

Gut Microbiota, Lipopolysaccharides, and Innate Immunity in the Pathogenesis of Obesity and Cardiovascular Risk

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Compelling evidence supports the concepts that gut microbiota actively promotes weight gain and fat accumulation and sustains, indirectly, a condition of low-grade inflammation, thus enhancing the cardiovascular risk. Fewer *Bacteroidetes* and more *Firmicutes* seem to characterize the gut microbiota of obese people as compared with that of lean individuals. This difference translates into an increased efficiency of microbiota of obese individuals in harvesting energy from otherwise indigestible carbohydrates. Furthermore, the microbiota also seems able to favor fat accumulation. Indeed, studies performed in germ-free animals have demonstrated that conventionalization of sterile intestine with gut microbiota is associated with an enhanced expression of various lipogenic genes in different tissues, *i.e.*, hepatic, adipose, and muscle tissues. Finally, the microbiota favors systemic exposure to the lipopolysaccharides (LPSs), large glycolipids derived from the outer membrane of Gram-negative bacteria. LPSs can cause a condition of "metabolic endotoxemia" characterized by low-grade inflammation, insulin resistance, and augmented cardiovascular risk. LPSs are a powerful trigger for the innate immune system response. Upon binding to the Toll-like receptor 4 and its coreceptors, LPSs trigger a cascade of responses ultimately resulting in the release of proinflammatory molecules that interfere with modulation of glucose and insulin metabolism, promote development and rupture of the atherosclerotic plaque, and favor progression of fatty liver disease to steatohepatitis. This review gives a comprehensive breakdown of the interaction among gut microbiota, LPSs, and the innate immune system in the development of obesity and promotion of an individual's cardiovascular risk. (*Endocrine Reviews* 31: 817–844, 2010)

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Abbreviations: AMPK, AMP-activated protein kinase; EU, endotoxin units; FIAF, fasting-induced adipocyte factor; HDL, high-density lipoprotein; IKK, inhibitor of NF- κ B kinase; iNOS, inducible nitric oxide; IRS, insulin receptor substrate; JNK, Jun N-terminal kinase; LBP, LPS binding protein; LDL, low-density lipoprotein; LPL, lipoprotein lipase; LPSs, lipopolysaccharides; MAMP, microbe-associated molecular pattern; MD-2, myeloid differentiation protein-2; MyD88, myeloid differentiation factor 88; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF- κ B, nuclear factor κ B; PPAR, peroxisome proliferator-activated receptor; PRR, pattern recognition receptor; ROS, reactive oxygen species; SCFA, short-chain fatty acid; T2DM, type 2 diabetes mellitus; TIR, Toll-IL-1 receptor; TLR, Toll-like receptor; Tollip, Toll-interacting protein.

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I. Introduction

In the last decade, microbiologists have invested much time and effort in trying to understand the contributions of gut microbiota to human health and disease. Until recently, relatively little was known about the biological potential of the gut microbiota because many constituent species could not be cultured. Nowadays, novel molecular approaches and high-throughput technologies are allowing the rapid identification of these species (1, 2) and a better comprehension of the relationship between microbiota and host (3). Gut microbiota may be described as an anaerobic “bioreactor” (see glossary in Table 1) within the host (2) that contributes to the host’s genomic profile and metabolic efficiency in a mutually beneficial association (2). Gut microbiota can affect the energy homeostasis of the host by modulating its capacity to harvest energy from given nutrients and to store it as fat (4). The first part of this review presents the basic concepts on gut microbiota.

The gut microbiota also seems able to promote systemic low-grade inflammation, insulin resistance, and enhanced cardiovascular risk through a mechanism that involves the increased exposure to bacterial products coming from the gut, and particularly to the Gram-negative-derived lipopolysaccharides (LPSs) (5–11). This condition is termed “metabolic endotoxemia” (5, 6).

Upon binding with the Toll-like receptor (TLR) 4, LPSs powerfully elicit the proinflammatory response of the innate immune system (12), resulting in target-organ damage (Fig. 1). Stimulation of TLRs by ligands leads to the recruitment of adaptor molecules such as the adaptor protein myeloid differentiation primary response gene 88 (MyD88) and the Toll-IL-1 receptor (TIR)-domain-containing adaptor inducing interferon- β through the interaction of the TIR (Fig. 2B). Indeed, TLR4 is located on the surface of immune cells (*i.e.*, monocytes, macrophages, Kupffer cells, and preadipocytes) and nonimmune cells (adipocytes, hepatocytes, and endothelial cells). Hence, a new set of problems emerge at the intersection of metabolism and immunity. Gaining a better insight into the bridge between the gut microbiota and the host’s metab-

TABLE 1. Glossary

Archaea	In biological taxonomy, this superkingdom or domain (highest taxonomic rank of organisms) corresponds to a group of single-celled microorganisms previously described as an unusual group of bacteria (Archaeobacteria). Because the Archaea have an independent evolutionary history and show many differences in their biochemistry from other forms of life, they are now classified as a separate domain in the current three-domain system, accordingly to the domain system of Carl Woese, introduced in 1990 (243).
Bacteria	In biological taxonomy, this superkingdom or domain corresponds to the large group of unicellular microorganisms, previously described as Eubacteria, which constitute the second domain of the current three-domain system (243).
Bioreactor	Any vessel (in this case the gut) in which chemical processes take place involving certain organisms and/or biochemical substrates derived from them.
Commensalism	A class of relationship in which one partner benefits and the other is apparently unaffected.
Conventionalization	Colonization of the intestine of germ-free animals with microbiota harvested from the colon of non-germ-free animals.
Eukarya	In biological taxonomy, this superkingdom or domain corresponds to single-celled or pluricellular microorganisms that constitute the third domain of the current three-domain system (243).
Metagenome Microbiome	The complex of host and microbiome (29). The microbial genome that encodes metabolic functions that the host has not had to evolve entirely on its own, including the ability to extract energy and nutrients from diet (244).
Microbiota	The community of living organisms assembled in a particular ecological niche of the host.
Mutualism	A class of relationship in which both partners experience increased ‘fitness.’
Taxonomic rank	Hierarchic taxonomic rank of gut microorganisms including first domain, then kingdom, phylum, class order, family, genus, and finally, species.

olism might open the way to novel therapeutic strategies for the treatment of obesity and associated morbidities.

