 1000 different bacterial species have already been identified in the human GIT (Garrett, Gordon, & Glimcher, 2010) (Turnbaugh, Ley, Hamady, & al, 2007) even if the intestinal microbiota reveals a relatively low biodiversity, at the higher phylogenetic level, with only 6 of 90-100 bacterial phyla present (Peterson, Frank, Pace, & al, 2008). The total diversity of a healthy human gut ecosystem is generally reported around 1.000-1.200 species-level phylogenetic types, named "phylotypes", defined as group of 16S sequences with a certain level (97-99%) of similarity

(Eckburg, Bik, Bernstein, & al., 2005) (Rajilic-Stojanovic, Smidt, & de Vos, 2007) (Xu, Mahowald, Ley, & al, 2007). Only a small percentage of the gut inhabitants correspond to fully characterized bacterial isolates, whereas 75-82% is estimated to remain uncultured (Eckburg, Bik, Bernstein, & al., 2005) (Rajilic-Stojanovic, Smidt, & de Vos, 2007) (Flint, Duncan, Scott, & al., 2007).

The vast majority of the bacterial inhabitants belongs to the dominant (50-80%) Firmicutes and Bacteroidetes, that arrange more than 90% of the gut bacterial community. All the other phyla such as Actinobacteria (3-15%) and Proteobacteria (1-20%) are generally well-conserved at a lower relative abundance, whereas Verrucomicobia (0.1%) and Fusobacteria are present only in some individuals (Eckburg, Bik, Bernstein, & al., 2005) (Frank, St Amand, Feldman, & al., 2007) (Costello, Lauber, Hamady, & al., 2009) (Muegge, Kuczynki, Knights, & al, 2011). In particular, the procaryotic phyla Fusobacteria, Cyanobacteria, Spyrochaetes, and Lentishaerae, as well as several eukaryotic fungal species (Candida, Aspergillus and Penicillium), were also reported as gut inhabitants in very small percentages (Rajilic-Stojanovic, Smidt, & de Vos, 2007). Furthermore, at lower taxonomic levels, each healthy human being has a peculiar subset of hundreds of species and no phylotype of the individual microbiota is present more than 0.5% and the 70% of the phylotypes are subject-specific (Turnbaugh, Hamady, & Yatsunenko, 2009).

1.1.2. Plasticity of GM

The human gut microbiota shows an astonishing individual variability, and it is considered as unique as a fingerprint in terms of species and strains composition (Eckburg, Bik, Bernstein, & al., 2005) (Lay, Rigottier-Gois, Holmstrøm, & al., 2005). Geographic origin, age, diet, and lifestyle influence the composition of the gut microbiota but also the impact of genotype may also be significant in shaping the gut bacterial ecosystem (Lay, Rigottier-Gois, Holmstrøm, & al., 2005) (Khachatryan, Ktsoyan, Manukyan, & al., 2008) (Zoetendal, Akkermans, Akkermans-van Vlietal, & al, 2001). Despite the remarkable host specificity in the gut community, a high degree of conservation in functions and metabolites has been reported (Mahowald, Rey, Seedorf, & al., 2009). This suggests that the gut microbiota may be characterized by a marked "functional redudancy" to ensure that the key functions of the microbial community remain unaffected by the individual variability in terms of species composition (Gill, Pop, Deboy, & al., 2006). The existence of a "human core gut microbiome", defined as those genes which are common to the gut microbiomes of all or the majority of humans, has been hypothesized to be responsible for the functional stability of the gut microbiota (Turnbaugh P. a., 2009). In the other hand, a "human core gut microbiota", defined as a number of species which are commons to all humans, could hardly be defined, since different combinations of species could fulfil the same functional roles (Turnbaugh & Gordon, 2009) (Tschop, Hugenholtz, & Karp, 2009).

Aside the core, the set of genes which are present in smaller subsets of human constitutes the "human variable microbiome". This large wide change from the core is the result of a combination of host-specific factors, such as genotype, host pathologies, lifestyle, diet, environment, genotype, and the presence of transient populations of microorganisms that cannot persistently colonize the human gut. in the end, variable and core components of the human microbiome influence different aspects of the human health, including immunity, health status and nutrient responsiveness (Turnbaugh, Ley, Hamady, & al, 2007).

The ecological disorder of the bacterial community is called "dysbiosis", and affects the structure of the microbiota at the level of order/phylum.

Several papers have shown how changes in the relative proportion of Firmicutes and Bacteroidetes are detected in metabolic disorders such as obesity (Ley, Turnbaugh, Klein, & al., 2006) (Turnbaugh, Hamady, & Yatsunenko, A core gut microbiome in obese and lean twins, 2009) (Turnbaugh & Gordon, The core gut microbiome, energy balance and obesity, 2009), type I diabetes (Wen, Ley, Volchkov, & al, 2008) and inflammatory disease (IBD) (Frank, St Amand, Feldman, & al., 2007) (Sartor, 2008). Bacterial overgrowth and imbalanced microbiota are also linked to the irritable bowel syndrome (IBS) (Othman, Aguero, & Lin, 2008).

As just explained, the human microbiota is involved in various and different aspects of human physiology and disease and represents a new frontier of human biology and medicine.

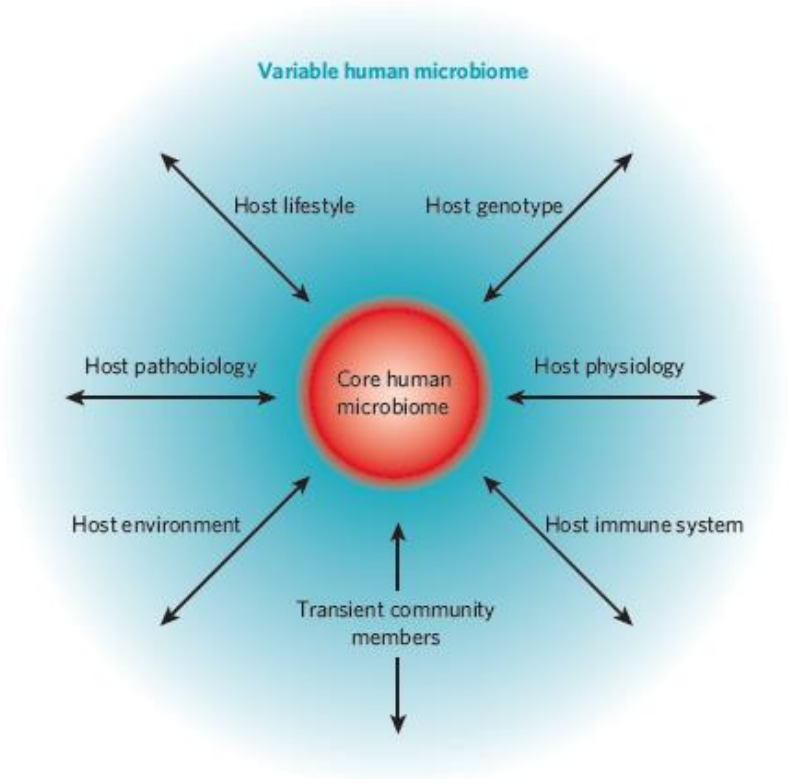

Figure 2: The concept of a core human microbiome. The core human microbiome (red) is the set of genes present in a given habitat in all or the vast majority of humans. The variable human microbiome (blue) is the set of genes present in a given habitat in a smaller subset of humans (Turnbaugh, Ley, Hamady, & al, 2007).

1.1.3. Dietary influences on GM composition and metabolites production

Mechanisms by which dietary, bacterial, and primary metabolites interact with the immune system have been largely discussed and topic of many papers along this last decade. Many of the food traditionally considered healthy, such as fibers, fish, vegetal olis, and other elements of the Mediterranean diet, have been connected to a better lifestyle and healthy

conditions because positively linked to molecular pathways that promote gut health and immune tolerance.

The nutritional change that has occurred in the Western world over the past few decades and the "Westernization" of many countries coincide with the increase in certain autoimmune diseases, asthma and allergy (Eder, Ege, von Mutius, & al., 2006). Indeed, the change in dietary habits and the correlation with disease are particularly evident in epidemiological studies correlating fibers and fat intake. Notably, rural Africans consume more fiber than Western individuals and rarely suffer from allergies, asthma, or colon cancer (Schnorr, Candela, Rampelli, & al, 2014). It is also well known that high intake of dietary fiber correlates with a lower risk of death from several conditions such as cardiovascular disease, infections and cancers (Park, Subar, Hollenbeck, & al, 2011).

Western individuals are used to eat an obesogenic diet characterized by energy-dense processed food and low comsumption of nutrient-rich foods, such as vegetables and fruits. Clearly, a bad food intake influences and strongly modifies our microbiota composition depriving ourselves from protective factors. A dietary basis for inflammatory diseases is most likely explained by interactions between dietary or bacterial metabolites and immune cells, or pathways for gut homeostasis.

The current findings leading the scientific community to focus on bacterial metabolites that actively play protective roles such as short-chain fatty acids (SCFAs), ω-3 fatty acids, and those derived from tryptophan catabolism.

Interestingly, only few bacterial species and their metabolites are already known for showing strong anti-inflammatory effects such as *Faecalibacterium prausnitzii* that, together with its metabolite (butyrate) inhibits NF-κB (Sokol, Pigneur, Watterlot, & al, 2008).

The major metabolites produced by bacteria in the gut are SCFAs. As more dietary fibers are ingested their production increases. An exhaustive example of the correlation between diet and SCFAs production has been provided by a study focused on the differences between the fecal microbiota of European children and rural African children. African children showed higher bacterial richness and a significant enrichment of bacteria from the genera Prevotella and Xylanibacter (that are efficient at digesting fibers and producting SCFAs), whereas these bacteria were absent from the European children (De Filippo, Cavalieri, Di Paola, & al., 2010). Notably, the microbiota composition of rural Africans is also different from that of African Americans because of an higher proportion of Prevotella and lower proportion of Bacteroides, associated with a higher production of SCFAs, such as butyrate (Ou, Carbonero, Zoetendal, & al., 2013).

1.2. Short-chain fatty acids

Humans lack the enzymes to degrade the bulk of dietary fibers therefore these nondigestible carbohydrates pass the upper gastrointestinal tract unaffected and are fermented in the cecum and the large intestine by the anaerobic cecal and colonic microbiota. Fermentation results in multiple

groups of metabolites of which SCFAs are the major group (Roy, Kien, Bouthillier, & al., 2006), required to balance redox equivalent production in the anaerobic enviroment of the gut (Van Hoek, Merks, & Merks, 2012). SCFAs are saturated aliphatic organic acids that consists of one to six carbons in lenght and are produced in the colon by bacterial fermentation of plant-derived nondigestible polysaccharides, such as cellulose (Cummings & Macfarlane, 1991) (Wong, de Souza, Kendall, & al, 2006); the most abundant (>95%) are acetate (C2), proprionate (C3) and butyrate (C4) and they are present in an approximate molar ratio of 60:20:20 in the colon and in the stool (Cummings, Pomare, Branch, & al., 1987).

The molecular mechanisms by which metabolites such as SCFAs, long chain fatty acids and tryptophan metabolites play a major role in the prevention of inflammatory disease and how they show a role in the interplay between diet, gut microbiota and regulation of host energy metabolism is target of a new active field of research.

The future phase of research might explain some of the dietary and microbiota-related associations with human disease and involves the identification of specific bacterial metabolites that could be associated with or could protect against human diseases.

It is important to consider when dietary metabolites influence the immune response and where they intersect with the immune system at different levels (gastrointestinal tract (GI), blood and fetal enviroment). Originally, the gut was considered the primary site where dietary metabolites exhert their effects, through either gut epithelial integrity o mucosal immunity. Indeed,

the distal colon is where fiber is fermented by commensal bacteria to produce large quantities of acetate, proprionate and butyrate (≈40, 20 and 20 mM, respectively) (Tan, McKenzie, Potamitis, & al., 2014). Several papers have shown that metabolites distribute systemically. Indeed, the exacerbated inflammatory reactions observed in germ-free mouse models of disease (Herbst, Sichelstiel, Schar, & al., 2011) (Maslowski, Viera, Ng, & al., 2009) are likely related in part to the absence of SCFAs in the gut, blood or tissues. In one recent study, the SCFA propionate was shown to affect DCs and macrophage biology in the bone marrow and affect Th 2 cell responses in the airways (Trompette, Gollwitzer, Yadava, & al, 2014). Then, SCFAs can have a profound effect on systemic macrophage and DCs biology illustrates the strong connection between dietary fiber intake and many different types of immune responses under the control of DCs or macrophages.

Various G protein-coupled receptors (Gpr) such as Gpr41 (FFAR3 gene), Gpr43 (FFAR2 gene), and Gpr109a (HCAR2/NCR1 gene) mediate SCFA activities, but the molecular and cellular events responsible for SCFAs-mediated beneficial effects in the intestine and in other districts are still unclear.

Althoughall those three receptors have been described and even others have not been studied yet, Gpr41 and Gpr43 are the most well-mentioned SCFA receptors (Brown, Goldworthy, Barnes, & al., 2003) (Le Poul, Loison, Struyf, & al., 2003) (Hirasawa, Hara, Katsuma, & al., 2008) (Feingold, Moser,

Shigenaga, & al.) (Maslowski, Viera, Ng, & al., 2009) (Samuel, Shaito, Motoike, & al, 2008).

Gpr41 is expressed in a variety of tissues and cell types including the colon, kidneys, sympathetic nervous system and blood vessels assessing how broad is the potential effect of bacterial-metabolites produced in the gut (Kimura, Inoue, Maeda, & al., 2011) (Tazoe, Otomo, Karaki, & al, 2009) (Xiong, Miyamoto, Shibata, & al, 2004).

Gpr43 is mainly expressed in vasculature and immune cells including lymphocytes, neutrophils, monocytes and peripheral blood mononuclear cells (PBMC) (Kimura, Inoue, Maeda, & al., 2011) (Tazoe, Otomo, Karaki, & al, 2009) (Xiong, Miyamoto, Shibata, & al, 2004). Gpr43 has been shown to regulate inflammatory responses of the host in response to SCFAs (Le Poul, Loison, Struyf, & al., 2003) (Maslowski, Viera, Ng, & al., 2009).

Gpr109a was initialli identified as a receptor for niacin (Wise, Foord, Fraser, & al, 2003), and subsequently was also found to respond to beta-D-hydroxybutyrate as well as butyrate (Taggart, Kero, Gan, & al, 2005) and its activation can apparently suppress carcinogenesis (Elangovan, Pathania, Ramachandran, & al., 2014) (Singh, Gurav, Sivaprakasam, & al, 2014).

A recent paper provides key evidence that bacterial-derived butyrate and dietary fibers attenuate intestinal inflammation through Gpr109a-mediated Treg cell differentiation. This study shows how diet, microbiota, and immune cells create a complex communication network essetial for the mainteinance of intestinal homeostasis. They observed that FoxP3 Treg cell number and frequency in the lamina propria of Gpr109a-/- mice were significantly lower

than in WT mice, matching an impaired immunosuppressive IL-10 secretion and enhanced production of pro-inflammatory IL-17 (Singh, Gurav, Sivaprakasam, & al, 2014). This inflammatory profile may be consequence of defective tolerogenic instruction provided by mononuclear cells, so they reported a failure in IL-10 release, class 1A aldehyde dehydrogenase (Aldh1a) production, and Treg cell generation by butyrate-treated splenic DCs and macrophages from Gpr109a-/- mice. Moreover, niacin, which is also a Gpr109a ligand and bacterial-derived product, reproduces butyrate effect on DCs, macrophages, and Treg cell activities. This highlight the central role of Gpr109a in capturing, processing signaling generated by microbial-derived metabolites and its essential role in mucosal immunoregulatory fucntions afforded by microbial metabolite butyrate. Comparing inflammation between GPR43-deficient and Gpr109a**-/-**mice would help understand the potentially divergent role of these receptors in mediating intestinal homeostasis. Furthermore, the cell type (epithelial cells, immune cells) responding to SCFA exposure may also dictate the extent of the protective response. This is clearly demonstrated in immune-cell-derived GPR43 signaling, which mediates an anti-inflammatory response (Maslowski, Viera, Ng, & al., 2009). Noteworthy, a recent report showed that butyrate-mediated expansion of Treg cells is Gpr109a independent, suggesting a complex impact of this SCFA on immune cell behaviors (Arpaia, Campbell, Fan, & al., 2013). A deep genetic dissetion of all the cell compartment for all the receptors involved would be necessary to fully capture the essence of the cellular network responding to microbial metabolites in mice and humans.

1.3. GM and the immune system

The intestinal epithelium at the interface between lymphoid tissue and microbiota plays a crucial role in the mucosa immune response. The fashinating IS's ability to co-evolve with the microbiota during the perinatal life allows the host and the microbiota to coexit in a dangerous relationship gaining mutual benefit which consists in orchestate, in a highly coordinated way, specific immune responses toward millions of foreign antigens and in discriminating false alarms triggered by benign antigens (Brestoff & Artis, 2013).

Recent studies have identified a critical role for commensal bacteria and their products in regulating the development, homeostasis and function of innate and adaptive immune cells (Chang, Offermanns, & al., 2014) (Smith, Howitt, Panikov, & al, 2013) (Trompette, Gollwitzer, Yadava, & al, 2014) (Atarashi, Tanoue, Oshima, & al, 2013).

Several recent reviews have described how commensal bacteria are recognized by the innate and how individual species or consortia of commensal bacterial species can influence distint subsets of the innate and adaptive immune rsponse.

However, an emerging area that has received little attention is how metabolites and nutrients derived from commensal bacteria regulate the host immune system. Commensal bacteria are key regulators of digestion, extraction, synthesis and absorption of many nutrients and metabolites including bile acids, lipids, amino acids, vitamins and short-chain fatty acids.

These metabolites derived from commensal bacteria are directly linked to diet and digestion and are considered to be diet-dependent microbial products (Tremaroli & Bachked, 2012).

Nutrients and metabolites derived from commensal bacteria may regulate immune cells via indirect and direct mechanisms. Commensal bacteria-mediated alterations in the availability or use of energy substrates may indirectly influence the development and function of immune cells (Backhed, Ding, Wang, & al., 2004) (Musso, Gambino, & Cassader, 2011).

The study of *germ-free mice* led to the discovery that the gut microbiota is required for the normal generation and/or maturation of GALTs. GALTs are immune structures in which antigen can be taken up and presented by antigen-presenting cells, and therefore these structures have important roles in lymphocyte functions that lead to inflammation or tolerance. GALTs include the Peyer's patches, crypt patches and isolated lymphoid follicles (ILFs) (Bouskra, Brezillon, Berard, & al., 2008). In the fetus, lymphoid tissue inducer (LTi) cells promote the development of Peyer's patches in the absence of resident bacteria, although Peyer's patches in germ-free mice are smaller in size than those in specific-pathogen-free mice (Moreau & Corthier, 1988).

Unlike Peyer's patches, the maturation of ILFs and crypt patches requires stimulation by the gut microbiota (Hamada, Hiroi, Nishiyama, & al., 2002) (Pabst, Herbrand, Friedrichsen, & al, 2006). Specifically, incomplete

maturation of ileal and colonic ILFs is observed in mice that are deficient in various pattern recognition receptors (PRRs).

Recently it has been recognized that the gut microbiota can influence immune functions beyond the gut in mice and humans. Specific bacterial species have previously been associated with the presence of certain T-cell subsets within the intestines. For instance, colonization of mice with a single commensal microbe, segmented filamentous bacteria (SFB), is sufficient for induction of the Th17 subset of CD4+ T cells (Ivanov, Atarashi, Manel, & al., 2009). These cells produce the cytokines IL-22 and IL-17, promoting the production of antimicrobial peptides and tissue repair, and enhance resistance to *Citrobacter* infections (Ivanov, Atarashi, Manel, & al., 2009) (Korn, Bettelli, Oukka, & al., 2009). Generation of Th17 cells can also be promoted via sensing of bacteria-derived ATP by dendritic cells (DCs), promoting the expression of Th17-inducing cytokines IL-6 and IL-23 (Atarashi, Nishimura, Shima, & al., 2008). Colonization of mice with *Clostridia* species from clusters IV, XIV, and XVIII isolated from human feces enhances Treg cell abundance and also increases the production of potent anti-inflammatory molecules such as the cytokine IL-10 (Atarashi, Tanoue, Oshima, & al, 2013) (Atarashi, Tanoue, Shima, & al., 2011). Further, addiction of cecal extracts from these mice to human and mouse IEC cell lines led to their production of TGF-β1, a major cytokine involved in Treg differentiation in the intestine. It also has been observed that cecal extracts contained high concentration of SCFAs and addition of a combination of purified acetate, propionate and butyrate to IECs also induced TGF-β1 in

vitro. All these evidences suggest that the production of SCFAs is likely responsible for the increase in the Treg cell numbers and they represent the link that previously was unkown by the scientific community.

Later on several papers have been published about the receptors and sensors that let possible the uptake of SCFAs from the environment and in how many districts far from the gut are sensitive to them.

Mice deficient in a single G protein–coupled receptor, Gpr3, have a profoundly altered inflammatory response. Gpr43-deficient mice (Ffar2–/–; called 'Gpr43–/–' here) have exacerbated and poorly resolving inflammation in the KxB/N serum-induced arthritis model and a model of allergic airway inflammation induced by ovalbumin plus aluminum hydroxide, as well as in colitis models, and Gpr43–/– neutrophils have an intrinsic hyper-reactive phenotype (Maslowski, Viera, Ng, & al., 2009). Gpr43 is expressed mainly on cells of the innate immune response and inflammatory cells, such as neutrophils, eosinophils and activated macrophages. SCFA can also bind other GPCRs, including Gpr41 (but with different affinity and SCFA preference) (Le Poul, Loison, Struyf, & al., 2003), and SCFA, particularly butyrate, inhibit histone deacetylases and inhibit activation of the transcription factor NF-**κ**B. Germ-free mice devoid of microbiota have very low concentrations of SCFAs and also show exacerbated or poorly resolving responses in many models (Maslowski, Viera, Ng, & al., 2009), similar to the response of Gpr43–/–. Several are the pathways by which the microbiota regulates inflammatory responses in the gut and more broadly. Bacteroidetes, for istance, use fiber for glycan synthesis and *Bacteroides*

fragilis one of our commensal bacteria belonged to this phylum, produce a particular glycan, polysaccharide A, which has strong anti-inflammatory effects protecting mice from inflammatory bowel disease through the local increase production of IL-10 by inducing T reg cells (Round & Mazmanian, 2010). An another example is represented by the peptidoglycan (PTGN), bacterial product that can modulate peripheral immune function spreading to the blood and priming the innate immune system to kill certain bacterial pathogens (Clarke, Davis, Lysenko, & al., 2010).