II. Gut Microbiota

A. Establishment of gut microbiota: temporal determinants of variability

For the purposes of this review, it may be important to understand how the composition of the microbial ecology is established during the human life. Several determinants, host- and environmental-related, greatly affect gut micro-

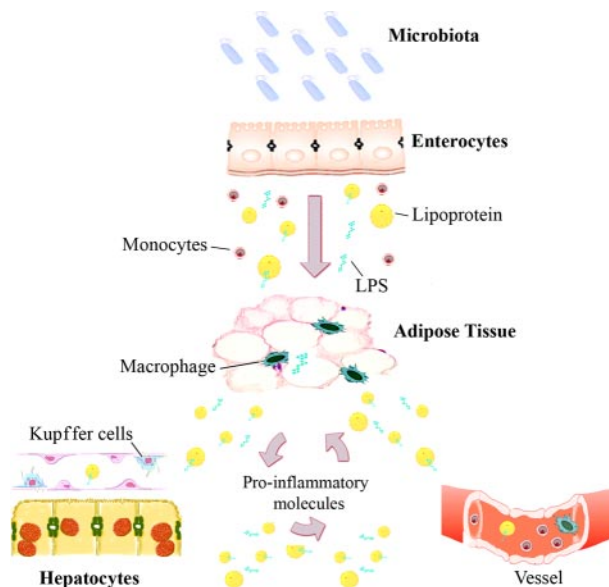


FIG. 1. Gram-negative-derived LPSs, embedded in freshly synthesized chylomicrons, pass across the intestinal barriers into the lymphatic system and then into the bloodstream. Hence, LPSs are vehicled by lipoproteins and LBP. Target tissues are the adipose tissue, the liver, and the endothelium. In the adipose tissue, LPSs trigger inflammation in local macrophages and preadipocytes with release of proinflammatory and chemotactic molecules. In the liver, LPSs are cleared by hepatocytes into the bile or activate inflammation in Kupffer cells. Proinflammatory and profibrotic molecules insult hepatocytes, leading to progression from simple fatty liver to steatohepatitis and hepatic fibrosis. In the endothelium, LPSs contribute to plaque formation and rupture.

flora components, inducing spatial and temporal variability. Until the moment of birth, the gastrointestinal tract of a normal fetus is sterile. During birth and rapidly thereafter, bacteria from the mother and the surrounding environment colonize the infant's gut. Rapidly after the birth, bacteria start to appear in the feces in a few hours and reach 10^8 to 10^{10} per gram of feces within a few days (13, 14). Immediately after vaginal delivery, babies have bacterial strains in the upper gastrointestinal tract derived from the mothers' feces (13). Infants born by cesarean section may also be exposed to their mothers' microflora, but the initial exposure is most likely to be from the surrounding environment such as the air, other infants, and the nursing staff, which serves as vectors for transfer (14). The gastrointestinal tract is colonized, first of all by facultative aerobes, then by anaerobes. When the facultative anaerobic bacterial populations expand, they consume the oxygen and create an anaerobic environment (14). During the first week of life, these bacteria create a reducing environment favorable for the subsequent bacterial succession of strict anaerobic species, mainly belonging to the genera *Bifidobacterium*, *Bacteroides*, *Clostridium*, and *Ruminococcus*. As anaerobes expand, the facultative bacteria cannot withstand the competition and environmen-

tal changes brought about by them and consequently decline in numbers (15).

Children born by vaginal delivery have a higher colonization rate and counts of *Bacteroides fragilis*, higher Bifidobacteria counts, and lower colonization rate of *Clostridium difficile* compared with infants born by cesarean section (16–18). The hospital environment itself only affects the colonization rate of *C. difficile* (16). Indeed, it is generally thought that infants are colonized with this spore-forming, anaerobic, microorganism mainly through the hospital environment. Hospitalization and prematurity have been consistently associated with higher prevalence and counts of *C. difficile*. Antibiotic treatment of the infant has been associated with decreased numbers of *Bifidobacterium* and *Bacteroides* (16). Other factors that influence the composition of the intestinal microbiota in neonates are the hygiene measures and the type of infant feeding (19). After birth, environmental, oral, and cutaneous bacteria are readily transferred from the mother to the infant through suckling, kissing, and caressing. All infants are initially colonized by large numbers of *Escherichia coli* and Group B *Streptococcus*.

Breast-fed children have a microbiota dominated by Bifidobacteria, possibly due to the presence of bifidobacterial growth factors in breast milk (20), and have rates of colonization with *E. coli*, *C. difficile*, *B. fragilis* group species, and Lactobacilli that are significantly lower than those found in formula-fed infants (16). After the introduction of solid food and weaning, the microflora of breast-fed infants becomes similar to that of formula-fed infants. The primary gut flora in infants born by cesarean delivery may be disturbed for up to 6 months after birth, whereas vaginally born infants take up to 1 month for their intestinal microflora to be well established. By the second year of life, the fecal microflora resembles that of adults.

Research into the developmental origins of obesity and associated metabolic disturbances has firmly established that pre- and perinatal developmental perturbations, which are however still unknown, increase the risk of developing not only obesity, but also type 2 diabetes mellitus (T2DM) and cardiovascular disease in adulthood. It is intriguing to speculate that the modality and the determinants of early-in-life variability of gut microbiota are pivotal because the evidence so far provided suggests a poor variability of the microbiota after its establishment. Longitudinal studies are needed to verify this hypothesis.

B. Establishment of gut microbiota: spatial determinants of variability

After its transformation to the adult-type, the microbiota remains remarkably constant over periods of months (21). Indeed, the recent study of Costello *et al.* (22) has ascertained that the gut community structure is highly

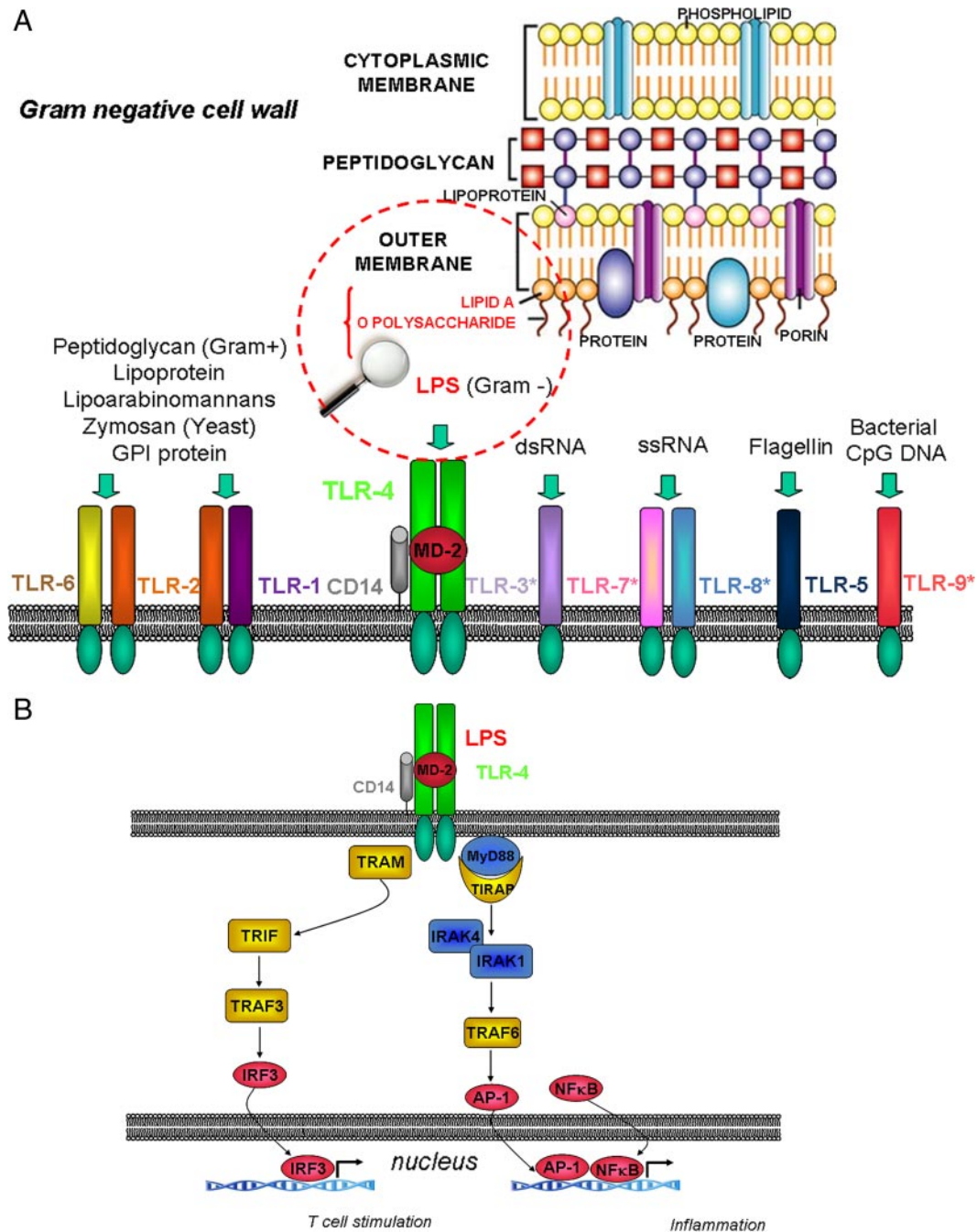


FIG. 2. A, The mammalian TLRs are germ line-encoded receptors, expressed by cells of the innate immune system, that are stimulated by structural motifs known as microbial or otherwise defined MAMPs expressed by bacteria, viruses, and fungi (238, 239), which are recruited to the TIR domain of the receptor. The MAMP molecule Lipid A of the LPSs of the outer membrane of the Gram-negative bacteria binds TLR4 and its coreceptors CD14 and MD-2. B, Interaction of the TIR domain of TLR4 and MyD88 triggers a downstream signaling cascade, leading to activation of the NF- κ B pathway. MD-2 physically associates with TLR4 on the cell surface and confers LPS responsiveness. CD14 is a glycosylphosphatidylinositol-anchored monocyte differentiation antigen, present also in soluble form (sCD14), which derives from both the secretion of CD14 and the enzymatic cleavage of the membrane form (238). Upon LPS recognition, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with the TIR domains (reviewed in Refs. 239 and 240). TIR domains contain three highly conserved regions that mediate protein–protein interactions between the TLRs and the signal-transduction adaptor proteins. There are four TIR domain-containing adaptor proteins: TIRAP [TIR domain-containing adaptor protein, also known as Mal (MyD88-adaptor-like)]; MyD88; TRAM (TRIF-related adaptor molecule); and TRIF (TIR domain-containing adaptor inducing IFN- β). The MyD88 adaptor protein activates the transcription factor NF- κ B via two IL-1 receptor-associated kinases (IRAK and IRAK-2). TLR4 signaling occurs via either MyD88-dependent or MyD88-independent (TRIF-dependent) pathways. The former pathway leads to expression of proinflammatory cytokines, whereas the latter mediates induction of type 1 interferon and interferon-inducible genes. In both pathways, the phosphorylation of the inhibiting protein I κ B leads to degradation of I κ B proteins and causes subsequent translocation of the transcription NF- κ B (reviewed in Refs. 241 and 242). AP-1, Activator protein 1; CpG, cytosine-phosphate-guanine; dsRNA, double-strand RNA; GPI, glycosylphosphatidylinositol; ssRNA, single-strand RNA; IRF3, interferon regulatory factor 3; TRAF3, TNF receptor-associated factor 3.

variable among people (*i.e.*, on a given day) but exhibits minimal variability within individuals over time (day to day). The microbiota comprises species that permanently either colonize the tract or transit temporarily through it. Permanent inhabitants seem to represent a restricted suite of highly adapted bacteria (23). Pioneer bacteria can either induce or modulate the expression of genes in host epithelial cells (24) to create a favorable habitat for themselves and to prevent the growth of other bacteria introduced later into the ecosystem. The initial colonization may therefore be very relevant for the final composition of the permanent flora in adults (25). Our burden of bacteria outnumbers our own cells. The density and the species of microorganisms vary as they progress along the adult gastrointestinal tract. The stomach and the duodenum harbor very low numbers of microorganisms, typically less than 10^3 microbes/ml of luminal content. The number progressively increases along the jejunum and ileum up to 10^{11} – 10^{12} microbes/ml of luminal content in the distal tract (26). The three domains of life (Table 1), namely Bacteria (the majority), Archaea, and single-celled Eukarya, are present in the adult intestinal tract. Bacteria predominate within the intestine and achieve the highest cell densities recorded for any ecosystem (27). Among them, *Bacteroidetes* and *Firmicutes* account for more than 90% of all phylotypes (28).

Within the gastrointestinal tract, differences exist between surface-adherent and luminal microbial populations. Furthermore, microniches of microorganisms can be identified along the intestinal tract (1). Eckburg *et al.* (1) evaluated 16S ribosomal DNA sequences, obtained by using broad-range bacterial and archeal primers, from mucosal and stool samples of three healthy volunteers. Mucosal samples were obtained during colonoscopy from the six major subdivisions of the colon. The working hypothesis of this study was that surface adherent and luminal microbial populations may be distinct and fulfil different roles within the ecosystem. Different percentages of *Firmicutes* and *Bacteroidetes* were observed between the mucosa and the stool of two out of three subjects. In detail, analysis of fecal and mucosal populations revealed that Phylum *Bacteroidetes* contains a relatively low number of phylotypes but a large number of sequences. Most of the *Bacteroidetes* sequences were classified as *B. vulgatus*, *Prevotellaceae*, *B. thetaiotaomicron*, *B. caccae*, *B. fragilis*, and *B. putredinis* phylotypes. Of note, the distribution of *Bacteroidetes* phylotypes demonstrated the most striking subject-to subject variability of any phylum. Proteobacteria and Verrucomicrobia sequences were detected rarely or not at all. Actinobacteria sequences were also rare, representing 0.2% of the total sequences and included bifidobacteria species. Single phylotypes of Fusobacteria

and unclassified near Cyanobacteria were detected, but in adherent microbial populations. The *Methanobrevibacter smithii*, a hydrogen-consuming methanogen, dominated the Archaea domain (1). Hence, the findings of this study demonstrated an interindividual variability that was significantly greater than the intraindividual variability among unrelated subjects; a significant bacterial diversity between intestinal mucosa and feces at the levels of both genus and species; and, in one individual, a certain degree of patchiness and heterogeneity in the distribution of microorganisms along the intestinal tract (1).