Although the gut microbiota may be one mechanism for the regulation of immune responses, it is also likely that dietary substances also directly affect immunity. The **ω**-3 fatty acids exert anti-inflammatory effects through binding to Gpr120 (Oh Da, Talukdar, Bae, & al., 2010). Gpr120 is expressed mostly by macrophages, and the binding of **ω**-3 fatty acids to this GPCR represses the production of tumor necrosis factor and interleukin-6 and macrophage induced tissue inflammation. Thus, the binding of fatty acids to Gpr120 and Gpr43 represent a new mechanism for the immune system regulation, and -up to date- these GPCRs are the two leading molecules that could be the mediators of the effects of diet on inflammatory response.

1.4. Immune system: a general overview

The immune system consists of innate and adoptive parts: the innate IS that is the sum of physical and chemical blocks, through reactivity of local nonspecific and specific cells recruited to the site of inflammation and the adaptive IS that acts as a specific second line, responding to antigen variability and producing immunological memory (Abbas, Litchman, & Pillai, 2012).

It is conceptually established that it can be divided in two basic components: the innate immune system and the adaptive immune system. The primary aim of the innate immune cells is to provide a rapid non-specific response to any pathogens or foreign aggressors possessing foreign antigens (Janeway & Medzhitov, 2002) (Steinman & Idoyaga, 2010). On the other hand, the primary aim of the adoptive immune cells is to provide a latent but higly specific response against those "non-self" antigens and to generate an "immune memory" against those ones to counter similar insults in the future more quickly (Vesely, Kershaw, RD, & al, 2011) (Matzinger, 2002).

Together these two parts of the immune system collaborate to initiate acute inflammation ultimately culminating in its resolution and healing after they have taken care of the "non-self" aggressors or insult.

Most notable innate immune cells include macrophages, natural killer (NK) cells, dendritic cells (DCs), various myeloid lineage subsets, neutrophils, basophils, and eosinophils (Janeway & Medzhitov, 2002) (Green, Ferguson,

Zitvogel, & al., 2009); while the most notable adaptive immune cells include T and B lymphocytes (Vesely, Kershaw, RD, & al, 2011).

The initial reaction orchestrated by innate immune cells consists of capturing, clearing up or destroying the source of injury, infections, or diseased cells, followed by priming of the adaptive immune cells against antigens derived from the "non-self" (Janeway & Medzhitov, 2002) (Steinman & Idoyaga, 2010). This adaptive immune cell priming helps to initiate more specific responses against the acquired antigens and leading to their eradication.

1.4.1. Dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells within the immune system. They are continuously produced from hematopoietic stem cells in the bone marrow and are widely distributed, as immature DCs, into both lymphoid and non-lymphoid tissues (Banchereau & Steinman, 1998) (Cella, Sallusto, & Lanzavecchia, 1997) (Reis e Sousa, Sher, & Kaye, 1999) (Moser & Murphy, 2001). Immature DCs, including epidermal Langerhan's cells, splenic marginal zone DCs and interstitial DCs within non-lymphoid tissues, continuously sample self-antigen to maintain T cell self-tolerance (Banchereau & Steinman, 1998).

DCs in general possess a diverse repertoire of surface receptors (and intracellular receptors) that help them in environmental sensing and to carry out rapid innate immunity-related functions (Steinman & Idoyaga, 2010)

(Mellman, 2013). Such receptors include various scavenging or phagocytic receptors like CD91, integrins, CD36, surface pattern recognition receptors (PRRs) like toll-like receptors (TLRs), and intracellular PRRs like NOD-like receptors (NLRs) (Lutz & Schuler, 2002) (Simmons, Wearsch, Canaday, & al, 2012) (Reis e Sousa C. , 2004).

DC-based PRRs help in detection and stimulation of danger signals like pathogen-associated molecular patterns (PAMPs) or damage-associated molecular pattern (DAMPS) (Matzinger, 2002).

Dendritic cells are also special in terms of their antigen processing machinery. Classically (for non-professional APCs and normal cells), antigens derived from intracellular sources are presented by the major histocompatibility complex (MHC) class I presentation system while extracellular antigens are preferentially processed for MHC class II presentation (Romao, Gannage, & Munz, 2013). This unique ability to cross-present antigen to adaptive immune cells is also behind DC's role as APCs.

DCs can exist in two main states, steady state immature dendritic cells (im-DCs) and fully mature DCs (m-DCs) (Mellman, 2013) (Reis e Sousa C., 2006). The distinction between immature and mature DCs is partly based on changes occurring on two crucial levels: phenotypic and functional.

Phenotypic maturation is attained when DCs up-regulate surface maturation ligands such as CD80, CD83, and CD86 along with MHC class II. DCs stimulated on the functional level exhibit the ability to secrete cytokines where the balance between inflammatory or immunostimulatory cytokines

(e.g., IL-12, IL-6, IL-1β) and immunosuppressive cytokines (e-.g., IL-10, TGF-β) is decided by the "environmental context" (Steinman & Idoyaga, 2010) (Reis e Sousa C., 2006) (Reis e Sousa C. , 2004).

Im-DCs are poor stimulators of T cell proliferation, as they lack the requisite MHC molecules and costimulatory receptors, and they are located in most tissues where they are capable of capturing antigens. Upon encountering a powerful immunological stimulus, im-DCs convert into m-DCs, thus switching from an antigen-capturing mode into a antigen-presenting and T cell-stimulating mode (Vander Lugt, Khan, Hackney, & al, 2014). The m-DCs express higher levels of costimulatory molecules, MHC molecules and proinflammatory cytokines than im-DCs thus it seems that while m-DCs induce a state of immune activation, im-DCs can induce a state of immune tolerance (Lechler, Ng, & Steinman, 2001) (Macagno, Napolitani, Lanzavecchia, & al., 2007) (Banchereau & Steinman, 1998) (Cella, Sallusto, & Lanzavecchia, 1997) (Reis e Sousa, Sher, & Kaye, 1999).

Cytokines and chemokines produced by m-DCs are important in determing the type of immune response, including Th1, Th2 and Th17 responses, leading to cellular, humoral and autoimmune responses, respectively (Gutcher & Becker, 2007).

Some studies suggest that DCs have the capacity to induce different types of T cell–mediated immune responses, depending on their lineage, maturation stage and activation signals. The first experimental evidence suggesting that DCs may direct the type of T cell–mediated immune response came from the observation that DCs could produce the T helper subset 1 (Th1)-polarizing

cytokine interleukin 12 (IL-12) (Macatonia, Hosken, Litten, & al., 1995) (Cella, Scheidegger, Palmer-Lehmann, & al., 1996) (Kotch, Stanzl, Jennewein, & al., 1996) (Sousa, C, Hieny, Scharton-Kersten, & al, 1997). In human blood there are two distinct types of DC precursor: these are myeloid monocytes (pre-DC1s) and plasmacytoid DC precursors (pre-DC2s) (Rissoan, Soumelis, Kadowaki, & al., 1999).

In humans, DCs showed different effector functions, which depended on multiple factors, in directing T cell responses. Whereas DC1s at a mature stage induce TH1 differentiation and strong cytotoxic T lymphocyte (CTL) responses (Rissoan, Soumelis, Kadowaki, & al., 1999), DC1s at an immature stage induce IL-10–producing CD4+ and CD8+ regulatory T cells (Jonuleit, Schmitt, Schuler, & al., 2000) (Dhodapkar, Steinman, Krasovsky, & al., 2001) (Gilliet & Liu, 2002). Pro-inflammatory and antiinflammatory factors also affect DC function. Immature DC1s derived from monocytes after 5–7 days of culture with granulocyte– macrophage colony-stimulating factor (GM-CSF) and IL-4 induce both Th1 and Th2 differentiation (Kalinski, Hilkens, Wierenga, & al., 1999).

Thus, the unique capacity of DCs to respond to microbial signals and to subsequently activate naive T cells enables these cells to determine the outcome of antimicrobial immunity and the streght of the immune response.

2. Haematopoietic stem cell transplantation and immuno reconstitution

2.1. Haematopoietic stem cell transplantation: overview

Haematopoietic stem cell transplantation (HSCT) is a widely used treatment for replacement of nonfunctioning organs and tissues with healthy organs or tissues. Technically, transplantation is the process of taking cells, tissues, or organs, called **graft**, from one individual and placing them into a different individual.

Transplant immunologists have developed a special vocabulary to describe the kinds of cells and tissues encountered in the transplant setting. A graft transplanted from one individual to the same individual is called an autologous graft. A graft transplanted between two genetically identical or syngeneic individuals is called a syngeneic graft (Abbas, Litchman, & Pillai, 2012). A graft transplanted between two genetically different individuals of the same species is called as allogeneic graft (or allograft).

Initially, the principal source of HSCs was bone marrow (BM) from an HLA identical sibling for transplantation in children and young adults. Subsequently, the choice of donors and the sources of HSCs have enlarged, extending transplant indications to more patients, especially adults. Today, transplant physicians must choose among stem cell sources between bone marrow, granulocyte colony-stimulating factor (GCSF)-mobilized peripheral blood stem cells (PBSC), or umbilical cord blood (UCB). The donor can be an

HLA identical sibling, an matched unrelated donor (MUD), a haploidentical family peripheral blood stem cell or bone marrow donor or, an HLA-mismatched unrelated UCB donor (EBMT, 2012).

Allogeneic hematopoietic stem cell transplantation (aHSCT) was initially developed for two purposes. First, it was a strategy to treat individuals with inherited anaemias or immune deficiencies by replacing the abnormal hematopoietic system with one from a healthy individual. Second, it allowed the delivery of myeloablative doses of radiation and/or chemo therapy to patients with cancer. Termed 'high-dose' therapy, myeloablative conditioning generates increased killing of tumor cells compared with conventional doses of radiation and chemotherapy. Myeloablative therapy can cure some patients who would otherwise relapse, but it results in the permanent loss of the patient's bone marrow function, requiring rescue with hematopoietic stem cells, which are administered as an intravenous infusion (Kolb, 2008).

Transplant carries a significant risk of morbidity and mortality, so the decision to proceed with HSCT must carefully balance risks and benefits.

Allogeneic transplants come with a risk of graft-versus-host disease (GvHD), but are also associated with lower rates of malignant relapse owing to an immune-mediated graft-versus-tumor (GVT) effect. The most impacting complications are infections, relapse, graft-versus-host disease (GVHD), and second malignancies which are due in part due to immune deficiency or deregulation of the immune system (IS). Studying immune reconstitution has it challenges and human studies are complicated as immune reconstitution

can be influenced by many factors, for which one cannot always control. These can be divided into pre-, peri- and post-transplant factors (

Figure 3). Pre-transplant (pre-HSCT) factors include underlying disease, prior chemotherapy or radiation, nutritional status, patient and donor age, and prior exposure to or infection with microorganisms. Peri-transplant (Peri-HSCT) factors include conditioning, stem cells source, graft manipulation, donor-recipient matching for human leukocyte antigen (HLA) and non-HLA antigens and peri-transplant infections. Post-transplant (Post-HSCT) factors include pharmacologic GVHD prophylaxis, donor lymphocyte infusions, antibiotic treatment and prophylaxis, the development of GVHD (Bosh, Khan, & Storek, 2012).

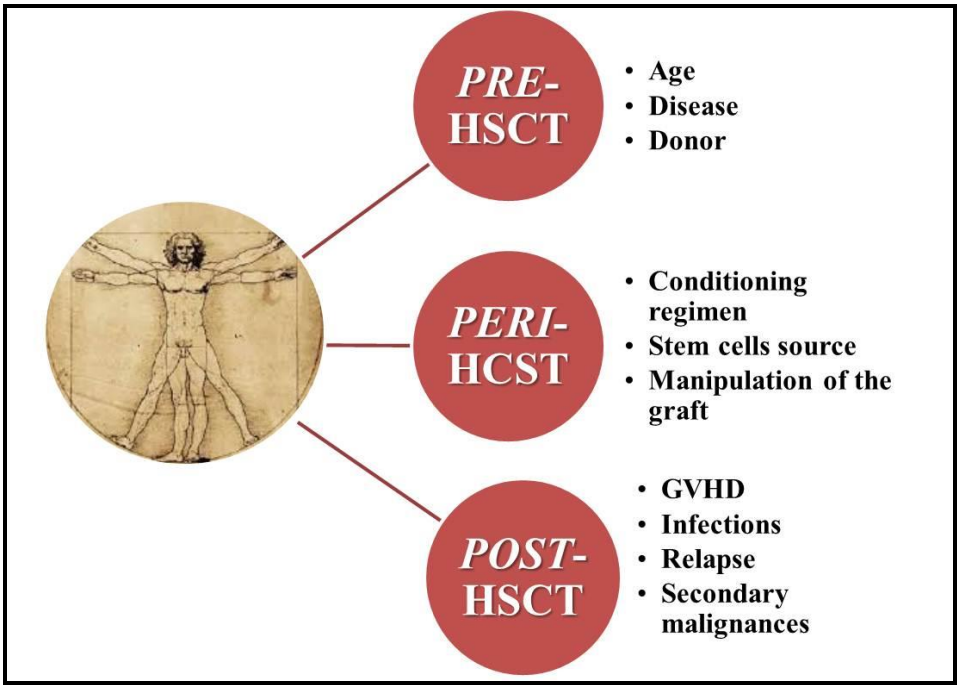

Figure 3: Schematic rapresentation of the most important factors that influence the trasplantation outcome considering the pre-, peri-, and post-HSCT periods.

2.2. Graft-versus-host disease

Graft-verus-host disease (GvHD) is a major cause of non-relapse morbidity and mortality, affecting up to 40-60% of transplanted patients (Jagasia, Arora, & Flowers, 2012) and accounting for 15% of death after aHSCT (Pasquini & Wang, 2013).

GvHD was initially reported by Barnes, Loutit and Micklem and classically defined by Billingham as a syndrome in which donor immunocompetent cells recognize and attack host tissues in immunocompromised allogeneic recipients (Barnes, Loutit, & Micklem, 1962) (Billingham, 1966–1967).

Many variables are correlated to the GvHD onset such as stem cell source, age of donor and recipient, preparative regimen and prophylaxis can impact the likelihood and severity of GvHD. Children risk less for GvHD than adults; however, that risk is still significant especially when using alternative donor sources.

Acute GvHD and chronic GvHD involve distinct pathological processes: acute GvHD has strong inflammatory components, whereas chronic GvHD displays more autoimmune and fibrotic features. Acute GvHD, typically occurring between the time of the engraftment through 100 days after transplant and can lead devastating consequences on the skin (81% of patients), gut (54%) and liver (50%) (Ferrara, Levine, & Holler, 2009).

Chronic GvHD typically occurs after 100 days, although this temporal distinction is blurring with strategies such as reduced-intensity conditioning, and an overlap syndrome is recognized that shares features of both.

Billingham formulated three requirements for development of GVHD: **(1)** the graft must contain immunologically competent cells; **(2)** the recipient must express tissue antigens that are not present in the transplant donor; and **(3)** the patient must be incapable of mounting an effective response to eliminate the transplanted cells.

The immunologically competent cells are T cells and hence GVHD can develop in various clinical setting when tissues containing T cells (blood products, bone marrow, and solid organs) are transferred from one person to another who is not able to eliminate those cells. Patients whose immune systems are suppressed and who receive white blood cells from another individual are at especially high risk for the disease. The pathophysiology of the GvHD is a complex but fast process that arises when donor T cells respond to genetically defined proteins on host cells, like human leucocyte antigens (HLA), which are highly polymorphic and are encoded by the major histocompatibility complex (MHC) (Krensky, Weiss, Crabtree, & al., 1990), (Peterson, Frank, Pace, & al, 2008) (Welniak, Blazar, & Murphy, 2007).

Despite HLA identity between a patient and donor, about 40% of recipients of HLA-identical grafts develop systemic acute GvHD that needs treatment with high-dose steroids. Moreover, polymorphisms in both donors and recipients of cytokines that have a role in the classic cytokine storm of GVHD have been implicated as risk factors for the disorders (Antin & Ferrara,

1992). In fact, some studies have showed a correlation between tumor necrosis factor alfa (TNF-α), interleukin 10 (IL-10) and interferon gamma (IFN-γ) variants with GvHD (Lin, Storer, Martin, & al., 2003) (Dickinson & Charron, 2005).

2.2.1. Pathophysiology of acute GvHD

For what concern the pathophysiology of acute GvHD, the disease is indicative of exaggerated but typical inflammatory mechanisms mediated by donor lymphocytes infused into the recipient and the recipient's tissues that stimulate donor lymphocytes have usually been damaged by underlying disease, previous infections, and the transplant conditioning regimen. As a result, these tissues produce molecules such as proinflammatory cytokines and chemokines, which increase expression of key receptors on antigen-presenting cell (APC), thereby enhancing cross-presentation of polypeptide proteins to the donor immune cells that mediate GvHD.

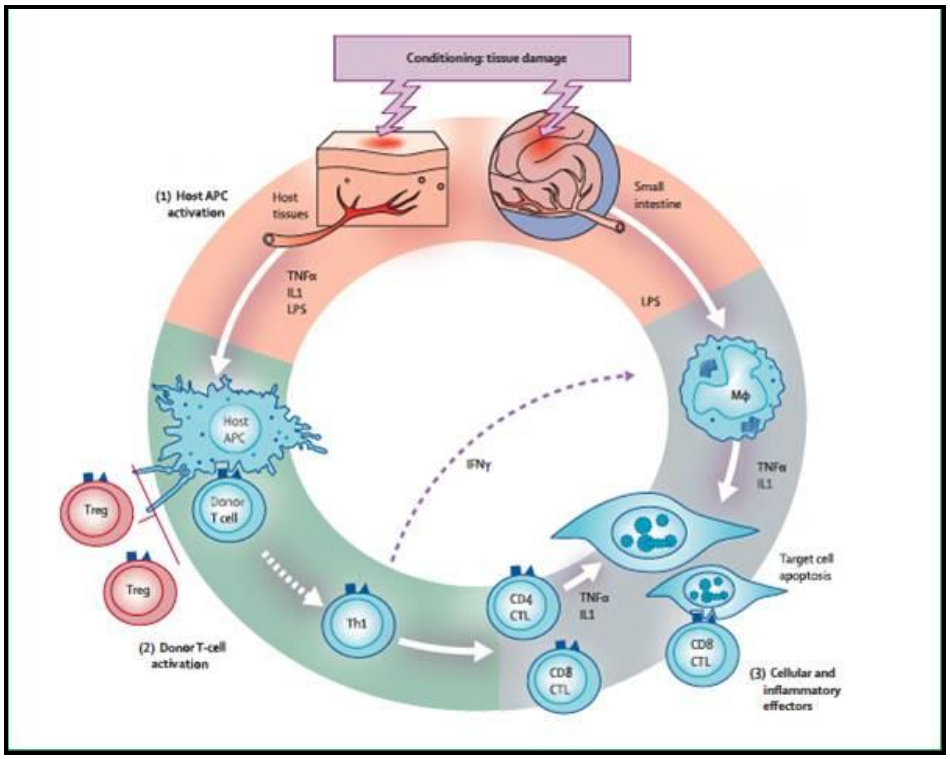

Figure 4: The three main phases of GvHD (by Ferrara, et al., 2009).

Severity of acute GvHD is ascertained by the extent of involvement of the three main target organs. According with the Seattle grading system, have been listed different grades: I (mild), II (moderate), III (severe), and IV (very severe) (Glucksberg, Storb, Fefer, & al., 1974).

Skin is most frequently affected and is usually the first organ involved, generally coinciding with engraftment of donor cells. The characteristic maculopapular rash is pruritic and can spread throughout the body. Gastrointestinal-tract involvement of acute GVHD usually presents as diarrhea but can also include vomiting, anorexia, abdominal pain, or a combination when severe. Liver disease caused by GVHD can be difficult to distinguish from other causes of liver dysfunction such as veno-occlusive disease, toxic drug effects, viral infection, sepsis, or iron overload; the

histological features of hepatic GVHD are endothelialitis, lymphocytic infiltration of the portal areas, and bile-duct destruction.

On the basis of this knowledge, the progression of acute GvHD can be summarized in three sequential steps or phases: **(1)** activation of APCs; **(2)** donor T-cell activation, proliferation, differentiation and migration; and **(3)** target tissue destruction (Figure 4).

- The ***first phase*** involves damage to host tissues by inflammation from the preparative chemo- and/or radiotherapy regimen that leads to the release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) and danger signals such as adenosine-5'-triphosphate (ATP) as well as extracellular matrix proteins such as biglycans that promotes activation and maturation of antigen-presenting cells (APCs) (Zeiser, Penack, Holler, & al., 2011). This is furthered by damage to the gastrointestinal epithelium, allowing translocation of lipopolysaccharide, which can activate innate immunity through Toll-like receptors, furthering the cytokine cascade (Hill & Ferrara, 2000) (**Figure 5**).
- In the ***second phase***, donor T-cell activation is triggered by recipient antigens presented by host APC and sustained by donor APC both recipient and donor antigen-presenting cells, as well as inflammatory cytokines trigger activation of donor-derived T cells, which expand and differentiate into effectors cells, that results in the transcription of genes for cytokines in a positive feedback. The activation is mediated by human leukocyte antigen (HLA) proteins encoded by the major

histocompatibility complex (MHC); in addition to the interaction between the T-cell receptor and MHC, T-cell activation requires signaling between costimulatory molecules such as CD28 (present on the T cell) and B7.1 or B7.2 (CD80 or CD86, present on the APC); other T-cell:APC costimulatory signaling pairs include inducible costimulator (ICOS) (CD278):B7H (CD275), OX40 (CD134):OX40L (CD252), CD40L (CD154):CD40, and 4-1BB (CD137):glucocorticoid-induced tumor necrosis factor receptor (GITR). The absence of these costimulatory signals, particularly CD28:B7.1/B7.2, can lead to anergy; furthermore, this interaction can be blocked by coinhibitory molecules such as CTLA4 (CD152), which competes with CD28 for B7.1/B7.2. Programmed death-1 (PD-1) (CD279): programmed death ligand 1 (B7H1, CD274) are another pair of inhibitory molecules that can induce anergy or tolerance. Models that block these costimulatory or coinhibitory interactions have been shown to reduce or exacerbate GVHD, suggesting possible therapeutic targets (Kwon, 2010).

- in the ***third phase***, activated T cells migrate from secondary lymphoid organs to target tissues through a combination of chemokine-receptor, selectin-ligand, and integrin-ligand interactions (Wysocki, Panoskaltsis-Mortari, & al, 2005); selectins and integrins mediate rolling and tethering of lymphocytes along high endothelial venules through interactions with their matching ligands. For example interactions between L-selectin (CD62L) and $\alpha 4\beta 7$ integrin expressed on T cells and peripheral node addressin and mucosal addressin cell

adhesion molecule expressed on secondary lymphoid tissue mediate homing to mesenteric lymph nodes and Peyer's patches and induction of gut GVHD (Dutt, Ermann, Tseng, & al., 2005). Once activated donor T cells reach target organs mediate cytotoxicity against target host cells through Fas-Fas Ligand interaction, perforin-granzyme B, production of tumor necrosis factor-α (TNF-α) (Braun, Lowin, French, & al., 1996). Cytokines such as TNF-α, IFN-γ, IL-2, IL-7, IL-10, and others also appear to be essential to regulating leukocyte recruitment and tissue destruction; these effects are dependent on strength, timing, and other interactions, making the effects of individual cytokines difficult to predict (Welniak, Blazar, & Murphy, 2007).

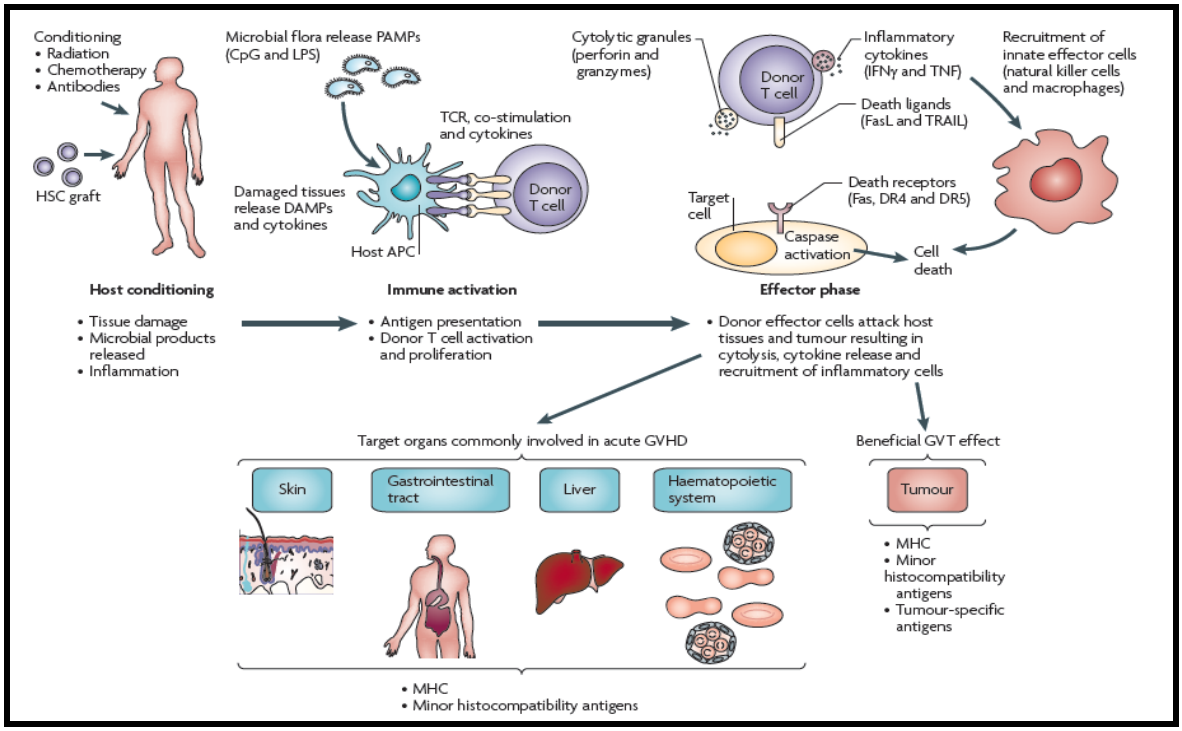

Figure 5: Pathopysiology of GvHD and GVT effects (modified by Jenq e Van der Brink, 2010).

2.3. Immune reconstitution after aHSCT

The restoration of a functional immune system is one of the main factors influencing the clinical outcome of aHSCT. The post-HSCT period is characterized by multiple immune defects that expose the patient to a high risk of opportunistic infections, second malignancies and disease relapse; all these complications are due in part to immune deficiency or dysregulation of the IS.

The duration of this period may vary according to several variables, including patient's age, the immune status before transplant, the intensity of the conditioning regimen, the source of stem cells, graft manipulation, the degree of donor compatibility, and pharmacological immune suppression.

Although often used interchangeably, it is therefore important to distinguish between *immune reconstitution*, which refers to qualitative immune cell repopulation, and *immune recovery*, which regards to their qualitative restitution (Shiobara, Harada, Mori, & al., 1982).

Studying and following immune reconstitution has its challenges. Observations in mice (typically inbred and relatively pathogen-free) cannot be applied to humans (outbred and harboring multiple pathogens). In fact, quantitative recovery of T cells following murine HCT takes weeks to months, whereas it takes months to years following human HCT. Furthermore, human studies are complicated as immune reconstitution can be influenced by many factors, for which one cannot always control. Although the quantitative aspects of immune reconstitution post-transplant and the qualitative features

of immune recovery have been the subject of several studies, a tight association between measurable immune biomarkers and the clinical outcome of HSCT, is currently missing. So far, the specific issue of validating thresholds of immune measurements that may help predicting the incidence of major post-transplant events has been approached by single-center with small-size studies. While sometimes sufficiently powered to obtain statistically significant results, these studies often failed to draw definitive conclusions that may be relevant to daily clinical practice. Conversely, the lack of harmonized methods for immune biomarkers measurements and the great heterogeneity of the transplant populations between the different studies have left more thoughts unresolved than meaningful unique results.

The normalization of granulocytes, monocytes and NK cell numbers usually occurs the first week and (with the possible exception of NK-cells) coincides with their full competence. In the other hand, the normalization of T and B cell numbers may take much longer and does not necessarily associate with their immediate functional restitution (Shiobara, Harada, Mori, & al., 1982).

The quantitative reconstitution of T cells post-transplant occurs through two main mechanisms: **(1)** the early peripheral expansion of donor-derived memory T cells present in the graft, that takes weeks **(2)** the late increase of host-tolerance naïve T cells originating from donor stem cells after thymic education, which, depending on donor age, occurs in months (Van der Brink, Alpdogan, & Boyd, 2004).

Studies on immune recovery after HCT have been performed extensively in adults (Lum, 1987) (Verma & Mazumder, 1993) (Storek, Witherspoon, &

Storb, 1995). It is well known that GvHD, its prophylaxis or treatment, and CMV infections influence the recovery of the immune system after allogeneic HCT in adults (Noel, WItherspoon, Storb, & al., 1978) (Meyers, Flournoy, & Thomas, 1980) (Parkman & Weinberg, 1999). Persistent CD4+ T-cell depletion post allogeneic HCT seems to be related primarily to age-associated thymic insufficiency (Mackall, Fleisher, Brown, & al, 1995) (Storek, Witherspoon, & Storb, 1995).

In children, however, only limited data on immune reconstitution post HCT with special regard to factors affecting the speed of recovery are available (Foot, Potter, Donaldson, & al., 1993) (Kook, Goldman, al., & al., 1996) (De Vries, Van Tol, Van der Bergh, & al., 2000). The role of age in CD4+ T-cell recovery was raised by a paediatric study, which showed no difference between T-cell recovery after allogeneic bone marrow transplantation (BMT) in children and in adults (De Vries, Van Tol, Van der Bergh, & al., 2000). Data on immune reconstitution after autologous HCT in children is extremely modest (Bengtsson, Smedmyr, Festin, & al., 1989) (Takaue, 1991) (Small, Papadopoulos, Boulad, & al, 1999) (Kamani, Kattamis, Carroll, & al., 2000).

The majority of studies were based on very low numbers of patients: the largest group of 41 children was analyzed by Takaue (1991) (Takaue, 1991). Furthermore, there was no large, single-center comparison of immune recovery between pediatric recipients of autologous and allogeneic grafts. Studies in adults show faster T-cell recovery after autologous HCT (Shiobara, Harada, Mori, & al., 1982), but some recent data suggest prolonged CD4+ T-cell depletion both in adults and in children post

autologous HCT (Nordoy, Kolstad, Endresen, & al., 1999) (Laurenti, Sica, Sorà, & al., 2000) (Mackall, Stein, Fleisher, & al., 2000). Another interesting study has been published comparing allo- and auto-transplanted children with regard to factors affecting the speed of recovery (Kalwak, Gorczynska, Toporski, & al., 2002) and some other informations about factors affecting lymphocyte subset reconstitution after cord blood transplantation children have been included in the literature (Niehues, Rocha, Filipovich, & al., 2001). More recently a study of 32 pediatric patients provided preliminary evidence that patients with slow recovery of cytotoxic T cells have a high risk of relapse or life-threatening infections (Koel, Bochennek, Zimmermann, & al., 2007). In 2010 has also has been published a innovative approach using a multivariate analysis methods in order to classify into high-risk and low-risk groups of children patients based on the speed of cellular immune reconstitution, offering new prospectives (Koening, Huenecke, Salzmann-Manrique, & al., 2010).

The progress of immune reconstitution is different in each patients and it creates several issues for biologists and clinicians to find reliable markers useful to predict risk of infections, relapse during the reconstitution period after HSCT.

2.4. GM and aHSCT

The impact of the microbiota on GVHD is known to be significant. Studies in mice have shown reduction of GVHD with gut-decontaminating antibiotics (Van Bekkum, Roodenburg, Heidt, & al., 1974) and transplantation in germ-free conditions (Jones, Wilson, & Bealmear, 1971). This led to efforts to eliminate bacterial colonization in allogenic bone marrow transplantation (BMT) patients, combining gut decontamination with a near-sterile environment (Storb, Prentice, Buckner, & al., 1983). Initial reports were promising, but subsequent studies could not confirm a benefit (Passweg, Rowlings, Atkinson, & al., 1998) (Russel, Chaundhry, Booth, & al., 2000). Other approaches include targeting anaerobic bacteria (Beelen, Elmaagacli, Muller, & al., 1999) and introducing potentially beneficial bacteria (Gerbitz, Schultz, Wilke, & al., 2004), with some reduction of GVHD. These initial studies, however, have been few in number, and no consensus exists between BMT centers regarding how to target the flora. Until recently, a reliance on microbiological culture techniques to characterize flora composition limited these studies. Culture-independent techniques such as ribosomal RNA (rRNA) gene sequencing have demonstrated that a large majority of the estimated 500–1,000 bacterial species present in the human intestinal tract are not detected by culture techniques (Manson, Rauch, & Gilmore, 2008). In one of the last study carried out by Jenq RR. et al. has been readdressed the relationship between GVHD and the microbiota in

murine and human allogenic BMT recipients.

The microbiota, in turn, can modulate the severity of intestinal inflammation. In mouse models of GVHD, has been observed loss of overall diversity and expansion of Lactobacillales and loss of Clostridiales. Eliminating Lactobacillales from the flora of mice before BMT aggravated GVHD, whereas reintroducing the predominant species of Lactobacillus mediated significant protection against GVHD. In parallel they then characterized gut flora of patients during onset of intestinal inflammation caused by GVHD and found patterns mirroring those in mice. Has been identified an increased microbial chaos early after allogeneic BMT as a potential risk factor for subsequent GVHD (Jenq, Ubeda, & Taur, 2012).

After two years the same research group have shown that the abundance of bacteria belonging to the genus Blautia, a commensal commonly found in the intestinal tract of humans, predicts for protection from life-threatening GvHD in allo BMT patients; furthermore, in murine models, introducing a species of Blautia of murine origin reduces GvHD severity. Interestingly, it appears to do so by inducing regulatory T cells with generation of short-chain fatty acid metabolic byproducts (Jenq & van der Brink, 2014). Recently it also has been shown that mortality outocomes following aHSCT were significantly worse in patients with lower intestinal diversity; in fact, overall survival at 3 years was 36%, 60%, and 67% for low, intermediate, and high diveristy group, respectively (Taur, Jenq, Perales, & al., 2015).

Taken together, these data support the hypothesis that the GM composition and diversity exert an important role also in transplanted patients and

suggest that a new research field on flora manipulation might open new clinical trials in order to reduce intestinal inflammation and improve outcomes for allogeneic BMT recipients.

3. **PROJECT OUTLINE**

The GM structure, as an active component of the human immune system - and the resulting pattern of immunological interaction with the host – can be of primary importance in governing the fate of the immunological reconstitution in patients who undergoing aHSCT, predisposing or protecting from the aGvHD onset and other complications.

The study carried out along three year of PhD course was primarily divided in three parts:

I. the phylogenetic and functionality study of the gut microbiota in pediatric patients undergoing allogeneic hematopoietic stem cell transplantation;

II. the transcriptome analysis of human DCs exposed to SCFAs in order to explore potential immunomodulatory effect of GM metabolites on one of the immune system's cell type that play the main role on the aGvHD onset;

III. the descriptive study of the early immune system's peripheral subsets reconstitution in terms of quantitative immune cells repopulation, and the analysis of a possible correlation between GM and IS during this delicate period of body recovery.

A common aim that has held all the three main goals pursued to investigate "GM predictor configurations" predisposing to or protective from aGvHD and to understand whether the composition of gut microbiota could exert an

immunomodulatory effect during the immune system's reconstitution after aHSCT.

Considering the state of art that strongly supports the idea that the GM exerts an important shaping role to the immune system, it has been considered that changes in dietary habits may modulate the composition of the gut microbiota leading to an increase of bacterial metabolites that could exert a protective role against the onset of aGvHD or to reduce the rate of this inflammatory response. The aim of this part was also to characterize *in vitro* the effect of SCFAs (acetate, butyrate, and propionate) on the immature and mature human monocyte-derived DCs, exploring the transcriptional response of one of the most important key regulators of the immune system upon exposure to these bacterial metabolites.

In parallel, the immune system reconstitution was monitored by phenotyping the lymphocytes populations circulating in blood in order to figure out their different trends of recovery and a potential correlation with several variables such as infections, aGvHD onset, and the final outcome. The aim of this part was to look over and find some reliable markers that might be useful for the clinicians to refine *ad hoc* the pharmacological approach improving also the children's lifestyle.

In fact, most of the available knowledge about the immuno recovery after aHSCT in pediatrics has been focused on the late period (after 100 days since the transplantation procedure) and very few studies have been published regarding the early period considering several challenges in

collecting samples for the delicate children status and a hard-to-get parental consent.

All this work has allowed to obtain several interesting results opening new perspectives for clinitians and researchers in this field; some of those have already been published in Bone Marrow Transplantation (Biagi, Zama, Nastasi, & al., 2015) and some others have been already elaborated and will be submitted to a peer-reviewed journal as Nastasi C., Candela M., Gaisler C., et al. "Short-chain fatty acids (SCFAs) effect on human monocyte-derived dendritic cells (DCs)".

4. Materials and Methods

4.1. I part: GM and SCFAs

Patients features

The study has been approved by the Ethical Committee of the Sant'Orsola-Malpighi Hospital-University of Bologna (ref. number. 19/2013/U/Tess). Ten pediatric patients (8 males), who have been chosen among the 23 children patients, with age ranging from 2 to 16 years (mean age 8.4 years), who underwent HSCT for hematological disorders (2 subjects, Blackfan-Diamond anemia (BDA); 2 subjects acute myeloid leukemia (ALM), 6 subjects acute lymphoid leukemia (ALL)), were enrolled at the Pediatric Bone Marrow Transplantation Unit of the Sant'Orsola-Malpighi Hospital, Bologna, Italy. Exclusion criteria were lack of informed consent, incomplete sample collection, and samples not evaluable for technical reasons. Demographic and transplant characteristics of the patients are summarized in (

Table 1). All children were in a HEPA filtered single room and received standard prophylaxis measures to prevent any complications due to infections. These include the use of non-absorbable antibiotics (levofloxacin) for gut decontamination since the start of conditioning regimen to the recovery of the enteral nutrition and neutrophil engrafment (Bucaneve, Micozzi, & Menichetti, 2005). No patient of the cohort had sepsis during the time of collection of the samples, so aGvHD and non-aGvHD patients had the same exposures to antibiotics. Acyclovir and fluconazole were administered for the antiviral and antifungal prophylaxis from day -1 and +2, respectively

(Saral, Burns, Laskin, & al, 1981). Neutrophil and platelet engraftment were defined as occurring on the first of the three consecutive days on which the neutrophil level was $0.5 \bullet 10^9$/l or higher and the blood platelets were above $20 \bullet 10^9$/l, respectively. All patients observed fasting since the day of the transplant and stopped it the day of the engraftment, had the clinical condition allowed. Only when per os intake was at least 50% of the caloric needs, parenteral nutrition was suspended. Foods were introduced gradually during the following 3-6 months after HSCT. Of the 10 patients, 5 developed aGvHD, 4 of which had skin aGvHD (Grade I-III) according with the Seattle grading system (Glucksberg, Storb, Fefer, & al., 1974). The patient who developed grade III skin aGvHD was treated with steroids and extracorporeal photoaferesis, the other 3 patients with grade I-II skin GvHD received only steroids. One (subject 15) developed severe grade IV intestinal aGvHD and received steroids, infliximab and extracorporeal phoaferesis.

Subject	Sex / Age	Diagnosis	Donor Type	Stem cell source	Conditioning regimen	GvHD Prophylaxis	TNC/Kg	Engraftment (day)		Acute GvHD				OUTCOME
								PMN	*PLT*	*Grade*	*Localization*	*Day*	*Therapy*	
4	F/12	AML	MUD	BM	BU+EDX L-PAM	ATG-CSA-MTX	$4.2x10^8$	+13	+20	I	Skin	+25	Steroid	ANED 2 ys
5	M/10	ALL	MFD	BM	BU+TT+EDX	CSA	$2.9x10^8$	+12	+17	III	Skin	+23	Steroid - PHEC	ANED 2 ys +1/12
6	M/2	BDA	MMUD	BM	BU+TT FLUDARA	ATG-CSA-MTX	$10x10^8$	+15	+16	II	Skin	+15	Steroid	ANED 2 ys +2/12
11	M/9	ALL	MUD	BM	BU+TT +EDX	ATG-CSA-MTX	$3.4x10^8$	+15	+27	II	Skin	+11	Steroid	Dead for relapse
15	M/8	ALL	MFD	BM	BU+ TT+EDX	CSA	$5.8x10^8$	+12	+17	IV	Skin Gastrointestinal tract	+11	Steroid - PHEC Infliximab	ANED 1ys +6/12
16	M/16	ALL	MFD	BM	BU+TT+EDX	CSA	$4.6x10^8$	+11	+30					ANED 1ys +6/12
19	F/10	ALL	MUD	BM	BU+TT +EDX	ATG-CSA-MTX	$7.1x10^8$	+34	+35					ANED 1 ys +3/12
20	M/7	ALL	MUD	BM	BU+TT +EDX	ATG-CSA-MTX	$4.8x10^8$	+12	+15					ANED 1 ys+4/12
22	M/2	BDA	MUD	BM	BU+TT +FLUDARA	ATG-CSA-MTX	$9.9x10^8$	+13	+14					ANED 1ys +3/12
26	M/8	AML	MUD	BM	BU+EDX+L-PAM	ATG-CSA-MTX	$7.2x10^8$	+12	+21					ANED 1 ys +9/12

Table 1: Anagraphical and clinical information of the enrolled patients.
Footnotes: F: female, M: Male, AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, BDA: Blackfan Diamond anemia, MUD: match unrelated donor, MMUD: mismatch unrelated donor, MFD: match family donor, BM: bone marrow, Bu: busulfan, EDX: cyclophosphamide, TT: cyclophosphamide, L-PAM: melphalan, Fludara: fludarabine, ATG: anti-thymocyte globulin, CSA: cyclosporine, MTX: methotrexate, GvHD: Graft verus Host Disease, TNC: total nucleated cells, PMN: polymorphonuclear leukocytes, PLT: platelets, ANED: alive no vidence of disease, ys: years, PHEC: extracorporeal phoaferes.

DNA extraction from fecal samples. Total bacterial DNA from fecal samples was extracted using DNeasy Blood&Tissue Mini Kit (Qiagen, Duesseldorf, Germany) with a modified protocol (Biagi, Nylund, Candela, & al., 2010). 250 mg of feces were suspended in 1.2 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA, 4 % SDS). Four 3 mm glass beads and 0.5 g of 0.1 mm zirconia beads (BioSpec Products, Bartlesville, OK) were added, and samples were treated thrice in FastPrep (MP Biomedicals, Irvine, CA) at 5.5 ms for 1 min with 5 min intervals in ice. Samples were heated at 95°C for 15 min to inactivate pathogens, and centrifuged for 5 min at full speed. Supernatants were added of 260 μl of 10 M ammonium acetate and kept in ice for 5 min, then centrifuged at full speed for 10 min. Supernatants were collected, 1 volume of isopropanol was added and incubated in ice for 30 min. DNA was precipitated by centrifugation for 15 min at full speed and washed with ethanol 70%. Pellets were resuspended in 100 μl of TE buffer and treated with 2 μl of DNase-free RNase (10 mg/ml) at 37°C for 15 min. Protein removal by Proteinase K treatment and DNA purification with Qiagen columns were performed following the manufacturer's instructions for Gram positive bacteria. Final DNA concentration was determined by using NanoDrop ND-1000 (NanoDrop® Technologies, Wilmington, DE).