The whole intestinal microbiota correspond to 1000 different species, mostly anaerobes, whose collective genome (“microbiome”) contains at least 100 times as many genes as our own genome (Table 1) (29). Such microbiome endows us with functions that we did not evolve ourselves. This community constitutes a “microbial organ,” composed of different cell lineages, using cell-to-cell and cell-to-host communications and signaling, self-repair, and situated within a host organ. The “fitness” of this microbial community exploits the host environment, but also provides the host with advantages such as energy salvage from indigestible dietary constituents (*e.g.*, cellulose) and synthesis of short chain fatty acids (SCFAs). Among SCFAs, propionate serves as substrate for gluconeogenesis in hepatocytes, and acetate serves as substrate for *de novo* lipogenesis in both hepatocytes and adipocytes (30). Microbiota acts as a barrier against colonization of pathogens and stimulates the development of the immune system (25). It is very likely that our knowledge of either health-promoting or pathogenic effects exerted by intestinal microbiota is quite incomplete. Table 2 summarizes the potential harmful and beneficial functions of microbiota *vs.* the host in a relationship that seems to be more mutualistic than commensal (see glossary in Table 1).

Remarkably, the community fitness may be affected by specific host factors, as reduced peristalsis, immune suppression or environmental challenges as antimicrobial drugs, irradiation, chemicals, alcohol, and burns. These factors may modulate the gut immunity homeostasis and trigger a perturbation in the bacterial niche composition leading to illness (Fig. 3).

C. Gut immunity modulation: homeostasis and perturbation

Commensalism dictates that “innocent” bacteria above the epithelial surface or within the mucus should be tolerated, but bacteria penetrating the epithelial barrier need to be rapidly eliminated. However, to survive with such a high number of organisms in very close proximity to host tissues, the intestinal mucosa and its immune system are highly adapted. Mucosal immune responses are induced by small numbers of live commensal organisms penetrat-

TABLE 2. Functions of gut microbiota

Metabolic functions
Extraction of energy from nondigestible carbohydrates (fermentation of resistant starch, cellulose, hemicelluloses, no-starch polysaccharides, pectins, and gums) and oligosaccharides and sugar alcohols.
Fermentation of carbohydrates into SCFAs (acetate, propionate, butyrate) and lactate, ethanol, succinate, formate, valerate, and caproate.
Conversion of dietary and endogenous nitrogenous compounds into ammonia and microbial protein.
Absorption of salt and water.
Proteolysis of amino acids to form branched chain fatty acids (isobutyrate, 2-methylbutyrate, and isovalerate) and NH ₃ , phenols, indoles, and amines.
Vitamin synthesis (vitamin B and vitamin K).
Breakdown of complex lipids and cholesterol.
Metabolism of xenobiotics.
Barrier function
Protection against penetrance of pathogens.
Trophic function
Modulation of cell proliferation, differentiation, and apoptosis.
Stimulation of intestinal angiogenesis.
Immune function
Migration and maturation of precursor lymphoid cell.
Development and maturation of IgA plasmocytes.
Modulation of local and systemic immune response (oral tolerance).
Regulation of the host fat storage
Modulation of lipogenesis and fatty acid oxidation.

ing specialized inductive sites such as Peyer's patches or isolated lymphoid follicles, where they are picked up by dendritic cells or phagocytosed and destroyed by macrophages, triggering host innate immunity (31). The dendritic cells induce mucosal IgA responses on the surface of the B cells, particularly, at the site of the Peyer's patches. Then, IgA plasmablasts recirculate, first, through the lymph and bloodstream and, successively, they go back to the intestine where they populate the lamina propria. Here, these B cells act as mature IgA-secreting plasma cells (32). Because dendritic cells loaded with commensal bacteria do not penetrate further than the mesenteric lymph nodes, immune induction to commensals is confined to the mucosa, whereas the systemic immune system remains relatively ignorant of these organisms (specific geography of the response to commensal bacteria). The gut epithelium itself can also directly sense commensal bacteria and pathogens: integral to this are the mammalian pattern recognition receptors (PRRs), which include TLRs and recognize conserved structures of bacteria, viruses, and fungi and generally activate proinflammatory pathways alerting the host to infection (33) (Fig. 2A). The TLRs are signaling PRRs that recognize several bacterial structures as LPSs of the Gram-negative outer membrane or the lipoteichoic acid of the Gram-positive cell wall. Both TLRs and other PRRs belong to the wide family of microbe-associated molecular patterns (MAMPs), molecules generally triggering the innate immune response through the following steps: 1) detection of a microbial organism; 2)

transduction of the recognition event; and 3) induction of appropriate effector responses.

An unresolved question is how the gut distinguishes between pathogens and commensal bacteria. The TLR-mediated bacteria recognition is relatively crude, in that TLRs are unable to identify bacteria at either the species or genus level, or to distinguish harmful pathogens from harmless members of the commensal flora. One means by which inappropriate responses to innate signals from commensals may be achieved is through the compartmentalization of the TLRs to the basolateral aspects of enterocytes or inside epithelial cells (34, 35). Indeed, bacterial-epithelial contact seems to be required to activate the antiinflammatory response of the host triggered by the commensal bacteria against the pathogens (reviewed in Ref. 36). This is the case of the *B. thetaiotaomicron*, which has been recently shown to markedly attenuate the inflammatory response of the enterocytes induced by the *Salmonella enteritidis* by selectively antagonizing the transcriptional nuclear factor κ B (NF- κ B) (37). The intimate contact between commensals and epithelial cells seems to play a central role in the tight host-commensal regulation against the pathogens. It is likely that antagonists for the TLR action, analogous to the single Ig IL-1R-related molecule (38), are involved in mediating the protective effects of the commensal flora on the gut. Furthermore, host-specific regulatory mechanisms limit inflammation response to pathogens at the levels of the single Ig IL-1R-related molecule, the Toll-interacting protein (Tollip) (39), ST2 (40) and Nod proteins (41). Tollip is known to bind to TLR2 and TLR4 and can suppress the activation of IL-1 receptor-associated kinase (Fig. 2B). Increased expression of Tollip has been demonstrated in gut epithelial cells that become hyporesponsive to LPSs and lipoteichoic acid after prolonged stimulations with the ligands (42). The membrane-bound form of ST2 has been shown to directly antagonize TLR4-mediated signaling by sequestering the adaptors MyD88 and TIR-domain-containing adaptor protein (36).

III. Gut Microbiota and Obesity

A. Modulation of the bacterial community composition: the association with obesity

Gut microbiota of obese people is characterized by fewer *Bacteroidetes* and more *Firmicutes* than the microflora of lean controls (21). Weight loss programs are associated with changes in the proportion of the two Phyla, as demonstrated in 12 obese individuals who followed a 1-yr weight-loss program with either a fat- or carbohydrate-restricted low-calorie diet. In this study, the relative proportion of *Bacteroidetes* increased from 3 to 15%,

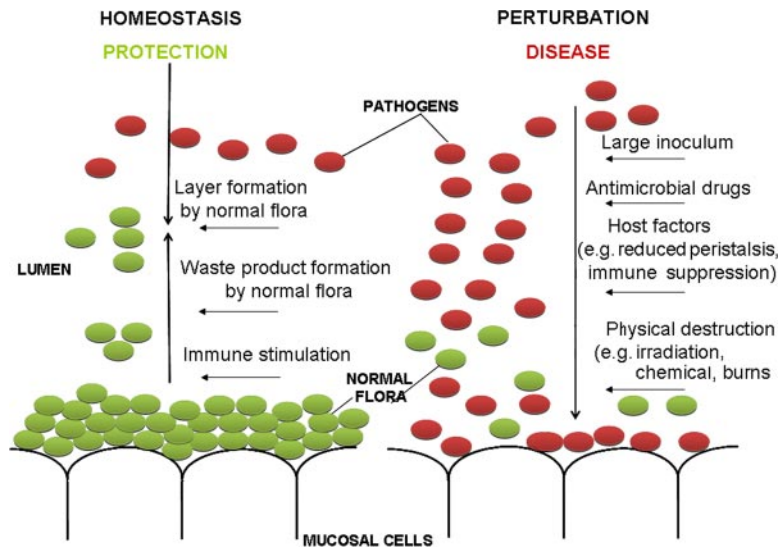


FIG. 3. Gut homeostasis and perturbation. Gut protection is performed by commensal microbiota (normal flora), which confer a protective physical layer to enterocytes, although occasional access of pathogen bacteria occurs within the gastrointestinal tract. The stratum is able to prevent penetration by external pathogens and provide the gut cells with helpful metabolic products and low immune stimulation. When an external stimulus (e.g., high bacterial inoculum) involves the gastrointestinal tract, an alteration of the gut balance is induced. Additionally, when host (reduced peristalsis, immune suppression, etc.) and environmental factors (irradiation, chemical, alcohol, burns, etc.) are induced, the immunity modulation is broken up, and the perturbation leads to disease.

whereas that of *Firmicutes* decreased. The change observed after weight loss was irrespective of the type of diet, division-wide and not due to blooms or extinction of specific bacterial species. Indeed, bacterial diversity remained constant over time. The change in the ratio between *Bacteroidetes* and *Firmicutes* correlated with the percentage of weight loss, which was in turn not associated with the change in calorie intake. Conversely, increased abundance of *Bacteroidetes* correlated with percentage loss of body weight. This correlation held only after the person had lost at least 6% of their body weight in the fat-restricted diet group and at least 2% in the carbohydrate-restricted diet group (21).

In keeping with these observations, more recent studies performed in monozygotic and dizygotic twins (43, 44) have provided insight into how host genotype and gut microbiota interact to promote weight gain. Microbiota of 31 adult female monozygotic and 23 dizygotic twin pairs, concordant for leanness or obesity, and of their mothers has been characterized. Family members shared microbiota but with a certain degree of variation in the specific bacterial lineages present in the microbial community. The degree of covariation did not differ between adult monozygotic and dizygotic twin pairs. There was no single abundant bacterial species shared by all 154 people of the sample because not all individuals shared more than 0.5% of the community. Conversely, there was a wide array of shared microbiomes (see glossary in Table 1) among the sampled population. Obesity was associated with

Phylum-level changes already demonstrated and with reduced bacterial diversity, altered representation of bacterial genes, and metabolic pathways (21). The *Bacteroidetes* bins were enriched in several carbohydrate metabolism pathways, whereas the *Firmicutes* bins were enriched in transport systems. Comparison of obese microbiome with the custom database of 44 human gut genomes revealed 273 genes enriched and 110 genes significantly depleted in the obese microbiome. Seventy-five percent of the obesity-enriched genes were from *Actinobacteria* and 25% from *Firmicutes*. However, 42% of the lean enriched genes were from *Bacteroidetes*. Their functional annotations indicated that many of them were involved in carbohydrate, lipid, and amino acid metabolism (43, 44).

Findings to date from both mice and humans have shown a poor microbial diversity within the gastrointestinal

tract. Nevertheless, this lack of variety can ensure the existence of an identifiable core gut microbiome, which is composed of genes encoding various signaling metabolic pathways. The few divisions existing within the microbiota probably reflect the strong host selection for specific bacteria whose emergent collective behavior is beneficial to the host. To benefit the host, bacteria are organized into a “food web” that aids in breaking down nutrients and provides the host with energetic substrates.

B. Energy harvesting and fat storage

Axenic animals are protected from diet-induced obesity and related comorbidities, whereas their conventionalization (see glossary in Table 1) with microbiota harbored in the colon of nonaxenic lean or obese mice results in a significant fat mass gain 2 wk after the conventionalization. Fat mass gain varied from 40% in mice conventionalized with microbiota from lean non-germ-free animals (4) to 60% in those conventionalized with the distal gut microbial community of *ob/ob* mice, respectively (45). The difference in the proportion of conventionalization-induced weight gain was probably due to differences in microbiota and associated microbiome of lean and obese mice. The microbiota of *ob/ob* mice was, in fact, characterized by a significant reduction of the ratio between *Bacteroidetes* and *Firmicutes* compared with that seen in lean mice (46), with no specific group being preferentially lost or amplified within these two divisions and independent of kinship and gender. The greater capacity to harvest energy