16S rRNA gene amplification and pyrosequencing. The V4 region of the 16S rRNA gene was amplified by using the primers 520F (5'-AYTGGGYDTAAAGNG-3') and 802R (5'-TACNVGGGTATCTAATCC-3') (Centanni, Turroni, Consolandi, & al., 2013). Primers included at their 5' end one of the two

adaptor sequences used in the 454-sequencing library preparation protocol (adaptor A and B), linked to a unique MID tag barcode of 10 bases allowing samples identification. PCR mix contained 0.5 μM of each primer, approximately 100 ng of template DNA, 2.5 U of GoTaq Flexi Polymerase (Promega, Milan, Italy), 200 μM of dNTPs and 2 mM of MgCl2. Thermal cycling consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 50 s, 40°C for 30 s, and 72°C for 60 s, with a final extension step at 72°C for 5 min. PCR amplifications were carried out in a Biometra Thermal Cycler T Gradient (Biometra, Göttingen, Germany). Amplicons were purified with MinElute PCR Purification Kit (Qiagen), quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Leek, Netherlands), pooled in equal amounts (creating five 7-plex and one 10-plex pools), and again purified by 454-Roche Double Ampure size selection protocol with Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) according to the manufacturer's instructions (454 LifeSciences, Roche, Branford, CT). Pools were fixed to microbeads to be clonally amplified by emulsion PCR following the GS-FLX protocol Titanium emPCR LIB-A (454 LifeSciences, Roche). Beads were enriched in order to keep only those carrying identical PCR products on their surface, and loaded onto a picotiter plate for pyrosequencing reactions, according to the GS-FLX Titanium sequencing protocol. Each pool was sequenced in one eighth of a plate.

Bioinformatic analysis of 16S rRNA gene sequencing data. Sequencing reads were analyzed using the QIIME pipeline (Schnorr, Candela, & al., 2014). Briefly, V4 sequences were filtered according to the following criteria: (i) read length 150 - 350 bp; (ii) no ambiguous bases (Ns); (iii) a minimum average quality score over a 50-bp rolling window of 25. For bacterial taxonomy assignment RDP-classifier (version 2.2) was used with 50% confidence threshold. Trimmed reads were clustered into OTUs at 97% identity level and further filtered for chimeric sequences using ChimeraSlayer (http://microbiomeutil.sourceforge.net/#A_CS). Alpha-diversity and rarefaction plots were computed using four different metrics: Shannon, PD whole tree, chao1 and observed species. Weighted and unweighted UniFrac distances and Euclidean distance of genus level relative abundance were used to perform Principal Coordinates Analysis (PCoA). The R packages Stats and Vegan were used to perform statistical analysis. In particular, data separation in the PCoA was tested using a permutation test with pseudo F-ratios (function Adonis in the Vegan package).

GC-MS determination of SCFAs in fecal samples Aliquots of dried fecal samples (about 250 mg) were briefly homogenized after the addition of 1 mL of 10% perchloric acid in water and centrifuged at 15,000 x g for 5 min at 4°C. 500µL of supernatant were diluted 1:10 in water, 10 µL of D8-butyrric acid (internal standard, IS) were added to the sample at the final concentration of 20 µg/mL (9). The calibration curves were prepared adding the internal standard to scalar amounts of the acids in diluted samples or water (for external standardization). All the standards (purity > 99%), acetic,

propionic, butyric, valeric acids and IS were provided by Sigma-Aldrich (Milan, Italy) and were used to prepare calibration solutions for quantitation (linear response) and identification. HS-SPME was performed by using a 75 μm CarboxenTM/polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA). The optimized final extraction conditions were: temperature 70°C, 10 min of equilibration time, 30 min of extraction time. The analytes were desorbed into the GC injector port at 250°C for 10 min, including fiber cleaning. GC-MS analysis was carried out on a TRACE GC 2000 Series (ThermoQuest CE Instruments, Austin TX, USA) gas chromatograph, interfaced with GCQ Plus (ThermoQuest) mass detector with ion trap analyzer, operating in EI mode (70 eV). The capillary GC column was a Phenomenex ZB-WAX (30 m x 0.25 mm ID, 0.15 μm film thickness), consisting of 100% Polyethylene Glycol. Helium (He) was the carrier gas at a flow rate of 1.0 mL min-1. An oven temperature program was adopted: initial 40 ºC (hold time: 5 min), then ramped by 10 ºC min-1 to 220 ºC (hold time: 5 min). The temperature of transfer line and ionization source was maintained at 250 and 200 ºC, respectively. The GC was operated in splitless mode; the injector base temperature was set at 250 °C. The mass spectra were recorded in full scan mode (34-200 amu) to collect the total ion current chromatograms. Quantitation was carried out by using the extracted ion chromatograms by selecting fragment ions of the studied analytes (43 and 60 amu for acetic acid, 55 and 73 amu for propionic acid, 60 and 73 amu for butyric and valeric acids and 63 and 77 amu for IS). The SCFAs concentration in fecal samples

was expressed in micromoles per gram (μmol/g) of feces. Limit of detection ranged from 4 to 68 nmol/g.

T helper cells, T reg cells phenotyping. Whole peripheral blood (PB) samples were obtained by venipuncture and were collected in correspondence of the fecal samples, when it has been possible. Each PB sample was received in heparinized tube (BD Vacutainer®) and the leucocyte counting was determined by SIEMENS ADVIA® 2120 Hematology System. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-density gradient centrifugation (Ficoll-Hypaque, GE Healthcare, Piscataway, NJ) and used immediately in assays following the isolation. In order to detect $CD4^+CD25^{++}CD127^-FoxP3^+$ T regulatory cells (T_{reg}), PBMCs were immunostained with conjugated monoclonal antibodies (mAb) for human CD4 (FITC, BD, clone RPA-T4), CD25 (PE, BD, clone M-A251), CD127 (PerCP-Cy5.5, BD, clone HIL-7R-M21) for 20 minutes at room temperature (RT) in the dark and then were washed and resuspended in fixation/permeabilization solution (BD, Cytofix/Cytoperm kit; BD Biosciences PharMingen) and intracellular staining was performed following the manufacturer's instructions using mAb FoxP3 (APC, eBioscience, clone 236A/E7) mAb. In order to detect $CD3^+CD4^+INF\text{-}\gamma^+$ T cells (Th1), $CD3^+CD4^+IL\text{-}4^+$ T cells (Th2), $CD3^+CD4^+IL\text{-}17^+$ T cells (Th17), PBMCs were stimulated for 5h with PMA(50ng/ml)/Ionomycin(1μg/ml) in the presence of BD GolgiStop™ Protein Transport Inhibitor for the last 2h. It has been used the Human Th1/Th2/Th17 Phenotytyping Kit (BD Pharmingen™, cat#5607751) and has been followed the main manufacturer's instructions

with the addition of CD4 (PeCy7, BD, clone SK3) and CD3 (APC-H7, BD, clone HIT3a) mAbs before permeabilization steps. Procedure was performed as recommended by protocol's kit and for the multicolor staining the kit's mAb cocktail (CD4 (PerCP-Cy5.5, clone SK3), IL-17A (PE, clone N49-653), IFN-GMA (FITC, clone B27) and IL-4 (APC, clone MP4-25D2)) was used. Stained cells were analyzed by flow cytometry on FACSCanto™ II (BD) and data were analyzed by FACSDiva software (BD). Prior to the analysis, PMT values were adjusted running unstained or unstimulated cells to exclude autofluorescence and to control the intensity of background. Absolute subset cell numbers were calculated by mutiplying the percentage of the indicated subset as obtained by flow cytomentry and absolute lymphocyte number as determined by the hematocrit.

General statistics. Significant differences among groups of samples in OTUs percentages, phylum or genus level abundances, as well as in SCFA fecal concentrations, were assessed by Mann-Whitney U tests. Where appropriate the paired version of the test was used. Kendall correlation test between SCFA or lymphocyte concentrations and the relative abundance of microbial groups was achieved using function cor.test (R package Stats). P values were corrected for multiple comparisons using the Benjamini-Hochberg method; False discovery rate (FDR) < 0.05 were considered as statistically significant.

4.2. II part: SCFAs effect *in vitro* on human DCs

Human monocytes-derived DCs generation and culture. DCs were generated from peripheral blood mononuclear cells (PBMC) from three different healthy volunteers. The mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation and subsequently CD14+ monocytes were isolated from PBMCs by negative selection using magnetic beads (Miltenyi Biotec, Germany), accordingly with manufacturer's instructions. Monocytes were cultured at 37°C in 5%CO_2 in media supplemented with 10% fetal bovine serum (FBS, Gibco) GM-CSF and IL-4 (50 ng/ml both, PeproTech) for 7 days to generate im-DCs and m-DCs, after LPS (100ng/ml, *E.coli* 055:B5) stimulation for the last 24h.

SCFAs treatments. Before all the SCFAs treatment has been performed a 7-AAD assay following the manufacturer's instructions to determine the concentration that does not affect on DCs viability and for the next steps has been chosen 1mM as final concentration for each SCFAs treatment. For both im-DCs and m-DCs the last 24h were used for the single exposure to acetate or butyrate or propionate (all purchased by Sigma-Aldrich).

7-Aminoactinomycin apoptosis assay. Cells were stained with 7-aminoactinomycin (Sigma-Aldrich), and subjected to flowcytometric analysis as described elsewhere (Telford, King, & Fraker, 1992).

FACS analysis for DCs activation state. Cells staining has been performed to assess the maturation stage of DCs using 100.000 cells for

each treatment and acquired 50.000 events on the live cells by LSR Fortessa (BD) at the CFFC (Core Facility for Flow Cytometry, Faculty of Health and Medical Sciences, University of Copenhagen) following their protocols and procedures. Anti human HLA-DR (PE, BD, clone L243), anti-human CD83 (BV421, Biolegend, clone HB15e) and anti-human CD86 (APC, Biolegend, clone IT2.2) were used. FACS data were analyzed by FlowJo v7.0.

LEGENDPlex and ELISA arrays. IL-6 production has been measured in the supernatants of DCs by ELISA using R&D kit. For simultaneous quantification of chemokines has been used the bead-based multiplex assay LEGENDPlex (BioLegend) and acquired by LSRFortessa (BD) at the CFFC. These two methods have been performed following the manufacturer's instructions.

RNA extraction and Affymetrix array. RNA was isolated and purified using the RNesy kit (Quiagen) according to the manufacturer's instructions then was assayed for quantity with NanoDrop spectrophotometer (NanoDrop, Wilmington, DE). Equally amount of RNA derived from the three donors were mixed together, each treatment with its own corrispondent.

Sample labelling, microarray hybridization and analysis. Using the WT_PLUS labelling kit (Affymetrix, Santa Clara) the total RNA was first reverse transcribed into double stranded cDNA, subsequently amplified using In Vitro Transcription. The cRNA were converted to cDNA, fragmented and end labeled with biotin (all according to the manufacturers instructions). The HTA 2.0 arrays were hybridized 16 h at 45°C and the arrays were scanned at a Affymetrix 3000 7G scanner. The raw data were RMA

normalized using the Affymetrix Expression Console Software ver 4.0.

Transcriptome analysis. Global gene expression analysis was conducted using Affymetrix GeneChip® Human Transcriptome Array HTA 2.0. containing 70.523 gene-level probe sets to detect known genes and ESTs, providing nearly a coverage of 285.000 full-length transcripts covered (>245.000 coding transcripts, 40.000 non coding transcripts, 339.000 probe sets covering exon-exon junctions) the expressed genes in the human genome (performed by AROS Applied Biotechnology A/S, Aarhus, DK). The array data were normalized using Robust Multichip Average (RMA) normalization as recommended by Bolstad et al. (Boldstad, Irizarry, Astrand, & al., 2003). Significance of DEG (2-fold change, $p < 0.05$) was assessed by ANOVA, and adjusted for multiple testing by estimating false discovery rates (FDR). Data visualisation, including principal component analysis (PCA), heat maps and clustering was performed in Qlucore Omics Explorer v.3.0 (Qlucore AB, Lund, Sweden). Functional analysis and network representation of DEG was performed in Ingenuity Pathway Analysis (IPA, Ingenuity® Systems).

RNA isolation, reverse transcriptase-PCR and qPCR. Total RNA was isolated using RNeasy Mini Kit (Qiagen) and cDNA was transcribed using the High Capacity cDNA Reverse Transcription Kit followed by PCR analysis using TaqMan® Gene Expression Assay method. All TaqMan probes were purchased by LifeTechnologies (GAPDH (Hs02758991_g1), FFAR2 (Hs00271142_s1), FFAR3 (Hs02519193_g1), HCAR2 (Hs02341584_s1), HCAR3 (Hs02341102_s1), IL6 (Hs00985639_m1), IL12B (Hs01011518_m1). Amplification was performed in an Mx3000P real-time thermal cycler

(Stratagene) on standard settings. Data presented here was obtained from three independent experiments. Each experiment included three technical replicates. Results are presented as relative quantity to the control sample determined by the ddCt method, using GAPDH as reference gene and untreated im-DC as calibrator.

4.3. III part: Immune system reconstitution

Patient features. Twenty three pediatric patients (9 males), with age ranging from 2 to 18 years (mean age 9 years), who underwent HSCT for onco-hematological disorders (16 subjects) and for hematological disorders (7 subjects), were enrolled at the Pediatric Bone Marrow Transplantation Unit of the Sant'Orsola-Malpighi Hospital, Bologna, Italy. After provision of parent's informed consent, the study has been approved by the Ethical Committee of the Sant'Orsola-Malpighi Hospital-University of Bologna (ref. number. 19/2013/U/Tess. 16 children were diagnosed with onco-hematological disease and 7 of them hematological diseases. 9 children were affected by aGvHD and the disease was graded according to the Seattle grading system, 15 were alive without any evidences of disease during the period of 100 days considered, 5 died before blood was sampled and for 3 of them the relapse of disease occurred. Demographic and general clinical characteristics of the patients are summarized in Table 2.

Patients enrolled, no.	23
Age at transplantation, years (median, range)	9 (2-18)
Sex (M= male, F= female)	9 M, 14 F
Underlaying disease, no. (%)	
Onco-hematologic diseases no. (%)	16 (69,6%)
LAL	13
LAM	3
Hematologic diseases no. (%)	7 (30,4%)
BDA	2
Talassemia major	1
ICL	1
Conditioning regimen no.	
TBI	21
Bu based	17
w/o Bu	2
w/o Bu + TBI	3
HCT type no. (%)	
Sibling	9 (39,1%)
MUD	15 (65,2%)
HLA Match	15 (65,2%)
HLA Mismatch	9 (39,1%)
Graft source no. (%)	
Bone marrow	21 (91,3%)
Cord Blood	1 (4,3%)
Peripheral blood (CD34+)	1 (4,3%)
GvHD no. (%)	9 (39,1%)
grade I-II	6
grade III-IV	3
Prophilaxys treatment GvHD no.	
ATG	15
CSA	22
cortisone	3
MTX	14
GvHD therapy no.	
Cortisone	9
PHEC	3
Infliximab	2
Infections/viral activation	
CMV reactivation	12
EBV reactivation	6
Adenovirus Reacivation	1
Sepsi	3
Fungal infection	1
Outcome no. (%)	
ANED	15 (65,2%)
Dead	5 (21,7%)
Relapse's disease	3 (13%)

Table 2: Anagraphical and general clinical informations of the enrolled patients - II part.
Footnotes: F: female, M: Male, AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, BDA: Blackfan Diamond anemia, ICL: idiopatic lymphocytopenia; Bu: busulfan ; TBI: total body irradiation; MUD: match unrelated donor, HLA: human leukocyte antigens; BM: bone marrow, Bu: busulfan, ATG: anti-thymocyte globulin, CSA: cyclosporine, MTX: methotrexate, PHEC: extracorporeal phoaferes, GvHD: Graft verus Host Disease, CMV: cytomegalovirus; EBV: Epstein Barr virus, ANED: alive no vidence of disease.

Lymphocytes population phenotyping. Whole peripheral blood (PB) samples were obtained by venipuncture and were collected every ten days, when it has been possible. Each PB sample was received in heparinized tube (BD Vacutainer®) and used immediately in assays for the leucocyte counting, determined by SIEMENS ADVIA® 2120 Hematology System. PBs were afterwards used for the immunophenotyping using a different mixes of monoclonal human-antibodies coniugated in different colors to detect all the subsets (all purchased from BD Biosciences): helper T cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+), naive T cells (CDRA+CD4+/CD8+), memory T cells (CDR0+CD4+/CD8+), B cells (pan CD19+), natural killer (NK) cells (CD3-CD16+CD56+). After 20 minutes of staining the samples were treated with lysis buffer (custumized by S.Orsola-Malphighi Hospital Pharmacy) to reduce the red cells debris during the flow cytometry detection.

In order to detect $CD4^{+}CD25^{++}CD127^{-}FoxP3^{+}$ T regulatory cells (T_{reg}) and Th1, Th2, and Th17 has been used the protocols described in "T helper and T reg phenotyping" in I part: GM and SCFAs.

Stained cells were analyzed by flow cytometry on FACSCanto™ II (BD) and data were analyzed by FACSDiva software (BD). Prior to the analysis, PMT values were adjusted running unstained or unstimulated cells to exclude autofluorescence and to control the intensity of background. Absolute subset cell numbers were calculated by mutiplying the percentage of the indicated subset as obtained by flow cytomentry and absolute lymphocyte number as determined by the hematocrit.

Statistical analysis. Patients were subdivided according to the type of HCT and aGvHD onset. The average values of each subset for each time point were compared between among all four groups to detect statistically significant differences by the Mann-Whitney two-sided. Significant differences among groups of samples for each lymphocyte subsets were assessed by ANOVA repeated-measurements. Further in order to evaluate all the time points as affecting variables on the recovery was used two-way ANOVA test for all the groups.

All the charts and statistical tests were obtained and performed by GraphPad Prism 6.0.

5. Results, discussion and conclusions

5.1. I part: Results and discussion

5.1.1. GM and SCFAs variations in pediatric patients undergoing aHSCT

HSCT procedures temporarily disrupt diversity, individual signature and SCFA production of the gut microbiota.

The 16S rRNA gene-based phylogenetic profile of a total of 53 stool samples, with a minimum of 4 time points per subject (**Figure 6**), were analyzed by 454 pyrosequencing of the V4 hypervariable region. A total of 384,106 reads were obtained, for a mean of 162 7,243±4,557 high-quality reads per subject. Reads were clustered in 6,895 operational taxonomic 163 units (OTUs, i.e. groups of sequences referring to the same species) at 97% of identity.

In order to assess the overall impact of HSCT and all the standard associated procedures on the gut microbiota of pediatric patients we investigated the richness and diversity of the ecosystem in the pre-HSCT sample, in the first post-HSCT samples available (approximately corresponding to the engraftment date), and in the last available samples (as reference point for the patient complete recovery), which varies in sampling date due to differences in the individual path to recovery.

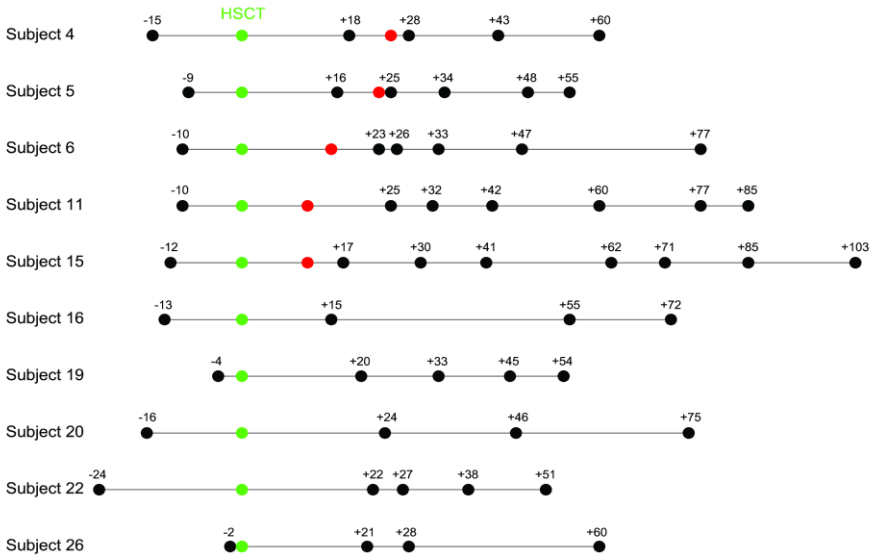

Figure 6: Clinical and sampling procedure. Schematic overview of the sampling time for each enrolled subject. HSCT (green), GVHD diagnosis (red) and fecal sample collection (black), with distance from HSCT expressed in days, are indicated.

We observed HSCT causes in all subjects a profound disturbance of the gut ecosystem that can be measured in a loss of the 30% in average of the pre-HSCT alpha diversity, expressed as Chao1 index for richness (mean ratio post-HSCT/pre-HSCT samples = 0.68, fdr corrected paired Mann-Whitney U test P = 0.0078). On the contrary, by comparing the alpha diversity of the pre-HSCT samples with the last samples available for each subject, it was evident that the ecosystem recovered the initial amount of diversity both in aGvHD and in non-aGvHD subjects (mean ratio = 0.94) (

Figure 7 A-B).