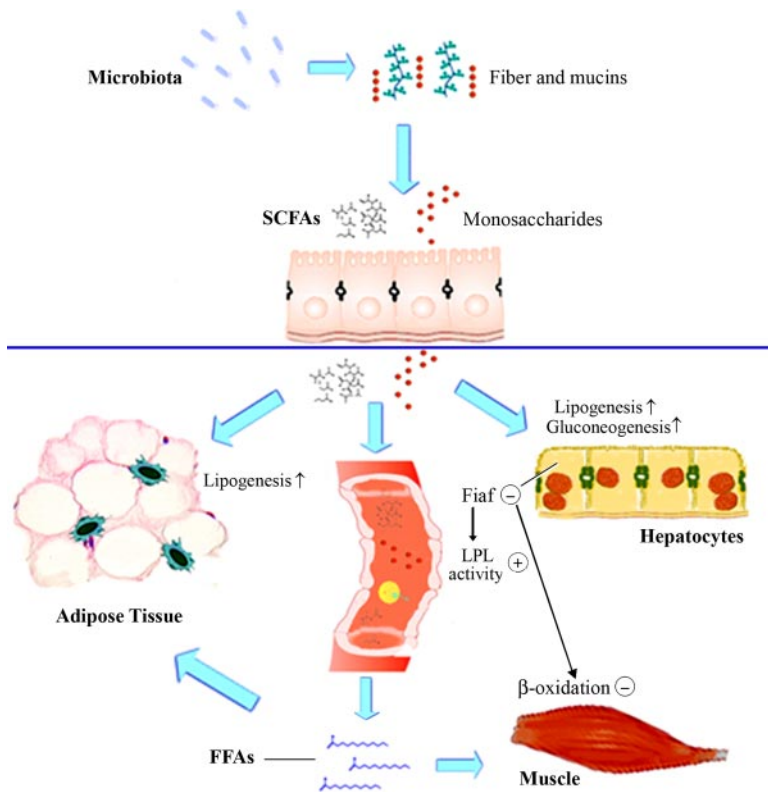


FIG. 4. Gene interaction between host and microbiota symbiotically enhances the metabolic capacity of the host, affecting energy harvesting and storage. The microbiota allows digestion of otherwise indigestible polysaccharides to monosaccharides and SCFAs, including propionate, the substrate for gluconeogenesis in hepatocytes, and acetate, which is the substrate for *de novo* lipogenesis in hepatocytes and adipocytes. Insulin and glucose induce *per se* the hepatic expression of lipogenic transcriptional factors such as sterol response element-binding protein type-1 and carbohydrate response element binding protein (48, 49). The microbiota down-regulates gut epithelium expression of the FIAF, also termed angiopoietin-like protein 4, a secreted LPL inhibitor. By suppressing FIAF expression, LPL activity is enhanced, with consequent hampering of fatty acid release from lipoprotein-associated triacylglycerols, and increased fat storage in adipose tissue. In turn, down-regulation of FIAF also reduces muscle expression of the peroxisomal proliferator activated receptor coactivator 1 α , in turn causing reduced mitochondrial fatty acid oxidation. Microbiota also affects fat storage via a FIAF-independent mechanism, involving the AMPK, a heterotrimeric enzyme, which functions as a “fuel gauge” monitoring cellular energy status and modulating β -oxidation in peripheral tissues (102–104). FFAs, Free fatty acids.

from nutrients observed in *ob/ob* mice seemed to be related to the presence of genes encoding enzymes that break down otherwise indigestible dietary polysaccharides with consequent enhanced production of more end-products of fermentation (mainly SCFAs). Accordingly, the calorie content in the feces of *ob/+* and *+/+* littermates as compared with lean mice was reduced (45). However, Bäckhed *et al.* (47) found that germ-free and conventionalized mice had energy content in their feces not statistically different despite a similar energy intake. This observation would suggest that other mechanisms, apart from a different capacity in energy harvesting, can be responsible for gut microbiota-induced weight gain. Indeed, conventionalization of germ-free mice resulted in significant changes in 1) the hormonal milieu and particularly in raised circulating levels of glucose, insulin, and leptin (4); 2) the density of

gut capillaries; and 3) more strikingly, the expression of genes regulating lipogenesis and fuel sensing (Fig. 4). Conventionalized animals also took up larger amounts of monosaccharides because of the higher density capillaries in the small intestine (4). In these animals, the *de novo* lipogenesis was up-regulated due to the increased availability of substrates (*i.e.*, SCFAs and monosaccharides deriving from the digestion of polysaccharides) as well as the higher concentrations of circulating glucose and insulin. Indeed, both glucose and insulin induce lipogenic genes, such as the carbohydrate response element-binding protein and the liver sterol response element-binding protein type-1 (48, 49), as also made clear by Bäckhed *et al.* (4) in their study. However, other pathways, mainly modulating the host’s lipogenesis, seemed to be affected in conventionalized animals. Conventionalization resulted in the reduced expression of the fasting-induced adipocyte factor (FIAF) in the gut epithelium (4). FIAF inhibits lipoprotein lipase (LPL) activity (50), hampering fatty acid release from lipoprotein-associated triacylglycerols. Hence, reduced levels of FIAF lead to increased activity of LPL and greater storage of triglycerides in adipocytes (4). Moreover, elevated levels of FIAF, as observed in axenic mice, also enhanced the expression of the peroxisomal proliferator-activated receptor coactivator 1 α , which up-regulates expression of genes encoding regulators of mitochondrial fatty acid oxidation (47). Finally, germ-free mice seemed to be resistant to diet-induced obesity (western-style, high-fat, sugar-rich diet) because they also express increased levels of phosphorylated AMP-activated protein kinase (AMPK) in the skeletal muscle and the liver (47). Indeed, AMPK regulates positively fatty acid oxidation and glucose uptake in the muscle tissue and inhibits fatty acid synthesis and gluconeogenesis in the liver. In the adipose tissue, AMPK inhibits fatty acid synthesis and lipolysis. Moreover, it inhibits fatty acid-induced insulin secretion. Hence, AMPK plays a pivotal role in the maintenance of body weight and in counteracting lipotoxicity (51–53).

Taken together, results of studies performed in axenic mice also spoke in support of an active role of microbiota

in modulating fat oxidation and accumulation (2, 4), and not only in harvesting energy from nutrients (2, 47). Interestingly, in the experiments of Bäckhed *et al.* (47), axenic mice fed with a high-fat diet did not gain body weight, suggesting that, contrary to earlier beliefs, dietary fats alone might not be sufficient to cause overweight and obesity.

IV. Lipopolysaccharides

LPSs are large glycolipids that consist of lipid and polysaccharide fractions joined by a covalent bond. They are found in the outer membrane of Gram-negative bacteria, act as endotoxins, and can elicit strong immune responses. The lipid A is a phosphorylated glucosamine disaccharide acylated with hydroxyl saturated fatty acids. The 3-hydroxyl groups of these fatty acids are further 3-O-acylated by saturated fatty acids, and particularly from lauric acid (54). Removal of these O-acylated saturated fatty acids or replacement with unsaturated fatty acids results in complete loss of endotoxic activity (54–56). Lipid A anchors LPSs into the bacterial membrane. It is responsible for much of the toxicity of Gram-negative bacteria and constitutes a major marker for the recognition of intruding Gram-negative bacteria by the host. The core oligosaccharide attaches directly to lipid A. The O antigen, a repetitive glycan polymer, is attached to the core oligosaccharide and comprises the outermost domain of the LPS molecule. The composition of the O chain varies from strain to strain. Thus, the ability of LPSs to promote low-grade inflammation and metabolic disturbances might be different depending mostly on lipid A or also on O-antigen chemistries. However, no data are available to confirm this hypothesis.

A. Intestinal absorption

A high-fat diet seems to favor absorption of LPSs across the intestinal barrier (5, 57), but the exact mechanism of this association has been debated for a long time. Very recently, Ghoshal *et al.* (58) demonstrated that the formation of chylomicrons promotes LPS absorption in an *in vitro* model of human epithelial colorectal adenocarcinoma cells. They incubated the cells with oleic acid, a long-chain fatty acid that induces chylomicron formation, or with butyric acid, a SCFA that does not induce chylomicron formation. They observed that cells incubated with oleic acid released more LPSs than cells incubated with butyric acid. The effect of oleic acid on LPS release was no longer evident when cells were incubated with an inhibitor of chylomicron formation, Pluronic L-81. Chylomicron formation was associated with basolateral secretion of cell-associated LPSs. Therefore, to confirm *in*

vivo that LPS absorption occurs via freshly formed chylomicrons, Ghoshal *et al.* (58) gavaged low-density lipoprotein (LDL) knockout mice first with radiolabeled LPSs, and then with triolein with or without the same inhibitor. Again, triolein enhanced LPS absorption, whereas Pluronic L-81 inhibited it. Mesenteric lymph node radioactivity was greater after triolein and reduced after Pluronic L-81, thus proving that LPSs were transported through mesenteric lymph nodes using freshly formed chylomicrons.

Ghoshal *et al.* (58) did not observe any effect of fatty acids on permeability when adenocarcinoma cells were incubated with fatty acids. Nevertheless, previous studies have found that 10-fold higher concentrations of fatty acids can also impair the integrity of the intestinal barrier in different (59, 60) and similar experimental models (61). In mouse models, endotoxemia after high-fat diet was associated with reduced expression of genes encoding for zonula occludens 1 and occludin (6).

An alternative mechanism of LPS absorption might include internalization by intestinal epithelial microfold cells (62) and enterocytes through a TLR4 (63) and myeloid differentiation protein-2 (MD-2)-dependent mechanism (63–65). Inside the enterocyte, LPSs are transported to the Golgi apparatus where chylomicrons are freshly synthesized (66).

B. Binding with lipoproteins

In the bloodstream, LPSs are transported by a specific transport 60-kDa acute phase response glycoprotein, the LPS binding protein (LBP), and by lipoproteins to hepatocytes (67). In the liver, LPSs are extracted into the bile for clearance (68).

All plasma lipoprotein subclasses can sequester LPSs (61), and the phenomenon seems dependent upon the number of phospholipids on the lipoprotein surface. Indeed, phospholipids bind LBP (69), which in turn is responsible for transporting LPSs to phospholipid vesicles (70), artificial phospholipid-rich particles (71), emulsions (72), and other lipoproteins (73). Under physiological conditions, high-density lipoproteins (HDLs) are the main acceptors of LPSs (74). Under conditions of acute inflammation and infection, plasma HDL cholesterol is reduced, and levels of triglycerides and chylomicrons are increased. Thus, LBP seems to deliver LPSs preferentially to LDLs and to favor the formation of complexes of LPSs with remodeled HDL and LDL (75, 76). By augmenting and redistributing the phospholipid content among the different lipoproteins, the immunostimulatory activity of LPSs can be attenuated. This mechanism of lipoprotein remodeling could represent an integral part of the innate host defense system against endotoxemia and Gram-negative bacterial infection (71). Supporting this view, it has been

observed that binding of LPSs with lipoproteins, and particularly with chylomicrons, partially prevents endotoxin-induced activation of monocytes/macrophages and secretion of proinflammatory cytokines (77, 78). The rate of binding between LPSs and monocytes is faster than the rate of binding between LPSs and lipoproteins (79, 80), and thus activation of monocytes in response to LPSs is likely to depend more upon the rate of LPS binding to lipoproteins than upon the total capacity of lipoproteins to bind LPSs (78).

It is worth mentioning that most of this evidence derives from studies in conditions of acute inflammation or sepsis, whereas few data are available on the role of lipoproteins in conditions of modestly increased levels of circulating LPSs, such as obesity, T2DM, and nonalcoholic fatty liver disease (NAFLD).

V. Metabolic Endotoxemia

A. High-fat diet and LPSs

The data provided in the first part of this review do not address the role of microbiota in the pathogenesis of low-grade inflammation and insulin resistance, which are frequently associated with obesity. Based on the observations of Bäckhed *et al.* (4), Cani *et al.* (5) looked for a microbial factor that could be responsible for concurrent fat-induced weight gain, low-grade inflammation, and development of insulin resistance. They hypothesized that LPSs might reasonably be a trigger for all these conditions because of the properties described above. As a first step, Cani *et al.* (5) wanted to rule out any relationship between dietary fat and LPSs. In their study, they fed wild-type mice with a high-fat diet and observed changes in circulating levels of LPSs. The diurnal rhythm of LPSs changed in relation to feeding. The LPS rhythm reached its zenith at the end of the dark hours, corresponding to the postfed condition, and presented lower concentrations during the light hours. When mice were fed with a high-fat diet for 4 wk, the LPS rhythm was disrupted and levels of the molecules were constantly 1.4-fold higher than the levels observed on a regular diet. In the same study, LPSs were administered orally with oil or water to wild-type mice. Serum levels of LPSs were greater in mice fed with the oil mixture, thus demonstrating a cause-and-effect relationship between fat absorption and increase in concentrations of endotoxin (5).

Thus, dietary fats definitely seem to enhance the absorption of LPSs, and there are at least three contributory factors: changes in gut microbiota, increased availability of chylomicrons, and reduced permeability of the gut epithelium. Microbiota from high-fat-fed animals was characterized by a significant reduction in Gram-negative

Bacteroidetes-like bacteria, in the *Eubacterium rectale-Clostridium coccooides* group, and in Bifidobacteria (1, 2). Of note, administration of Bifidobacteria has been shown to reduce levels of LPSs in mice (81) and to improve the mucosal barrier function (82). Indeed, Bifidobacteria do not degrade intestinal mucous glycoproteins as other pathogenic bacteria do, and they promote a healthier microvillus environment by preventing permeability and bacterial translocation (82).

B. The triad of LPS-induced weight gain, insulin resistance, and low-grade inflammation

The next step for Cani *et al.* (5) was to gain insight into the *in vivo* causative relationship between systemic exposure to mildly increased LPS levels and the development of weight gain, insulin resistance, and low-grade inflammation, a condition referred to by the researchers as “metabolic endotoxemia.” To accomplish their aim, wild-type mice were implanted with a sc minipump and small amounts of LPSs (from *E. coli* 055:B5; $300 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$; Sigma, St. Louis, MO). Animals were infused continuously for 1 month to achieve the same circulating levels of LPSs as those observed in mice fed with a 72% fat-enriched diet. Mice that were chronically infused with LPSs and those that were fed a high-fat diet gained the same amount of body weight and accumulated intrahepatic triglyceride similarly, although significance compared with controls was observed only in high-fat-fed mice. As compared with control mice, both mice models showed similarly increased levels of circulating glucose and insulin. Mice chronically infused with LPSs had increased hepatic insulin resistance, heavier liver weight, and normal peripheral insulin sensitivity as compared with high-fat diet-fed mice. Conversely, high-fat diet-fed mice had peripheral but not hepatic insulin resistance. Noteworthy, they had reduced insulin secretion compared with controls.