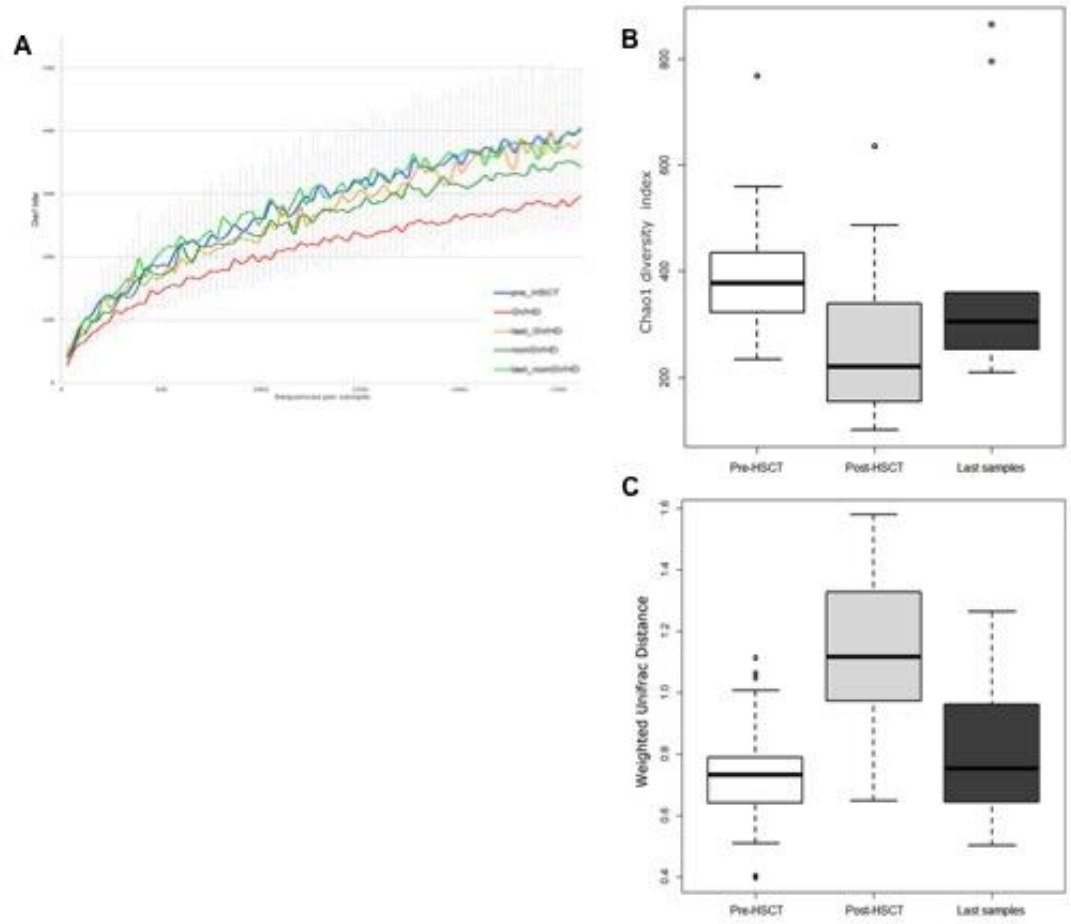

Figure 7: GVHD-related microbiota signatures. (A) Superimposition of the rarefaction curves of chao1 α-diversity metrics obtained for pre-HSCT samples (blue), GVHD samples (red), non-GVHD samples (dark green), as well as for the last available samples of the GVHD (orange) and non-GVHD (light green) subjects. Dynamics of intestinal ecosystem diversity and variability shown by Chao1 index (B), and the variability among subjects (beta-diversity) expressed as weighted Unifrac distance (C).

The mean value of weighted UniFrac distances among pre-HSCT samples was significantly lower than that obtained among post-HSCT samples (**P**<0.001), demonstrating an increase of the inter-subject gut microbiota diversity after transplantation. On the other hand, the last available samples showed a comparable degree of inter-subject diversity respect to pre-HSCT samples, further suggesting a progressive ecosystem recovery following HSCT (

Figure 7 C).

The dramatic events surrounding HSCT (immunological reconstruction above everything else, but also the prolonged fasting, antibiotic usage and other drugs administration) have been also associated to a loss of the individual signature of the GM composition.

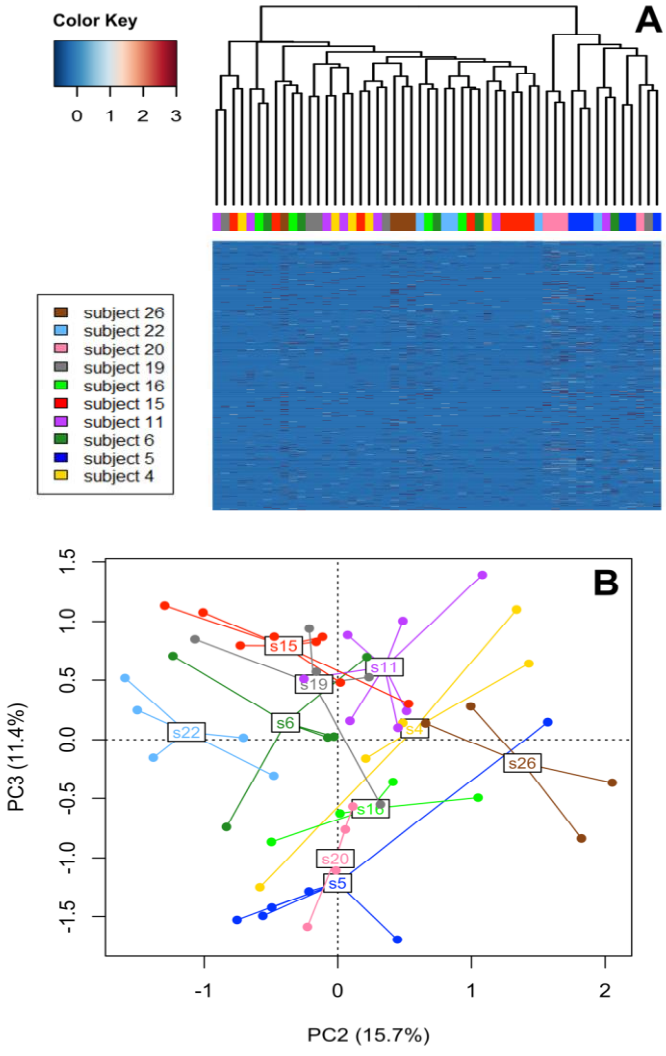

Figure 8: Loss of individual signature in gut microbiota composition. (A) Hierarchical Ward-linkage clustering based on the Eisen distance between OTU count profiles. (B) Weighted UniFrac distance PCoA of the fecal microbiota of all enrolled subjects. Percentage of variance in the dataset shown by the second and third principal component (PC) is reported.

In order to quantify the effect of this upsetting event on the ecosystem architecture, we quantified the loss and subsequent recovery of OTU detected. HSCT was associated to a loss of the individual signature of the gut microbiota composition - samples from the same subjects did not cluster together in multivariate analyses (Figure 8 A-B). Indeed, only 8.3±4.9% of the OTUs in the pre-HSCT samples were conserved in the post-HSCT samples, a very small number of which "survived" through the last available samples of each subject (2.3±2.6% of the OTUs in the pre-HSCT samples), mostly assigned to the phyla Firmicutes (66.7%) and Bacteroidetes (27.5%). A small percentage of OTUs was under the limit of detection in the post-HSCT samples but "reappeared" in the last available samples (5.9±4.7%); again, these sequences were assigned mostly to Firmicutes (73.6%).

The 89.3±4.6% of the OTUs in the post-HSCT samples was new with respect to the pre-HSCT ecosystem, but 80.6±9.9% of those was only transient (i.e. not present in the last available sample of each patient). The fecal microbiota of the last available sample of each subject (i.e. taken at least 51 days after HSCT) was made for 86.1±9.7% of OTUs that were not present in the pre-HSCT samples in both aGvHD and non-aGvHD patients (Figure 9).

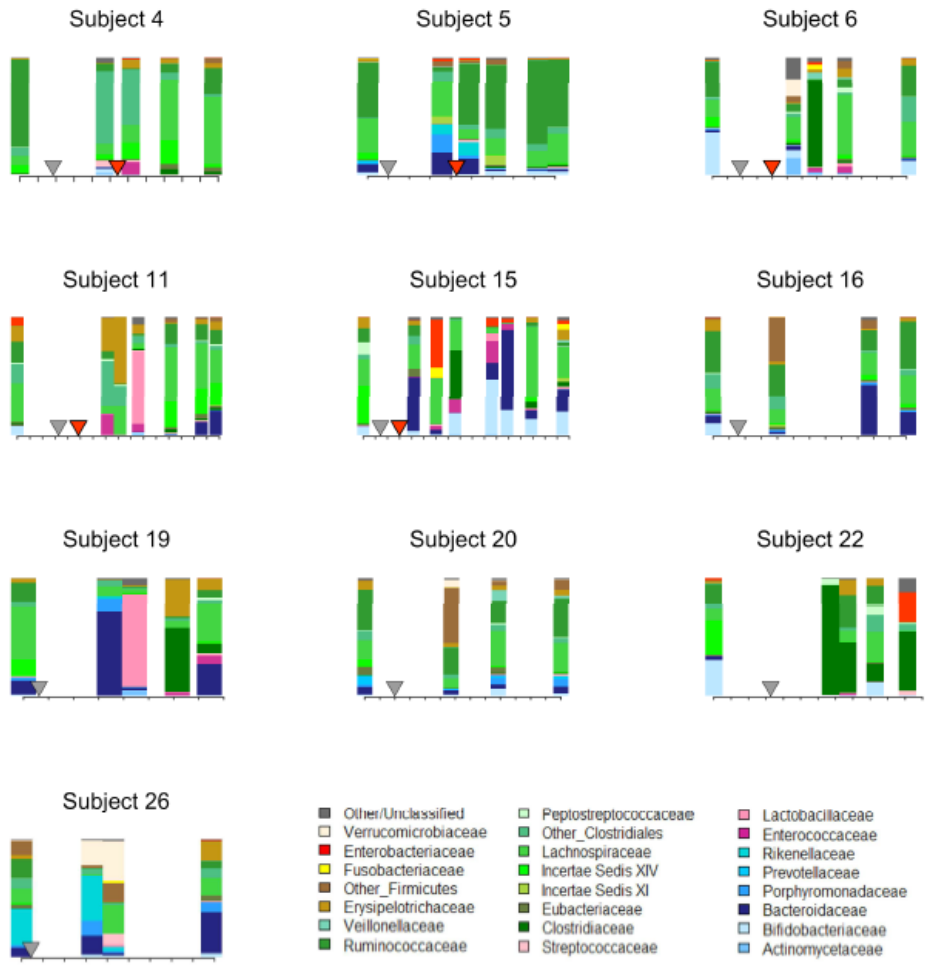

Figure 9: Individual trajectory of microbiota profiles at family level for each enrolled subject. Relative abundance profiles are plotted on a timeline (weeks) with indication of HSCT (grey triangle) and eventual aGvHD diagnosis (red triangle).

aGvHD-associated gut microbiota signatures

In order to highlight peculiarities of the post-HSCT reconstruction process of the gut microbiota in subjects developing and non-developing aGvHD, the respective gut microbiota temporal trajectories were constructed and compared. To this aim, samples were grouped in four time intervals: (I) pre-HSCT; (II) 0-35 days after HSCT (interval in which engraftment occurred

and, in our study, the diagnosis of the aGvHD was performed); (III) 35-65 days after HSCT; (IV) >65 days after HSCT.

PCoA analysis of the weighted UniFrac distances showed that samples from aGvHD subjects taken in the time interval between 0 and 35 days after HSCT, during which aGvHD emerged, clustered in the right part of the PCoA plot (**P**<0.05), showing that the aGvHD onset is associated to higher values on the PCo2 axis. This is even clearer when samples from each group are ordinated by PCo2 coordinates, as inspired by *Smith et al*. : aGvHD samples taken within the 0-35 days interval shows significantly higher values of PCo2 coordinates than all the other samples (P<0.05).

Interestingly, PCo2 was positively correlated (P<0.05) to members of the Enterococcus genus and unassigned Clostridiales, while Faecalibacterium and Ruminococcus showed a negative correlation with PCo2 (P<0.05). This suggests that aGvHD involves the overgrowth of Enterococcus and Clostridiales and a correspondent decrease of Faecalibacterium and Ruminococcus. Confirming these finding, Enterococcus members were found in significantly higher relative abundance in aGvHD samples, within 0-35 days after HSCT, with respect to non-aGvHD samples (median 1.9% for aGvHD, 0.01% for non-aGvHD; P=0.016); Enterococcus abundance in this time interval was also almost significantly higher than in pre-HSCT samples of aGvHD subjects (median 0.03%, **P**=0.06), confirming the association with the aGvHD diagnosis (data not showed).

An opposite trend was found for the known health-promoting Faecalibacterium in aGvHD subjects (median values: pre-HSCT 12.5%, 0-35 days 0.5%; **P**=0.06), but not for non-aGvHD subjects.

To better understand the capability of aGvHD to affect the overall structure of the microbiota, phylum-level temporal dynamics of gut microbiota in non-aGvHD and aGvHD subjects were constructed. Samples collected >65 days after HSCT were not taken into account in this analysis because they were available only for a small subset of subjects.

Different trends emerged for the two most abundant phyla in the intestinal ecosystem: Firmicutes and Bacteroidetes. aGvHD subjects showed a drop in Firmicutes abundance after HSCT, then they recovered higher abundances of Firmicutes than the initial ones; such distinctive trend was not observed in non-aGvHD subjects (Figure 10 A). More interesting, aGvHD subjects showed lower abundances of members of the Bacteroidetes in all the considered time interval than non-aGvHD subjects, and the difference was significant in pre-HSCT samples (median 0.11% for aGvHD, 14.8% for non-aGvHD; P=0.05) (Figure 10 B).

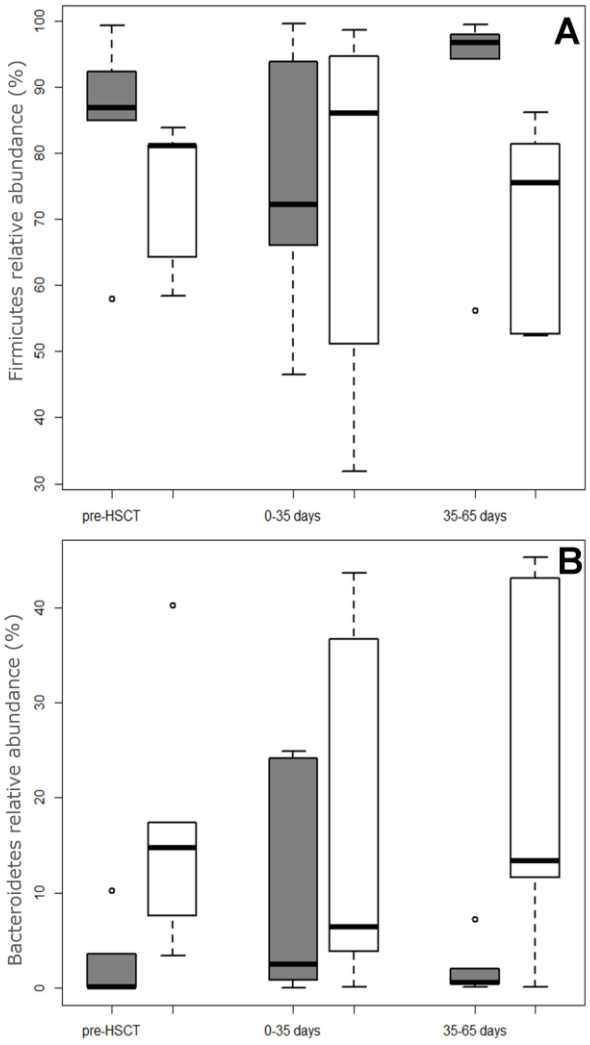

Figure 10: Phylum-level gut microbiota temporal dynamics. Box plots of relative abundance of Firmicutes (A) and Bacteroidetes (B) for aGvHD (grey) and non-GvHD (white) subjects at three time intervals (pre-HSCT, 0-35 days and 35-65 days after HSCT).

5.1.2. GM and SCFAs

In order to obtain an indication of the maintenance or disruption of the functionality of the intestinal ecosystem, we quantified the short chain fatty acids content in fecal samples. The fecal amount of short chain fatty acids decreased by 76% after HSCT in both aGvHD and non-aGvHD subjects (mean ratio post-HSCT/pre-HSCT 0.23, fdr-corrected paired Mann-Whitney U test P=0.016). Acetate and butyrate decreased by 64 and 77%, respectively (mean ratio post-HSCT/pre- HSCT 0.17 and 0.16, respectively; fdr-corrected paired Mann-Whitney U test P=0.023 and 0.011, respectively), while propionate was the most reduced, with a mean loss of 86% in the first post-HSCT sample with respect to the pre-HSCT (mean ratio post-HSCT/pre-HSCT 0.14, fdr-corrected paired Mann-Whitney U test P=0. 201 018). By comparing the short chain fatty acids amount in pre-HSCT samples with the last samples available for each subject, the gut microbiota seemed to recover the initial functionality both in terms of total short chain fatty acids (mean ratio 1.23; paired Mann-Whitney U test P=0.73) and propionate (mean ratio 1.09; paired Mann-Whitney U test P=0.82) production.

Indeed, the relative abundances of Bacteroides and Parabacteroides in the pre-HSCT samples were found significantly correlated with the amount of the immunomodulatory SCFA propionate (Kendall tau correlation coefficients: Bacteroides-propionate, 0.61, P = 0.028; Parabacteroides-propionate, 0.61, P = 0.025). Also, pre-HSCT samples in subject who did not develop aGvHD

tended to show higher amounts of both total SCFA (mean 4.3 µmol/g vs 2.5 µmol/g, P = 0.1) and propionate (mean 0.56 µmol/g vs 0.27 µmol/g, P = 0.06) (

Figure 11).

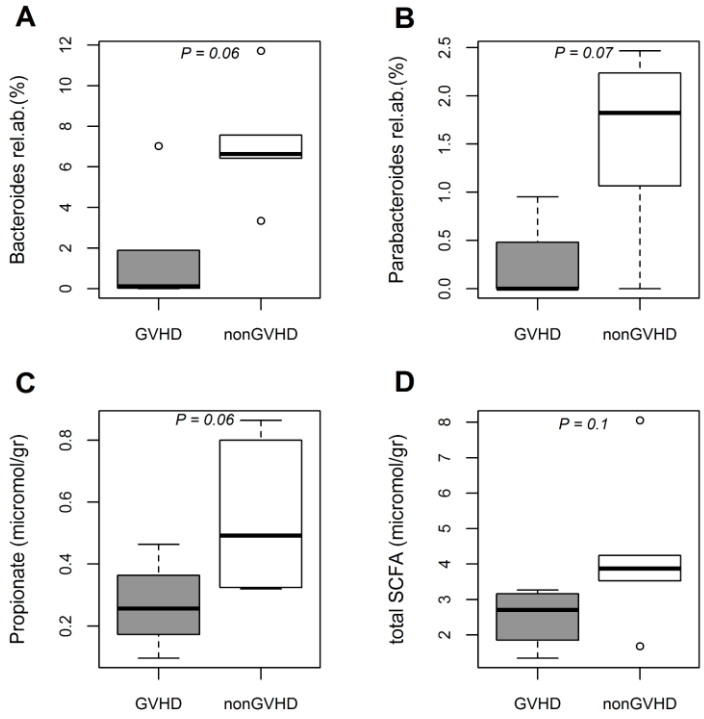

Figure 11: Pre-HSCT compositional and functional microbiota signatures that could exert a protective role against GVHD. (A) Pre-HSCT relative abundance of OTUs assigned to the genus *Bacteroides* in GVHD (grey) and non-GVHD (white) subjects. (B) Pre-HSCT relative abundance of OTUs assigned to the genus *Parabacteroides* in GVHD (grey) and non-GVHD (white) subjects. (C) Pre-HSCT fecal concentration of propionate (µmol/gr of feces) in GVHD (grey) and non-GVHD (white) subjects. (D) Pre-HSCT fecal concentration of total SCFA (µmol/gr of feces) in GVHD (grey) and non-GVHD (white) subjects.

5.1.3. The pre-HSCT gut ecosystem could influence the immune system.

Among the 53 available samples five groups were defined: (I) pre-HSCT samples of all subjects (n = 10); (II) non-GVHD samples, including all samples from the 5 non-GVHD patients with the exception of pre-HSCT samples and the last available sample of each subject (n = 12); (III) GVHD samples, including all samples from the 5 GVHD patients with the exception of the pre-HSCT samples and the last available sample of each subject (n = 21); (IV) last samples of non-GVHD subjects (n = 5); (V) last samples of GVHD subjects (n = 5).

Pre-HSCT samples were significantly enriched in OTUs assigned to the genera *Blautia* (mean 11% in pre-HSCT group, 0.8% in non-aGvHD, 4.5% in aGvHD; pre-HSCT vs. non-aGvHD, P < 0.01; pre-HSCT vs. aGvHD, P = 0.04) and *Faecalibacterium* (mean 17% in pre-HSCT group, 5.1% in non-aGvHD, 5.5% in aGvHD; pre-HSCT vs. non-aGvHD, P = 0.04; pre-HSCT vs. aGvHD, P < 0.01), with respect to both aGvHD and non-aGvHD samples. Analogous tendency was found for the genus *Roseburia* (mean 8.7% in pre-HSCT group, 0.7% in non-aGvHD, 2.9% in aGvHD; pre-HSCT vs. non-aGvHD, P = 0.09; pre-HSCT vs. aGvHD, P = 0.2). For these genera no significant differences were found between pre-HSCT and the last available samples of each subject, both in aGvHD and non-aGvHD cases. Both *Faecalibacterium* and *Blautia* tended to positively correlate with Treg and Th17 lymphocytes population counts in peripheral blood (Kendall *tau* coefficients: *Blautia*-Treg, 0.22, P = 0.03; *Blautia*-Th17, 0.24, P = 0.02; *Faecalibacterium*-Treg, 0.25; P

= 0.01). Moreover, *Faecalibacterium* was the most frequent genus among those who decreased until under the detection limit after HSCT but were present in the last available samples of each subjects (17.1% of the "reappearing" OTUs, in 8 out of 10 subjects).

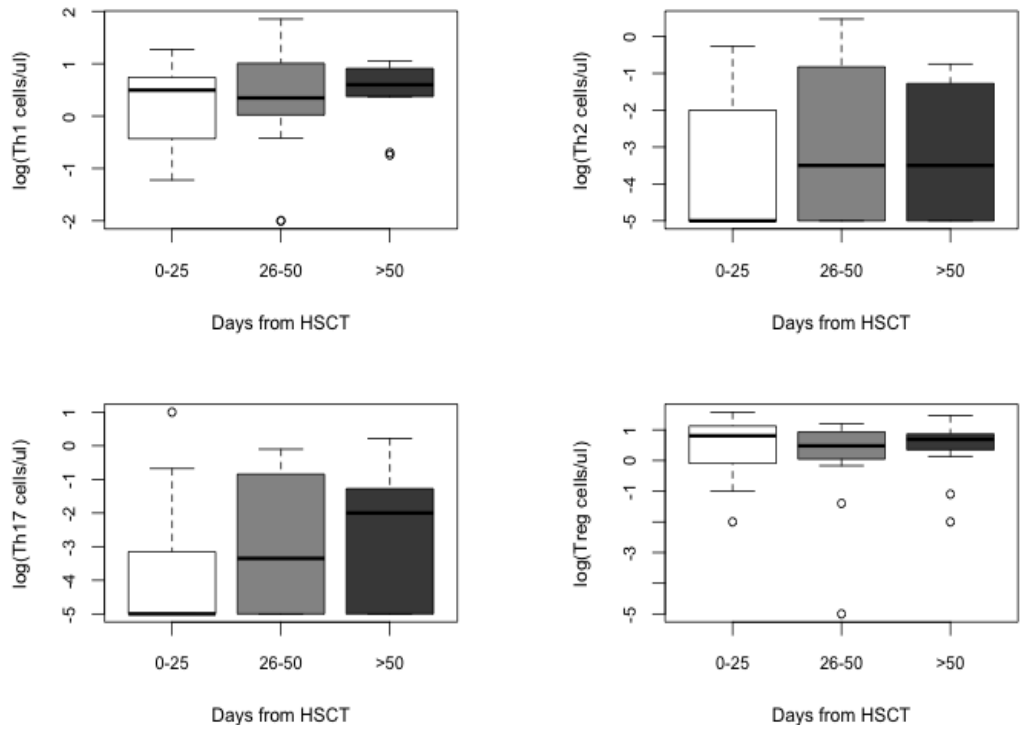

Figure 12: Early recovery of lymphocytes populations in peripheral blood after HSCT. Box plot of the Th1, Th2, Th17 and Treg count binned in 3 intervals, from day 0 to 25 (white), from day 26 to 50 (light grey) and from 50 to 100 (dark grey).

5.2. I part: Conclusions

In this study we demonstrated, to our knowledge for the first time, that in pediatric patients allogeneic HSCT is associated to a profound modification of the gut bacterial ecosystem with a disruption of its mutualistic asset. The pre-HSCT GM structure of the enrolled subjects well approximated the commonly reported healthy-like profile (Eckburg, Bik, Bernstein, & al., 2005) (Dethlefsen, Huse, Sogin, & al., 2008), in terms of diversity and relative abundance of the most abundant Firmicutes, Bacteroidetes and Actinobacteria families. Moreover, the pre-HSCT GM was found to be efficient in terms of SCFA production, which is the biomarker of GM functionality (Tremaroli & Bachked, 2012). After HSCT, we witnessed a disruption of the gut ecosystem with only a very small percentage of conserved OTUs and an "invasion" of new OTUs. This deep modification was accompanied by a marked reduction in the ecosystem diversity, confirming the available literature on adult patients (Holler, Butzhammer, Schid, & al., 2014) (Taur, Xavier, Lipuma, & al., 2012), and a damage in the mutualistic layout of the microbiota, as shown by the impaired transgenomic host-microbiota metabolism – here measured as the ability of the gut microbiota to produce SCFA– found in post-HSCT samples. These dramatic shifts bring to a loss of the individual fingerprint, with samples from the same subject that are not more compositionally similar to each other than samples from different patients. The great majority of the newly acquired phylotypes is only

temporarily invading the ecosystem and is replaced later on during the ecosystem recovery.

After approximately 2 months from the day of HSCT, the ecosystem recovers the initial richness and metabolic capability, as well as a phylogenetic architecture that mirrors a healthy-like asset, as demonstrated by the ability of well-known gut mutualists, such as *Faecalibacterium*, *Roseburia* and *Blautia*, to regain abundances comparable to the pre-HSCT values after the strong HSCT-induced decrease. Interestingly, a small percentage of OTUs resisted HSCT and are conserved throughout the whole longitudinal survey, or lower their abundance under the detection limit as a consequence of the ecosystem upsetting and "reappeared" later on. These persisting species could act as "founders" to reconstruct a healthy-like ecosystem once conditions allow. Indeed, *Bacteroides* species, that were the most represented among the persisting ones, are known to be able to penetrate the colonic mucus and reside deep within crypt channels where they act as a bacterial reservoir to maintain long-term colonization or repopulate the gut after ecosystem disruption (Lee, Donaldson, Mikulski, & al., 2013). Moreover, they are recognized as the most stable members of GM over lifetime (Faith, Guruge, Charbonneau, & al., 2013), probably thanks to their greater adaptability to different energy sources with respect to other symbionts, and are known to be able to utilize mucins as a fermentation substrate (Fisbach & Sonnenburg, 2011), an ability that could be of use during the prolonged post-HSCT fasting period. Finally, Bacteroidetes were found to be only marginally affected by chemotherapy in oncology patients

(Zwielehner, Lassl, Hippe, & al., 2011), confirming their resilience and capability of niche adjustment even in prohibitive conditions.

According to our data, aGvHD and non-aGvHD subjects showed differences in the process of gut microbiota recovery. aGvHD samples showed a lower diversity, confirming recent data obtained in adults and mice (Jenq, Ubeda, & Taur, 2012), and higher abundances of unassigned OTUs, that could be indicative of a higher invasion of the ecosystem by opportunistic bacteria. Moreover, aGvHD samples were characterized by higher abundances of *Granulicatella* and *Enterococcus,* the latter confirmed in adults by Holler et al. (Holler, Butzhammer, Schid, & al., 2014).

On the contrary, non-aGvHD subjects showed a higher abundance of members of the phylum Bacteroidetes (*Bacteroides* and *Parabacteroides*) not only after HSCT but also in the pre-HSCT composition, letting hypothesize that these gut persisters can somehow contribute in protecting from aGvHD. Indeed, these microbes are known to produce propionate from dietary fiber (Fisbach & Sonnenburg, 2011), and were correlated with the pre-HSCT fecal concentration of propionate in this study.

Propionate is generated in the gut but can enter the blood circulation and disseminate systemically, where it exerts immunomodulatory functions, such as promotion of extrathymic Treg generation and homing to the gut (Arpaia, Campbell, Fan, & al., 2013), as well as enhancement of hematopoiesis of dendritic cell precursors with a low ability to activate the allergy-related Th2 cells (Trompette, Gollwitzer, Yadava, & al, 2014), that might contribute to aGvHD protection.

The challenging nature of the enrollment of ill children, for whom parents' consent and strong motivation are required, and the difficulty to obtain compliance for the collection of fecal samples during a highly sensitive period of illness, limited the number of patients studied in the present paper, that should be considered as a pilot study in the field. Nonetheless, the relevance of this study, as well as the importance of future development of the same approach, become evident considering the increasing number of pediatric patients who can be cured by allogeneic HSCT and the relevance of aGvHD as a potential life-threatening event limiting this procedure (Dignan, Potter, Ethell, & al., 2013).

Our study indicates, with the needed caution, that the pre-existing GM structure can be protective against aGvHD onset, and we propose a model in which specific gut mutualist microorganisms, belonging to Bacteroidetes phylum and able to produce immunomodulatory protective metabolites, resist the ecosystem damages by HSCT and can act as founders for the ecosystem recovery, preventing the occurrence of future complications. In this scenario, it could be important to seek for strategies to reduce the probability of aGvHD in pediatric patients by manipulating the pre-HSCT dietary habits. Indeed, diet might be the most important determinant of the pre-HSCT microbiota composition and functionality, and indirectly control the production of immunomodulatory and protective metabolites, such as propionate (Biagi, Zama, Nastasi, & al., 2015).

5.3. II part: Results and discussion

5.3.1. Immature and mature DCs generation and SCFAs treatments

DCs were generated from monocytes isolated from peripheral blood mononuclear cells (PBMC) of three different healthy donors. The mononuclear cell fraction was isolated by Ficoll-Hypaque density gradient centrifugation. CD14+ monocytes were isolated from PBMC by negative selection using MACS magnetic beads and were cultured in media supplemented with GM-CSF (50ng/ml) and IL-4 (50 ng/ml) for 6 days. Immature dendritic cells (im-DCs) were treated with 1mM of sodium acetate (im-DC_A), or sodium butyrate (im-DC_B), or sodium propionate (im-DC_P) for the last 24h of culture in order to investigate the effect of the these three SCFAs on the maturation process; mature dendritic cells (m-DCs) were exposed to SCFAs in the same way and for the same time period together with lipopolysaccharide (LPS) (100ng/ml, *E.coli* 055:B5) obtaining the following treatments: m-DC_A, m-DC_B and m-DC_P; im-DC SCFA-untreated (im-DCs) and m-DCs SCFAs-untreated (m-DCs) were used as controls for the immature and mature state, respectively. The viability of DCs under the different treatments was examined, and no increase in non-viable cells numbers was observed when cells were exposed to 1 mM SCFA (Figure 13).

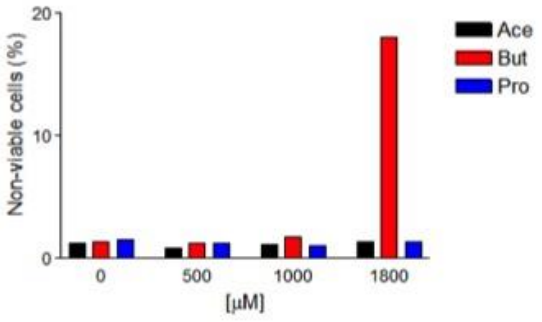

Figure 13: 7AAD vitality assay shows the percentage of non viable DC cells after each exposure at different concentrations with acetate or butyrate or propionate.

In order to validate the DCs maturation state have been performed flow cytometry analysis with particular attention on HLA-DR, CD83 and CD86, surface markers that are usually overexpressed only on the surface of m-DCs but not on im-DCs (Figure 14 A).

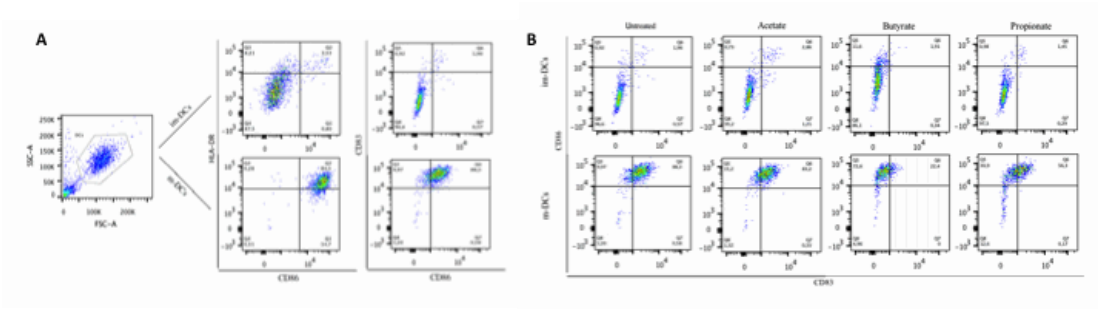

Figure 14: (A, B) Flow cytometry DCs gating and SCFAs effect. The figure is representative of the gating strategy for DCs for HLA-DR, CD83, and CD86 markers for both im-DCs and m-DCs. It also shown an example of SCFAs effect on im-DCs and m-DCs.

For all three donors monocytes-derived DCs has been checked for the maturation state through all the eight treatments considered to assess the reliability for the next steps. As expected, their surface expression was increased after the addition of LPS into the culturing media (Figure 14 A).

Next we investigate the impact of SCFAs on DCs maturation makers. Interestingly, the presence of SCFA did not affect the expression of either

HLA-DR or CD86, whereas the LPS-induced expression of CD83 was significantly reduced by the exposure to both sodium butyrate and sodium propionate when compared to normally matured DCs (unpaired t test with Welch's correction, p-value: 0.025 and 0.01, respectively) (Figure 14 B) (Figure 15).

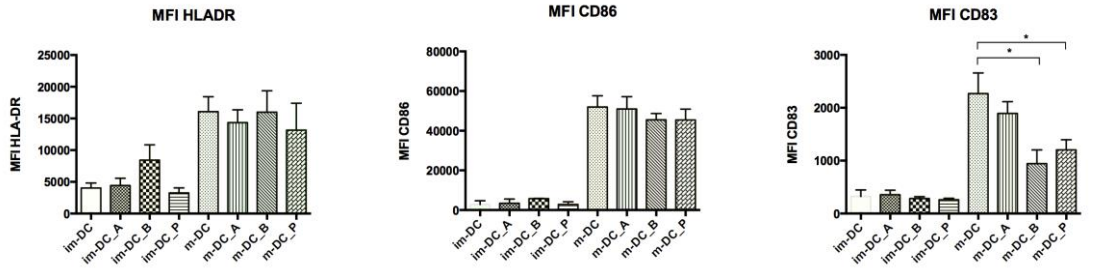

Figure 15: Rate of the DC's activation analyzed by FACS shown as mean fluorescence intensity (MFI). Each bar corresponds to the average between three different donors values ± SD; (*) P value<0.05.

5.3.1. SCFAs receptors expression by DCs

Several G-protein-coupled receptors (GPCRs), including Gpr41 (*FFAR3*), Gpr43 (*FFAR2*), Gpr109a (*HCAR2*), and Gpr109B (HCAR3) have been shown to be activated or sensitive to SCFAs (Soga, Kamohara, Takasaki, & al., 2003) (Taggart, Kero, Gan, & al, 2005) (Irukayama-Tomobe, Tanaka, Yokomizo, & al., 2009).

To determine the expression pattern of SCFAs receptors on human DCs, we performed qPCR assays for these genes using MCF-7 cell line as a positive control as it expresses high level of FFAR2 and much lower levels of FFAR3 gene (Yonezawa, Kobayashi, & Obara, 2007). As shown in Figure 16 A we found that basically human monocyte-derived im-DCs express FFAR2 and FFAR3 at lower and higher level, respectively, compared to the positive

control. In contrast we observed very low levels of HCAR2 and HCAR3 expression on MCF-7 cells and even higher for DCs. Interestingly, the expression of HCAR2 and HCAR3 is affected by the LPS stimulation thus that the m-DCs do not express anymore those receptors. Afterwards the treatments with butyrate or propionate we observed a recovery of these receptors expression, not observed after acetate exposure Figure 16 B. This evidence opens new perspectives on the regulation of SCFAs receptor expression on DCs respect other cellular types.

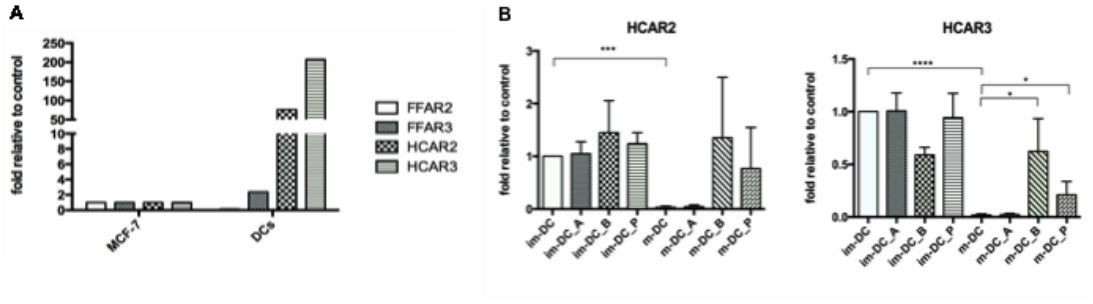

Figure 16: Gene expression of SCFAs receptors (A) FFAR2, FFAR3, HCAR2, and HCAR3 on MCF-7 and DCs and (B) HCAR2 and HCAR3 only on DCs un-/treated with acetate, propionate, or butyrate.

5.3.2. SCFAs effect on immature and mature DC.

In order to investigate the DCs gene expression profile have been used total-RNA extracted from the above mentioned samples and analyzed on Affymetrix GeneChip® Human Transcriptome Array HTA 2.0.

Were used 70.523 probe sets to detect known genes and ESTs, providing nearly a coverage of 285.000 full-length transcripts covered (>245.000

coding transcripts, 40.000 non coding transcripts, 339.000 probe sets covering exon-exon junctions) the expressed genes in the human genome.

The array data were normalized using Robust Multichip Average (RMA) normalization as recommended by Bolstad et al.

Significance of DEG (2-fold change, $p < 0.05$) was assessed by ANOVA, and adjusted for multiple testing by estimating false discovery rates (FDR). In order to characterize those genes specifically affected by SCFAs the transcription profiles have been normalized by subtracting the genes similarity affected by unconditioned culture medium from genes affected by SCFAs in the case of im-DCs, and by subtracting the gene similarity affected by LPS-conditioned medium from genes affected by SCFAs together a LPS-medium in the case of m-DCs. Then data were visualized in Qlucore Omics Explorer 3.0 (Qlucore AB), and network analysis was performed by Ingenuity Pathway Analysis (IPA, Qiagen).

Comparing untreated im-DCs to untreated m-DCs revealed 1752 differentially expressed genes (DEG) (cut off criteria: >2-fold change, $p<0.05$) due to the maturation stimuli, while exposure to SCFAs resulted in the modulation of the m-DC gene expression response to LPS and in a slightly variation of im-DC trascriptome (Figure 17).

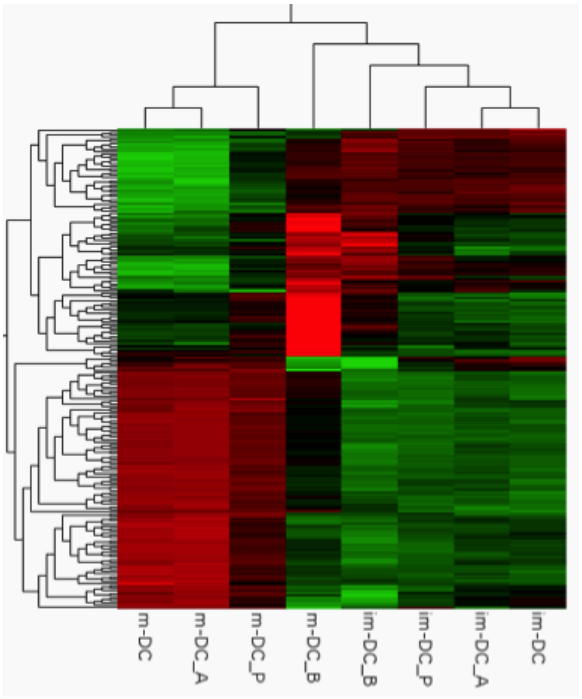

Figure 17: Heat-map and unsupervised hierarchical clustering based on the top 200 differentially expressed genes (DEG).

Visualization of the data by an unsupervised clustering model (principal component analysis) [PCA] further supported the specific and stronger effect of butyrate and propionate on the clustering of expressed genes of m-DCs. The same behavior was observed for im-DC, with an overall lower variation in the expression profile. In contrast, the effect of acetate treatment is similar to the one shown by untreated controls (both for im-DC and m-DCs) (Figure 18).

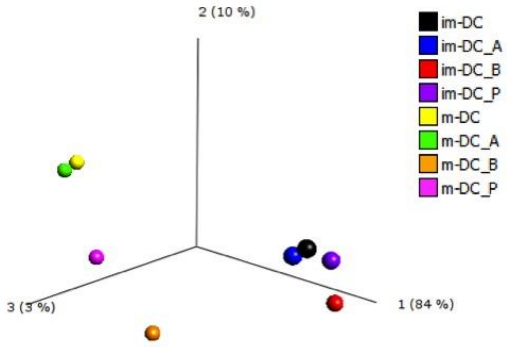

Figure 18: PCA representing the individual effect exerted by acetate, butyrate, and propionate on im-DC and m-DC among 737 most variables genes.

Indeed, the transcriptome analysis revealed that butyrate and propionate, more than acetate, elicit a specific change of response by both treated im-DCs, and m-DCs; the general effect exerted by SCFAs can be ranked as: acetate < propionate < butyrate. In fact, acetate only induced minor changes in gene expression (8 DEG) in im-DCs, and none in m-DCs.

Further, acetate seems not to share many affected genes with either butyrate (none and 3, up- and down-regulated genes, respectively) or propionate (2 and 0, respectively) in immature DCs. Regarding m-DCs, acetate treatment also has the same weak effect on mature DCs not sharing any genes in common with butyrate and propionate. Propionate affects few genes (14 up-, and 10 down- regulated) while butyrate shows the biggest effect up-regulating 467 and down-regulating 327 genes. Furthermore, butyrate and propionate treatments share the largest number of affected genes 233 up- and 44 down-regulated among all the comparisons mentioned above (Figure 19).

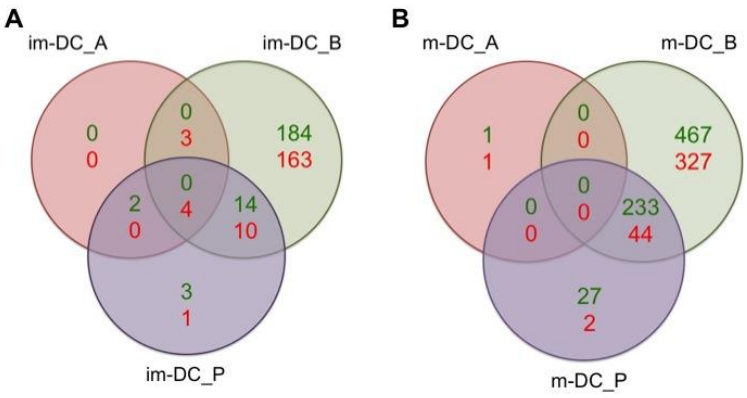

Figure 19: Venn diagrams representing the up-(green) and down-(red) regulated genes by im-DC and m-DC after exposure to A (acetate) or B (butyrate) or P (propionate).

Ingenuity pathway analysis (IPA) based on these DEG identified no significant networks or pathways involved by acetate treatments magnifying the effect exerted by butyrate and propionate. In fact, IPA analysis revealed "granulocytes adhesion and diapedesis" as one of the top altered canonical pathway involved by the exposure to butyrate, for both im-DC and m-DC; the same has been proposed by IPA regarding the effect of propionate shown only by m-DC. Indeed looking at the down-regulated genes have been shown that the expression of some of the chemokines have been affected by butyrate and propionate, especially in the mature stage of DCs (Table 3)**.** The top molecules down-regulated in the m-DC cells after butyrate treatment when compared to the untreated control are CXCL9 (-5.8), CXCL10 (-5.0), CXCL11 (-5.3), CCL19 (-5.4) (Table 4) and the top molecules that are affected by propionate treatment includes CXCL9 (-2.0), CCL19 (-1.7), IL-6 (-2.3), IL12B (-2.0) (Table 4) indicating an overall anti-inflammatory effect exerted by both SCFAs. Among the up-regulated genes by butyrate in both im-DCs and m-DCs we noticed Aldh1a (aldehyde dehydrogenase 1 family, member 1), that codes for an enzyme involved in metabolizing retinoic acid

(RA) and that exerts an immunomodulatory role in mice (Singh, Gurav, Sivaprakasam, & al, 2014).

Comparison	Top canonical pathway	P value	Top diseases and bio-functions	P value	num. molecules	Top networks	Score
m-DCs vs im-DCs	dendritic cell maturation	1.57E-16	immunological diseases	9.25E-39 - 6.83E-08	486	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	34
im-DCs_A vs im-DC	no effect						
im-DC_B vs im-DC	granulocyte adhesion and diapedesis; agranulocyte adhesion and diapedesis	3.48E-09; 5.63E-09	Inflammatory response	4.64E-16 - 2.81E-04	90	Antigen Presentation, Lipid Metabolism, Small Molecule Biochemistry	33
im-DCs_P vs im-DCs	eicosanoid signaling	1.15E-04	Inflammatoy response; hematological system development and function; cell-to-cell signaling and interaction	5.9E-08 - 1.16E-02; 2,44E-06 - 1.16E-02; 2.4E-06 - 1.2E-02	14; 15; 16	Cardiovascular Disease, Inflammatory Response, Cell-To-Cell Signaling and Interaction	32
m-DCs_A vs m-DCs	no effect						
m-DCs_B vs m-DCs	granulocyte adhesion and diapedesis; role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses; dendritic cell maturation	6.4E-13; 3,3E-10; 9.48E-10	Cell movement; Cellular function and maintenance; hematological system development and function	1.38E-25 - 2.9E-06; 4.88E-25 - 2.16E-06; 4.4E-27 - 2.73E-06	257; 284; 278	DNA Replication, Recombination, and Repair, Nucleic Acid Metabolism, Small Molecule Biochemistry	39
m-DCs_P vs m-DCs	granulocyte adhesion and diapedesis; dendritic cell maturation; graft-versus-host disease signaling	7.32E-12; 1.05E-10; 1.12E-07	Immunological disease; inflammatory response; cell-to-cell signaling and interaction; hematological system development and function	3.82E-20 - 1.40E-04; 4.33E-19 - 1.62E-04; 4.04E-19 - 1.9E-04;1.76E-19 - 1.9E-04	102; 118; 116; 117	Cellular Function and Maintenance, Cellular Development, Hematological System Development and Function	30
m-DCs_B vs m-DC_A	granulocyte adhesion and diapedesis; role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses; dendritic	6.7E-13; 9.62E-11; 4.93E-09	Inflammatory response; Cellular function and maintenance; hematological system development and function	8.31E-24 - 1.14E-06; 1.22E-27 - 7.33E-07; 7.69E-31 - 1.23E-06	275; 278; 268	Cell Morphology, Cellular Development, Embryonic Development	38

	cell maturation						
m-DCs_P vs m-DC_A	granulocyte adhesion and diapedesis; dendrititc cell maturation; Communication between Innate and Adaptive Immune Cells	2.11E-13; 1.47E-08; 2.17E-08	Immunological disease; Cell-to-cell signaling and interaction; immune cell trafficking; hematological system development	6.04E-22 - 1.15E-04; 2.72E-18 - 1.49E-04; 1.44E-19 - 1.5E-04; 1.48E-18 - 1.5E-04	88; 95; 76; 100	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	30
m-DCs_P vs m-DC_B	granulocyte adhesion and diapedesis; agranulocyte adhesion and diapedesis	6.21E-11; 1.52E-10	Immunological disease; Cell-to-cell signaling and interaction; hematological system development	2.57E-24 - 4E-05; 2.54E-16 - 7E-05; 2.54E-16 - 6.8E-05	88;75;77	Antimicrobial Response, Inflammatory Response, Infectious Disease	37

Table 3: IPA analysis report relative to each comparison.

Comparison	Top molecules up-regulated	log ratio	Top molecules down-regulated	log ratio
m-DCs vs im-DCs	CXCL11, CXCL10, CXCL9, IFI44L, IL2RA, IDO1..	7.6, 7.4, 7, 6.0, 5.5, 5.2	F13A1, SCN9A, CLEC10A, MRC1, CD209..	-5.7, -5.4, -5.0, -5.0, -4.5
imDCs_A vs im-DC	CXCL10, CCL2, CD40, IL12B, IL1A..	1.6, 0.6, 0.5, 0.5, 0.4	PTPLB, TLR7, TLR8..	-1.0, -0.5, -0.3
imDC_B vs im-DC	MIR3143, mir-146, IL1B, IL3RA,ALDH1A1..	3.5, 3,3, 3.0, 3.0, 2.5	NFXL1, PRR11, PTGER3, MAP2K6, CXCL13..	-3.3, -2.2, -2.2, -2.2, -2.0
imDCs_P vs im-DCs	CCR2, LRP1, CCL1, CXCL10..	1.8, 1.7, 1.6, 1.5	NFXL1, IFI6, PRR11, PTGER3..	-1.5, -1.5, -1.2, -1.2
mDCs_A vs mDCs	--	--	--	--
mDCs_B vs mDCs	MRC1, STEAP4, MPEG1, IL1A, CD36, IL1RAP, HCAR2,ALDH1A1..	4.8, 4.6, 4.2, 4.0, 3.8, 3.7, 3.5, 2.3	CXCL9, CXCL11, CXCL10, CCL19, GBP4, IL12B, HERC6, BRIP1, ANKRD22, CYP7B1..	-5.8, -5.3, -5.0, -4.0, -4.0, -3.4, -3.4, -3.3, -3.0, -3.0
mDCs_P vs mDCs	CD207, MRC1, CCL1, IL1RAP	3.3, 3.2, 3.2, 3.0	TNFSF18, IL6, CXCL9, IL12B, TNFSF4, HERC6, CCL19..	-2.6, -2.3, -2.0, -2.0, -2.0, -1.7, -1.7
mDCs_B vs mDC_A	STEAP4, MRC1, IL1A, CD36, MPEG1, IL1RAP, HCAR2, HCAR3	4.5, 4.5, 4.0, 4.0, 3.6, 3.5, 3.4, 3.3	CXCL9, CXCL11, CXCL10, CCL19, HERC6, IL12B..	-5.5, -5.3, -5.2, -4.0, -3.3, -3.3
mDCs_P vs mDC_A	CCL1, MRC1, CD207, IL1RAP, HCAR2, CCR1	3.0, 2.8, 2.8, 2.7, 2.3, 2.3	CXCL9, IL6, IL12B, TNFSF18, GBP4, TNFSF4, HERC6, CCL19..	-2.0, -1.9, -1.7, -1.7, -1.6, -1.6, -1.6
mDCs_P vs mDC_B	CXCL10, CXCL11, CXCL9, ANKRD22, CCL13, CCL2, CCL19	3.8, 3.8, 3.4, 2.8, 2.55, 2.5, 2.4	IL1A, GATM, CD70, STEAMP4, MMP10..	-3.0, -2.6, -2.0, -2.0, -2.0

Table 4: Top molecules up- and down-regulated shown by each comparison and relative log ratio.

5.3.3. Immunomodulatory effects of butyrate and propionate on m-DCs.

The transcriptome analysis revealed that butyrate and propionate, but not acetate, exert an immunomodulating effect on mature DCs. For LPS-treated DCs the butyrate effect is most pronounced in "normalizing" the gene expression levels back to those observed in the PBS-treated DCs down-regulating the *CXCL9, CXCL10, CXCL11*, and *IL12B* genes. The propionate effect is similar but not as strong as the butyrate one and likewise it decreases the expression of *CXCL9, IL6* and *IL12B*. To assess the microarray data we performed qPCR for *IL6* and *IL12B* and ELISA assays for IL6. Both butyrate and propionate significantly reduced LPS-induced IL-6 mRNA expression (P-value <0.05) (Figure 20 A), whereas the effect of on IL-12B gene expression was significant (P-value<0.05) only after butyrate treatment but not for propionate, as seen in (Figure 20 B).

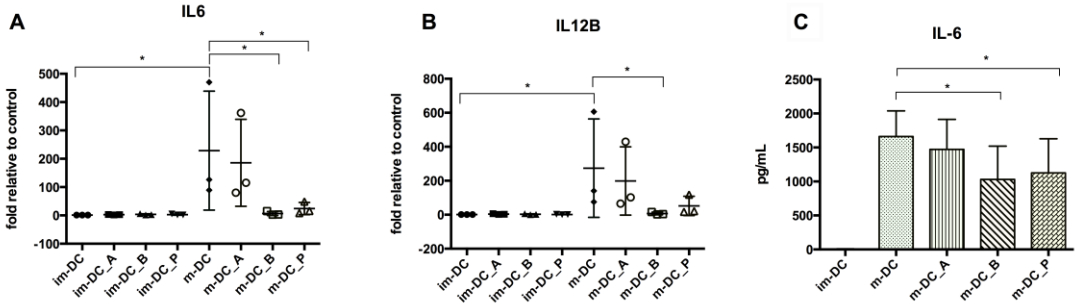

Figure 20: qPCR (A, B) and ELISA (C) profile by m-DCs treated with acetate, butyrate, propionate. Shown are the averages ± standard deviations (SD) (n=3); Mann-Whitney t test, *P < 0.05.

The effect on LPS-induced IL-6 was confirmed by ELISA for IL-6 release by

mature DCs exposed to butyrate and propionate as protein levels were significantly reduced (P-value < 0.05, respectively) (Figure 20 C).

Further, the chemokines secreted into the media were measured through LEGENDPlex™ array confirming a significant reduction of CXCL9, CXCL10, and CXCL11 production after butyrate and propionate exposure by m-DCs (Figure 21 PanelA).

In order to investigate deeper the effect of SCFAs on DCs chemokines pattern we measured ten chemokines by LEGENDplex™ array after the same treatments. This analysis revealed that im-DC are not responsive to the addiction of SCFAs instead of the mature DCs whose chemokines production change peculiarly depending on what SCFA is added into the media. As mentioned above, butyrate and propionate, but not acetate, significantly reduce the secretion of CXCL9, -10, -11 by m-DCs (Figure 21 Panel A). Further, CCL3 release is significantly reduced by acetate and propionate, but not by butyrate; CCL4 is reduced only by butyrate but not by acetate neither by propionate; CCL5 production decreases after butyrate and propionate exposure but is not modified by acetate (Figure 21 Panel B). CCL2, CCL11, CXCL11 are not significant affected by SCFAs and CXCL5 is not produced at all by m-DCs (Figure 21 Panel C).

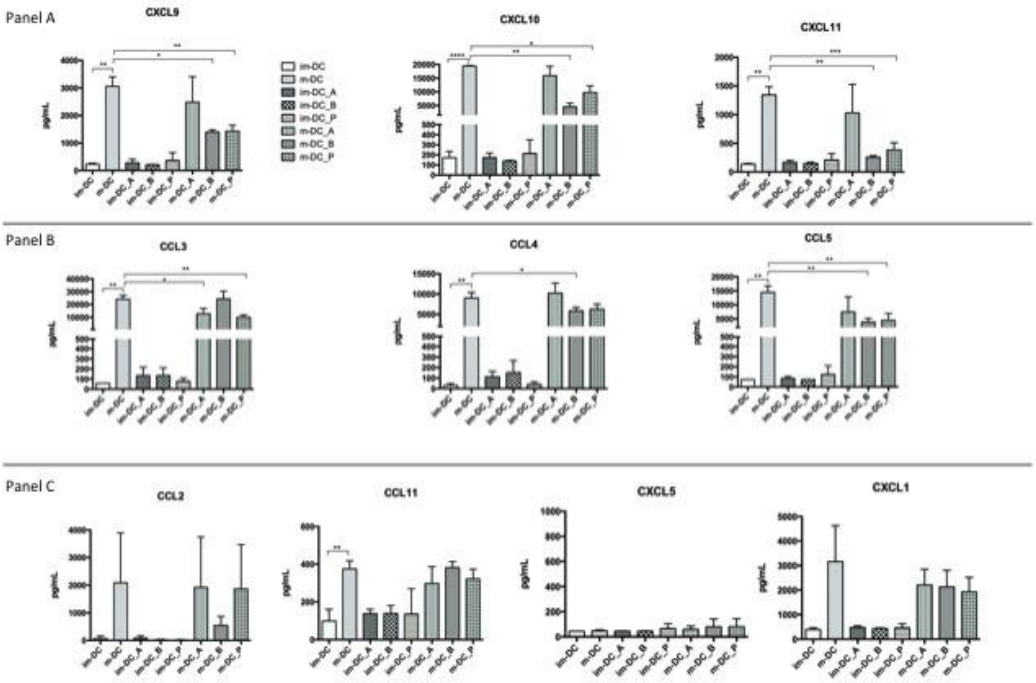

Figure 21: Chemokines production profile by im-DCs and m-DCs treated with acetate, butyrate, propionate. Shown are the averages ± standard deviations (SD) (n=3); Unpaired t test with Welch's correction, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

It's known that the chemokines CCL3, CCL4, CCL5 and CXCL10 are peculiarly secreted by m-DCs but not by imDC (Sallusto, Palermo, Lenig, & al, 1999) and from our assays has been seen the same trend confirming the maturation state of the DCs; interestingly, has been demonstrated that butyrate and proprionate affect on their secretion and thus on the normal maturation DC's chemokines pattern.

We hypothised that the reduction of CCL3, CCL4 and CCL5 mediated by butyric and proprionate acids may impact the normal maturation of DCs and hence their ability to modulate the T cells polarization.

5.4. II part: Conclusions

Human beings have been recently reconsidered as super-organisms in co-evolution with an immense microbial community living in the in the gastrointestinal tract (GIT), the human intestinal microbiota (Gill, Pop, Deboy, & al., 2006) (Ley, Hamady, Lozupone, & al, 2008). Its collective genome provides functional features that humans have not evolved by their own, and several of our metabolic, physiological and immunological features depend on the mutualistic association with our intestinal microbial community such as enhancing our digestive efficiency by degrading otherwise indigestible polysaccharides, influencing the synthesis and absorption of many nutrients and metabolites including short-chain fatty acids (Neish, 2009) (Lee & Marzamian, 2010). Further, the link between the immune system and the GIT microbial community is essential for the development, education and functionality of our immune system (Garrett, Gordon, & Glimcher, 2010) (Hooper & Macpherson, 2010). Dendritic cells behave as sentinels of the immune system and their function is to sample antigen in inflamed tissue and migrate to the local lymph nodes where they besides the presentation of these antigens to T naive cells also produces cytokines, thus influencing the polarization into different T-helper-cell subsets eg. Th1, Th2 or Th17.

In this study we hypothesized that SCFAs, secreted in to the gut and distributed through blood to tissues, have immunomodulatory effects on

human dendritic cells that, as APCs, have the ability to shape the immune system's response.

DCs use specific receptors to sense and respond to bacterial metabolites. According to the literature, one largely used mechanism is via metabolite-sensing GPCRs such as, GPR43, GPR41, and GPR109A, all of which can act as receptors for SCFAs. Indeed, many of the actions related to SCFAs, and linked to gut homeostasis, are ascribed to GPR43 and GPR109A that are also both expressed by inflammatory leukocytes (such as neutrophils and macrophages) and by Treg cells (Smith, Howitt, Panikov, & al, 2013) (Singh, Gurav, Sivaprakasam, & al, 2014). However, SCFAs effects are not restricted to GPR43 and GPR109a, given that a recent study established a role for propionate and the SCFA receptor GPR41 in the generation of macrophages and DC precursors (Trompette, Gollwitzer, Yadava, & al, 2014). Here we observed that GPR43 and GPR41 are not expressed by human im-DCs. Instead, these cells express relative high levels of GPR109A and GPR109B, indicating that these receptors more than the traditional ones, such as GPR41 and GRP43, can mediate the uptake by human DCs.

These observations made us propose a new hypothesis, namely that human DCs use different SCFA receptors compared to the other immune cells e.g. macrophages. This hypothesis is supported by the fact that GPR109A has recently emerged as a major regulator of gut homeostasis binding the SCFAs butyrate but also the tryptophan metabolite nicotinic acid which anti-inflammatory properties upon monocytes, macrophages and epitelial cells are

well known (Digby, Martinez, Jefferson, & al., 2012) (Gambhir, Ananth, Veeranan-Karmegam, & al., 2012).

Besides, the analysis of the transcriptome revealed that butyrate and propionate, more than acetate, elicit a specific change of response by human DCs; further, butyrate and propionate share the largest number of host dendritic cell genes that were affected by SCFAs exposure as well as has been showed by *Lokovac et al.* for the murine gut organoids model (Lukovac, Belzer, Pellis, & al., 2014).

Among these observations we focused on the potential immunomodulatory effect exerted by SCFAs on human DCs, previously reported on another type of APC cells such as bone marrow-derived macrophages in a murine model (Chang, Offermanns, & al., 2014). In fact, it is known that macrophages, once activated by LPS- stimuli, have the important role to prime and enhance the inflammasome producing large amounts of CCL2, TNF-α, IL-12p40 and IL-6; has been shown that mainly butyrate suppresses the production of those inflammatory mediators by monocytes and macrophages (Cox, Jackson, STanton, & al., 2009) (Fukae, Amasaki, Yamashita, & al., 2005) and further in one study seems to enhance the release of the anti-inflammatory cytokine IL-10 (Saemann, Bohmig, Osterreicher, & al, 2000). Accordingly with these results, we observed that among the SCFAs analyzed, only butyrate and propionate, but not acetate, play a crucial role in modulating immune responses on human dendritic cells. In particular, in our study, propionate shows the ability to reduce IL-6 expression and release, more than butyrate and both butyrate and propionate to reduce the

expression of IL-12B (IL12p40), a shared component for IL-12 and IL-23 formation (Oppmann, Lesley, Blom, & al., 2000). This latter data lets us to hypothesize that both SCFAs compromising the right IL-12 and IL-23 production could shape the T naive polarization reducing the pro-inflammatory Th1 and Th17 phenotypes and therefore favoring the balance towards anti-inflammatory populations such as Tregs, as already shown in mice (Arpaia, Campbell, Fan, & al., 2013). In addition, it also appears that the activity of butyrate and propionate are selective because they do not affect primary LPS response genes such as TNF-α and CCL2.

Another aspect of our study was to explore how the DCs chemokines pattern was influenced by SCFAs taken that the chemokine production is instrumental for DCs to regulate their own migratory capacities and to organize recruitment of different cell types for both the afferent and efferent limb of the immune response (Sallusto, Palermo, Lenig, & al, 1999). It is already known, in addition to their chemotactic effect on neutrophils, SCFAs also modulate production and release of chemokines and expression of adhesion molecules in neutrophils (Vinolo, Rodrigues, Hatanaka, & al, 2009) and endothelial cells (Miller, Zaloga, Hoggatt, & al, 2005), which may be relevant to their effect on leukocytes recruitment. We observed that in the first 24h after the exposure to SCFAs each of them showed a peculiar individual effect, acetate reduces the release of CCL3, butyrate decreases CCL4, CCL5 and CXCL9,-10,-11 and propionate CCL3, CCL5 and CXCL9,-10,-11 in terms of gene expression and protein production. Early on Sallusto et al. has shown that inflammatory chemokines, such as CCL3, CCL4, and CCL5,

can be induced during maturation/ activation of human monocyte-derived DCs (Sallusto, Palermo, Lenig, & al, 1999) and, together with the other pro-inflammatory chemokines CXCL9, CXCL10, CXCL11, our results strongly support the idea that SCFAs exert an immunomodulatory effect *per se* directly on DCs. It is likely that inflammatory chemokines regulate cell traffic within secondary lymphoid tissues and, thus, it may influence T cell development during antigen recognition (Moser & Loetscher, 2001).

We observed the up-regulation of ALDH1A1 gene in both im-DCs and m-DCs after butyrate treatments. Aldh1a1 is an enzyme involved in metabolizing retinoic acid that is also produced from Vitamin A and is crucial for the development of food-specific Fop3+ Tregs. The expression of Aldh1a1 is directly dependent on GPR109a and regulated by butyrate, as already observed in butyrate-treated mouse colonic DCs and macrophages (Singh, Gurav, Sivaprakasam, & al, 2014). Our data clarify that among the three SCFAs studied, butyrate and propionate exert the main immunomodulatory role directly influencing DCs in gene expression profile. These findings open a new perspective on the ability of SCFAs to modulate the human m-DCs response to an inflammatory stimulus and guide them to a fine regulation of leukocyte polarization and recruitment.

5.5. III part. Results and discussion

5.5.1. Lymphocytes subsets circulating in peripheral blood

The process of the early immune reconstitution of 23 pediatric patients was followed by measuring the kinetic of relapse of the twelve immune system's populations circulating in blood one time point before and subsequent after the transplantation, from the time of the engraftment (neutrophil and platelet engraftment were defined as occurring on the first of the three consecutive days on which the neutrophil level was $0.5 \bullet 10^9$/l or higher and the blood platelets were above $20 \bullet 10^9$/l, respectively) every ten days for three months, covering the time of the potential risk of aGvHD onset.

Some of the children enrolled at the beginning were excluded for lack of informed consent, incomplete sample collection, and sample not evaluable for technical reasons.

The challenging nature of the enrollment of all children, for whom parents consent and strong motivation required due to obtain compliance for the collection of blood samples during a highly sensitive period, limited the patients studied in this latter part of the thesis and it should considered one of the few ongoing studies about IS reconstitution involving children and collecting data among twelve different subsets.

Absolute counts of different lymphocytes subsets were determined in peripheral blood by flow cytometry (FACS CantoII, BD) after staining with

monoclonal antibodies for cell surface antigens or intracellular markers; have been considered several potential factors affecting the IS's speed of recovery including age, type of donor, diagnosis, conditioning, source of stem cells that can impact on final reconstitution.

Considering the small cohort of patients, has been evaluated to split the patients only regarding the type of transplantation (MUD vs. SIBLING) and the aGvHD onset (aGvHD vs. no-GvHD) for the analysis; in fact, had not been possible to consider other factors affecting the speed of the recovery because further subdivisions would have been carried out in even smaller subgroups not comparable between each other.

Owing to low total lymphocyte counts in the early phase of immune recovery post HSCT, we calculated the absolute counts of lymphocyte subsets and not their relative frequencies.

As expected, each subset has its peculiar trend (Figure 22, Figure 23):

Natural killer cells (NKs - CD16+CD56+), related to innate immunity, usually take 1-2 months to normalize, although NK are the first lymphocyte subset to return to a normal level just before 100 days (average cell/ul [range cell/ul]) (MUD: 317 [116-646] vs. Sib: 170 [13-437]) especially considering the MUD group.

B cells (pan CD19+), involved in adaptive immunity, are at very low values during the first 2 months following transplant and those cells that are present are of donor origin; they usually start to be detectable after 3-5 months and completely reconstitute at least 1-2 year post transplant.

T helper and cytotoxic cells (CD3+CD4+/CD8+) reconstitution is

markedly different than of B cells and it does not mimic ontogeny: memory/effector T cells originating from mature T cells present in the graft relatively more abundant than naive cells; it might be because the thymus is not completely mature in some children and also it can be damaged after the conditioning.

In general, given the lack of **naive T cells** (both CD4 and CD8), the T cell repertoire is limited and furthermore, for unknown reasons, peripheral expansion of **memory T cells** (CD45R0+) is slightly more effective for CD8 than CD4 considering the late reconstitution, as largely reported; instead, for example, looking at day 100 MUD group CD8+ (210[0-588]) vs CD4+ (174[64-388]) and no-GvHD group CD8+ (306[50-588]) vs. CD4+ (258[112-388]) it seems not possible to discriminate a clear predominance of memory CD8+ cells than CD4+ underlying that the two population are still unbalanced and their reconstitution requires more time to reach normal values.

T helper 1, 2 and 17 cells are very low or undetectable since the engraftment and during the first 100 days, their relapse clearly overcomes the period considered; in fact for all the four groups that have been considered, Th17 and Th2 cells/ul values do not increase during 100 days. Regarding the Th1 cells values there is not a strong difference between all the four groups: MUD (9[1-17]), Sib (2[0-6], aGvHD (4[1-9]), no-GvHD (7[0-17]).

Treg cells are relatively abundant respect the T helper cells 1 and 17, confirming their immunomodulatory role. Has been observed a significant

difference between aGvHD vs. noGvHD groups at 100 days' time point (3.5[1.35-5.71] vs. 43[12-99]) (Mann-Whitney test, p value<0.05) that may contribute to a better recovery in the patients and prevents the GvHD onset.

In general, the comparison between those two big groups analysis do not show a real and substantial difference among the lymphocytes subsets trends.

For each subset comparison, between the four groups considered, has also been performed the two-way ANOVA statistical test to assess whether, considering all the time points, a better reconstitution was due to the GvHD onset or the type of HCT; the analysis revealed that only the GvHD onset has a statistic impact (P value<0.05) on all the subset's reconstitutions trends but not the type of HSCT.

Furthermore if we not exclude the pre-transplantation values to the data set, the two-way ANOVA test reveals the time as one of the most important factor affecting on the reconstitution (p value<0.001); in the other hand, if we excluded those values only the factor "GvHD" seemed to affect on each trend. This latter observation is reasonable taken that any recovery *per se* requires time especially and considering that the pre-transplantation values, healthy-like, consistently increase the difference in average and standard deviation respect all the other values following the transplant.

We also noticed, among the four groups, that splitting our cohort in MUD/SIBLING groups leads to even enhance the standard deviation for each individual average value (st. dev. not shown in charts) making us think that it

is strongly necessary to enroll more patients to reduce the variability, in order to deeply analyze the type of transplantation as a influencing factor on the early IS's reconstitution.

Unfortunately, up to date, all the data we obtained did not lead us to extend the analysis in order to consider possible any correlation between IS subsets with infections and GvHD incidence or post-transplant morbidity and mortality.

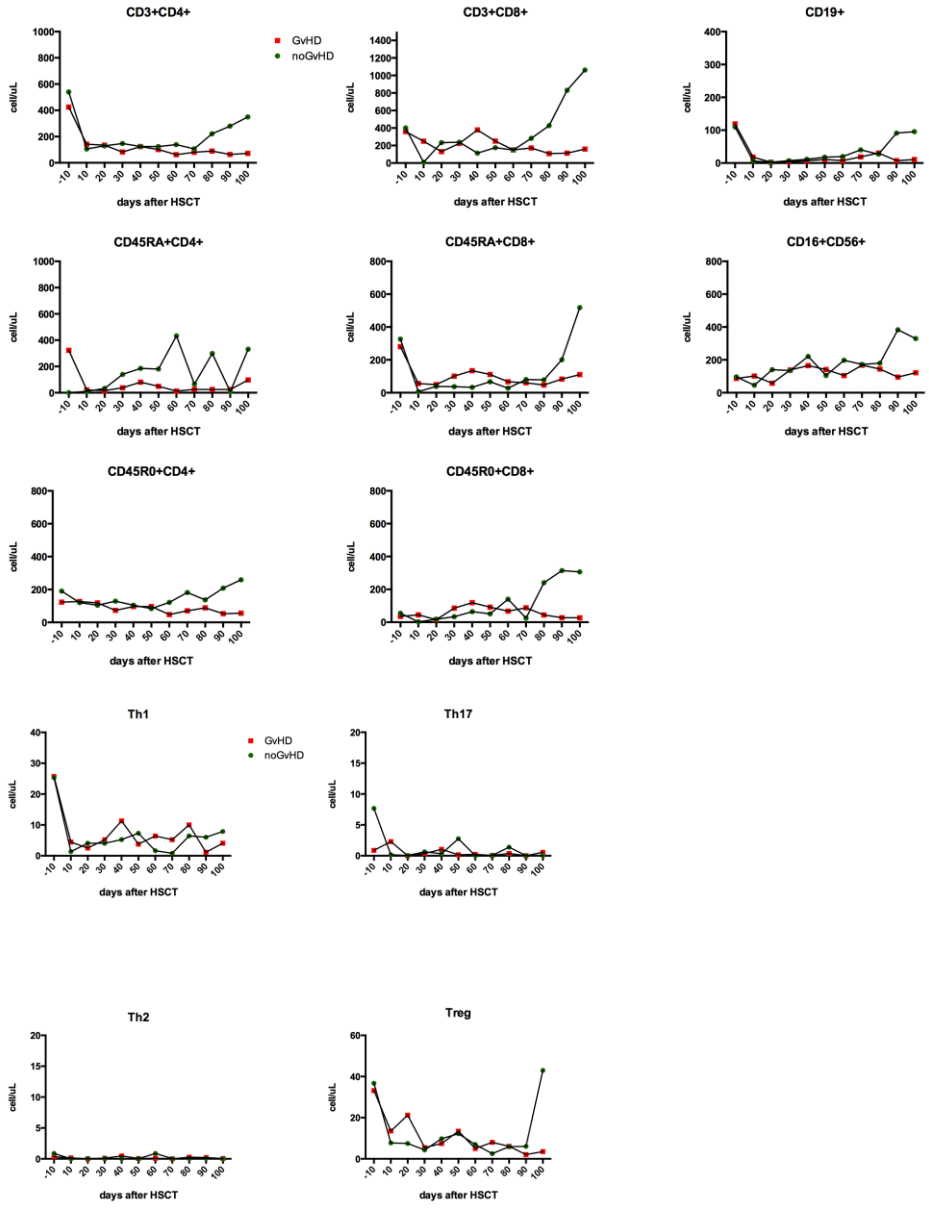

Figure 22: Lymphocytes trends among aGvHD and no GvHD groups. Averages of Absolute lymphocyte counts (ALC) are shown and connected by a time-line for 100 days after HSCT. Footnotes: T helper cells (CD3+CD4+), Cytotoxic T cells (CD3+CD8+), B cells (pan CD19+), naive T cells (CD45RA+ CD3+CD4+/CD8+), memory T cells (CD45R0+CD3+CD4+/CD8+), Th1 (CD3+CD4+IFN-γ), Th2 (CD3+CD4+IL-4+), Th17 (CD3+CD4+IL-17+), Treg (CD3+CD4+CD25++CD127-FoxP3+).

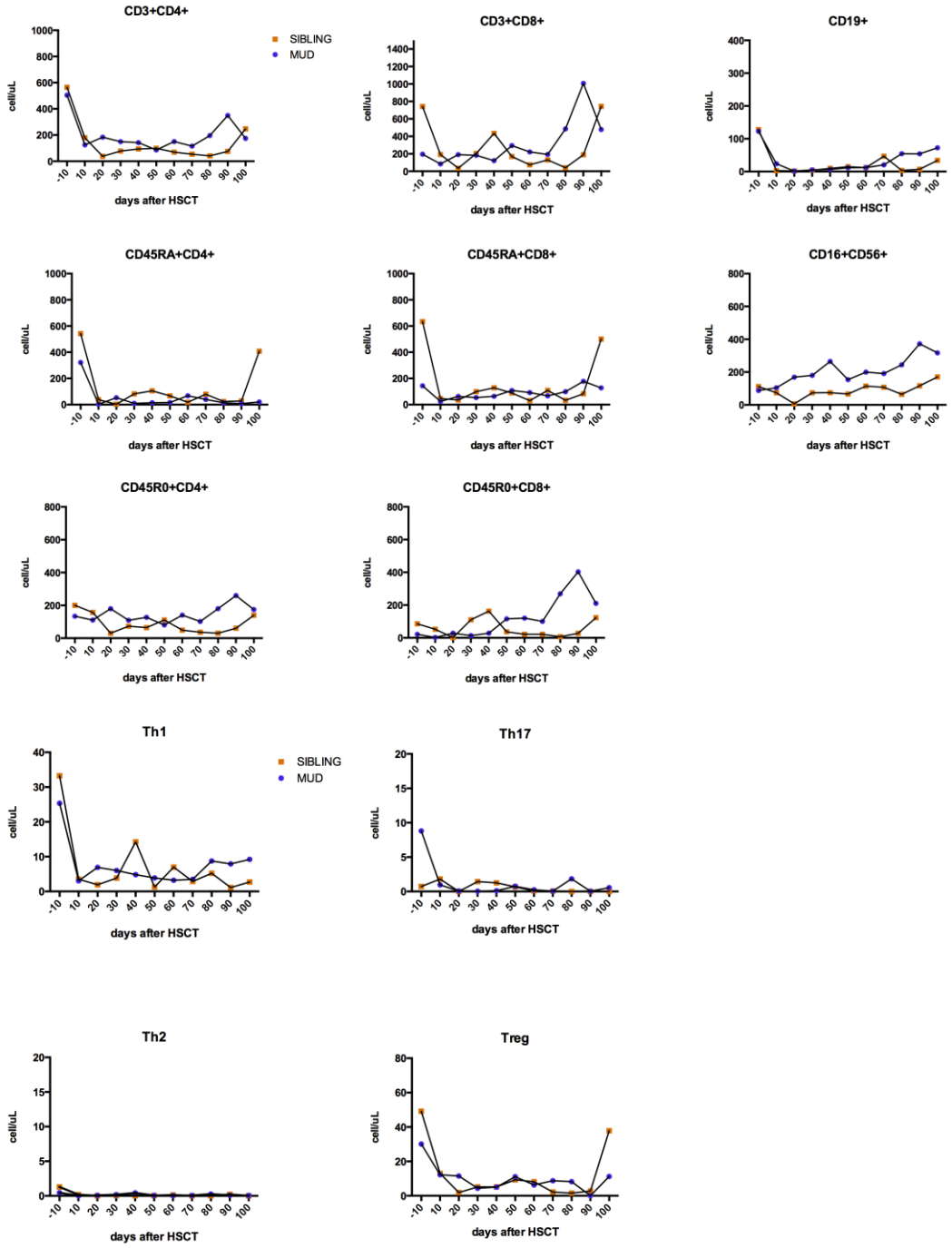

Figure 23: Lymphocytes trends among MUD and SIBLING groups. Averages of Absolute lymphocyte counts (ALC) are shown and connected by a time-line for 100 days after HSCT. Footnotes: T helper cells (CD3+CD4+), Cytotoxic T cells (CD3+CD8+), B cells (pan CD19+), naive T cells (CD45RA+ CD3+CD4+/CD8+), memory T cells (CD45R0+CD3+CD4+/CD8+), Th1 (CD3+CD4+IFN-γ), Th2 (CD3+CD4+IL-4+), Th17 (CD3+CD4+IL-17+), Treg (CD3+CD4+CD25++CD127-FoxP3+).

5.6. III part: Conclusions

A rapid immune reconstitution post-HSCT is of central importance to protect the patients from relapse and severe infections (Koel, Bochennek, Zimmermann, & al., 2007) (Kim, Kim, Sohn, & al., 2004) (Kalwak, Gorczynska, Toporski, & al., 2002). An adequate immune recovery can effectively eliminate a recipient's residual malignant cells in the early phase post SCT, therby reducing the risk of relapse (Powles, Singhal, Treleaven, & al, 1998). Patients with delayed immune reconstitution show an enhanced probability of relapse and late infections remain the major cause of non-relapse mortality (Fry & Mackall, 2005). In patients with impaired immune reconstitution the introduction of additional cell therapies may be helpful to augment the immune functions (Kim, Kim, Sohn, & al., 2004). To date, intervention in pediatric patients has mainly based on minimal residual disease diagnostic and donor/recipient chimerism (Bader, Niethammer, Willash, & al., 2005).

This aspect emphasizes the importance of studying the immune system reconstitution as a mirror of potential risks of complications that -with the appropriate therapies- could be avoided or prevented.

Adult patients show a very slow immune reconstitution reaching normal values 2-3 years post transplantation (Storek, Dawson, Storer, & al, 2001). This is due to the thymus, which rapidly involutes after puberty (Dumont-Girard, Roux, van Lier, & al., 1998) (Roux, Dumont-Girard, Starobinski, & al,

2000). In contrast, in children immune reconstitution, cell function and T-cell receptor repertoire recover much faster within the first one and half year post SCT (Kook, Goldman, al., & al., 1996) (Kook, Goldman, Giller, & al., 1997) (Kalwak, Gorczynska, Toporski, & al., 2002).

Among the factors known to influence immune reconstitution are the stem cell source, the amount of CD34+ cells and the content of CD3+ cells in the graft (Kook, Goldman, al., & al., 1996) (Kalwak, Gorczynska, Toporski, & al., 2002) (Roux, Dumont-Girard, Starobinski, & al, 2000) (Fallen, McGreavey, Madrigal, & al., 2003), the cytomegalovirus status before and after SCT (Kalwak, Gorczynska, Toporski, & al., 2002) (Storek, Dawson, Storer, & al, 2001) (Kook, Goldman, Giller, & al., 1997), the relationship and disparity between donor and recipient (Niehues, Rocha, Filipovich, & al., 2001) (Kalwak, Moson, Cwian, & al., 2003), the age of patients (Fallen, McGreavey, Madrigal, & al., 2003) (Kook, Goldman, Giller, & al., 1997), and the development of GvHD (Roux, Dumont-Girard, Starobinski, & al, 2000) (Kook, Goldman, Giller, & al., 1997).

Among all the factors already mentioned above we have considered the type of transplantation regarding the disparity between donor and recipient, and the development of GvHD; moreover we focused only at the early reconstitution, so evaluating the lymphocytes reconstitution during the first 100 days after the transplantation.

The limited period of time and the small number of patients enrolled forced to do not split further the cohort for subsequent analysis, so the main big four groups evaluated were: GvHD vs. no-GvHD and MUD vs. Sibling.

From the comparison between GvHD vs. no-GvHD and MUD vs. Sibling we observed that there were not real differences in each trends of recovery between all four groups suggesting that, up to date, our study is not enough abounding in patients in order to allow more analysis and statistical discrimination power.

Neverthless, we observed that each peripheral subset has its own trend and even if we have not obtained substancial statistical validation they showed a similar tendency already observed in literature.

We noticed that NK cell counts recover rapidly within 1 month post-transplantation, followed by cytotoxic CD3+CD8+ T cells 2-3 months and CD19+ B cells even after the period considered, as has already been shown by Koel, Bochennek, Zimmermann, & al., 2007. Initially, mostly of the cytotoxic T cells are memory CD45R0+CD8+, whereas the naive CD45RA+CD8+ regenerate in the second half year post SCT.

CD3+CD4+ T helper cells emerge very slow reaching normal values 6-10 months post SCT (Kook, Goldman, al., & al., 1996) (Kalwak, Gorczynska, Toporski, & al., 2002) (De Vries, Van Tol, Van der Bergh, & al., 2000) (Kim, Kim, Sohn, & al., 2004) (Koel, Bochennek, Zimmermann, & al., 2007) and as we observed Th1, Th2, Th17 and Treg cells were absent or barely detectable during the first 3 months.

6. General conclusions

Allogeneic HSCT is a potential curative therapy for children with hematologic disorders, primary immunodeficiency and metabolic disease. The main complications of HSCT are represented by infections, secondary malignances and GVHD, an immunological disorder which can be lethal and limits the use of this important procedure. GVHD arises when donor T cells respond to genetically defined protein expressed on host cells APC such as DCs.

Human beings share a close mutualistic relationship with the GM; in particular, the interaction with the gut microbiota GM is crucial for a balanced immune system development. Indeed, GM regulates the adaptive immune response, exerts a key role in the education of the immune system to tolerance and influence the functionality of the intestinal epithelium. Moreover, GM can impact the host susceptibility to enteropathogen infection by exerting the well-known barrier effect.

As an active component of the human immune system, the GM structure - and the resulting pattern of immunological interaction with the host – can be of primary importance in governing the fate of the immunological recovery in patients who undergoing aHSCT, predisposing or protecting from the aGVHD onset.

Following this hypothesis, by providing the first monitoring of the GM changes occurring during the early recovery of immune defenses in children undergoing allogeneic HSCT, the GM has been sequenced in ten enrolled children.

The GM composition's analysis have shown a peculiar trajectory of reconstruction of the individual microbiota profile after HSCT, which is temporary interrupted by the occurrence of GvHD; even though the strong clinical pathway (transplantation, pharmacological treatment, fasting, GvHD) the gut microbiota from different subjects tends to group with itself and, differently, the subject who developed intestinal aGvHD maintains his individuality and his distance from the others. For all the patients it has been shown that the GM regains its own composition similar to the one showed before transplantation.

We also showed that the pre-HSCT samples of the subjects who did not developed GvHD tended to show higher abundance of *Bacteroides* and *Parabacteroides*, with respect to the pre-HSCT samples of GvHD patients; further, the relative abundances of *Bacteroides* and *Parabacteroides* in the pre-HSCT samples were found significantly correlated with the amount of the immunomodulatory SCFA propionate. Also, pre-HSCT samples in subject who did not develop GvHD tended to show higher amounts of both total SCFAs and propionate suggesting that SCFAs may exert a protective role versus aGvHD onset.

Our study indicates, with the needed caution, that the pre-existing GM structure can be protective against aGvHD onset, and we propose a model in which specific gut mutualist microorganisms, belonging to Bacteroidetes phylum and able to produce immunomodulatory protective metabolites, resist the ecosystem damages by HSCT and can act as founders for the ecosystem recovery, preventing the occurrence of future complications.

In this scenario we hope that our study emphasizes the importance to seek new and less invasive strategies to reduce the probability of aGvHD in pediatric patients by manipulating the pre-HSCT dietary habits.

Taken the difficulty to find a valid biomarkers among the lymphocites subsets that might help routinely the clinicians to predict the GvHD onset (or other complications) and taken the lack of a statistic correlation between the immune system lymphocites's trends and the GM composition probably due to the high biological variables between different human beings or to the few patients considered in this study, we focused on *in vitro* experiments trying to find out a possible cross-talk or interactions between this two worlds considering the bacterial metabolites SCFAs and DCs cells as the main characters of this connection.

We observed that among the SCFAs analyzed, only butyrate and propionate, but not acetate, play a crucial role in modulating immune responses on human dendritic cells. In particular, in our study, propionate shows the ability to reduce IL-6 expression and release more than butyrate does, and both butyrate and propionate to reduce the expression of IL-12B (IL12p40), a shared component for IL-12 and IL-23 formation.

We also have demostrated that butyrate and proprionate, more than acetate, influence the DCs maturation process affecting the chemokines profile and the consequent DCs ability to shape the T cells polarization, that may be the way through SCFAs shape the immune system balancing between tolerogenic and inflammatory behaviour.

At the light of all these findings we think is interesting to emphasize a final aspect that might be promising for further studies. As mentioned in the second part of this thesis, we noticed a strong immunomodulatory effect exherted by both butyrate and propionate on the chemokine release CXCL9, CXCL10, CXCL10. Those chemokines were recently pointed as biomarkers of chronic GvHD because increased in the serum of patients with chronic skin manifestations (Croudace, Inman, Abbotts, & al., 2012) and especially the CXCL10, together with IL-6 and IL12, have been also considered as a candidates aGvHD biomarkers with diagnostic and prognostic significance (Piper, Horlock, Curnow, & al, 2007) (Malone, Leisenring, Storer, & al., 2007) (Mohty, Blaise, Faucher, & al, 2005).

These findings together with our observations let us to hypothesize that changing the dietary habits of transplanted patients in favour of species that produce more immunomodulatory metabolites, such as SCFAs, could be useful in order to indirectly protect from the onset or reduce the manifestations of the graft-versus host disease.

7.Bibliography

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- **Nastasi, C**., Candela, M., Bonefeld, MC., Gaisler, C., Krejsgaard,T., Biagi, E., Brigidi, P., Odum, N., Litman, T and Woetmann A. - "Short-chain fatty acids (SCFAs) effect on human monocyte-derived dendritic cells (DCs)" - Paper in submission.

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