Infusion of LPSs and a high-fat diet elicited a similar inflammatory response in muscle and adipose and hepatic tissues, as determined by the increased expression of TNF- α , IL-1, IL-6, and plasminogen activator inhibitor 1. However, the proinflammatory response was blunted in knockout CD14 mice perfused iv with LPSs for 3 h as compared with wild-type mice, which had a dramatic increase of proinflammatory cytokines expression, phosphorylated NF- κ B, and inhibitor of NF- κ B kinase (IKK) isoforms in sc fat. When chronically infused with LPSs, knockout CD14 mice developed no hyperglycemia, hyperinsulinemia, or intrahepatic accumulation of triglycerides. These animals were extremely sensitive to insulin. When the same animals were chronically fed with a high-fat diet, they developed hyperinsulinemia and insulin resistance, but significantly later than wild-type animals. Nevertheless, both wild-type and CD14 knockout mice

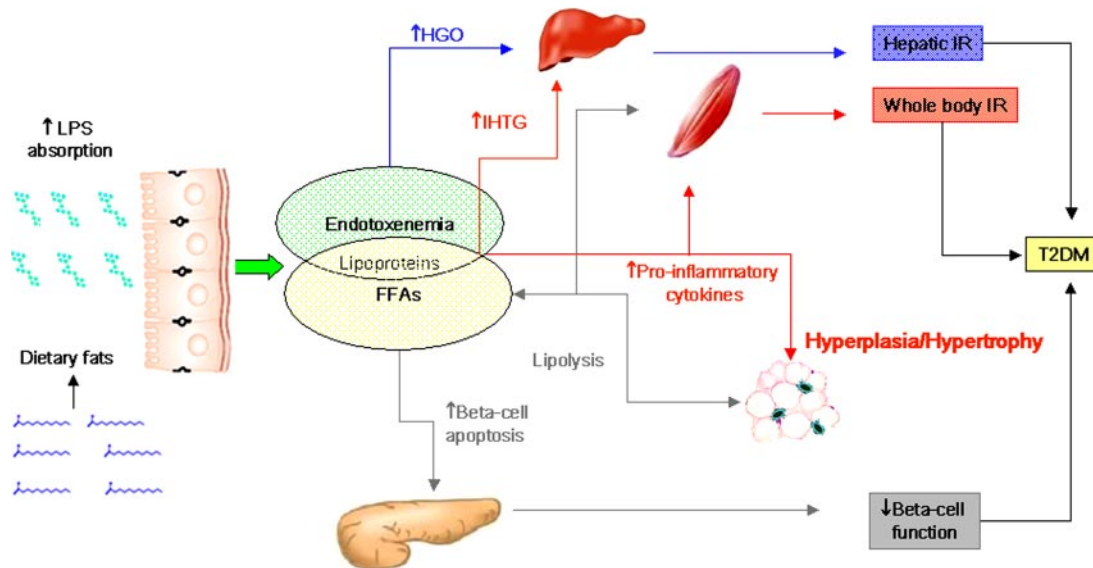


FIG. 5. Evidence emerging from the experiments of Cani *et al.* (5) supports the notion that both LPS and dietary fats contribute to the weight gain and the pathogenesis of insulin resistance (IR)-associated conditions that result in the development of T2DM. Red lines indicate the pathogenic pathways common to both; blue lines indicate the pathways where the effect of LPS seems to be prevalent; and gray lines indicate the pathways induced by free fatty acids (FFAs). IHTG, Intrahepatic triglycerides; HGP, hepatic glucose production.

developed carbohydrate intolerance after 24 wk of high-fat diet. Of note, intrahepatic accumulation of triglycerides was totally blunted in CD14 knockout mice. In Fig. 5, based on the experiments of Cani *et al.* (5), we summarize the mechanisms by which LPSs and dietary fats are likely to promote development of impaired carbohydrate metabolism up to T2DM. It seems that endotoxemia exacerbates and accelerates the proinflammatory and prodiabetogenic effects of fatty acids, which, in turn, modulate the LPS-induced activation of TLR4 (83). The hypothesis of the metabolic endotoxemia also reinforces the concept of diabetes as disorders of lipid metabolism (84).

Moreover, a high-fat diet and LPSs exerted different effects on adipocyte size. Whereas chronic infusion of LPSs was associated with reduced mean adipocyte size but with the highest number of macrophages embedded within the adipose tissue, high-fat diet caused an enlargement in the adipocyte size of wild-type mice. CD14 knockout mice chronically challenged with high-fat diet or LPS infusion showed a mean adipocyte size ranging from normal to slightly increased and no markers of inflammation within the adipose tissue (5). Thus, it might be interesting to evaluate whether chronic exposure to LPSs is associated with hyperplasia of adipose tissue and enhanced differentiation of preadipocyte to adipocyte, whereas chronic high-fat feeding may cause mostly hypertrophy of the tissue. The balance between hyperplasia and hypertrophy may influence the individual risk to gain fat mass and develop insulin resistance and cardiovascular disease (85).

Concentration of endotoxin was approximately 4.5 endotoxin units (EU)/ml in mice chronically infused with

LPS and approximately 5 EU/ml in high-fat-fed mice. These concentrations are 10–50 times lower than that obtained during septic shock (86). However, the amount of LPS administered in the study might be sufficient to induce a reduction in food intake. It was not the case, probably because long-term exposure to LPSs causes tolerance to the anorectic effect of the endotoxin. Furthermore, species sensitivity to LPSs varies considerably. For instance, in rats that are insensitive, ip injected LPS doses of 100 $\mu\text{g}/\text{kg}$ body weight or less mimicked many clinical features of Gram-negative bacterial infection including abnormalities in glucose metabolism (87). The same amount when infused chronically through an ip-implanted osmotic minipump caused a reduction of food intake in lean and obese (Fa/Fa) rats of approximately 42 and 43%, respectively, after the first day and of approximately 43 and 27% after 4 d of infusion. The reasons and the mechanisms for a different responsiveness and tolerance to the anorectic effect of LPSs are not known. They may be related to between-species difference in the genotype, in the hormonal milieu (*i.e.*, leptin and ghrelin) (87), or in the chemistry of LPSs.

VI. LPSs and Insulin Signaling

A. Molecular mechanisms

Induction of the immune response by LPSs activates pathways leading to inflammation that intersect and thus may inhibit insulin signaling at various steps.

Insulin affects cells through binding to its receptor on the surface of insulin-responsive cells (Fig. 6). The stimulated

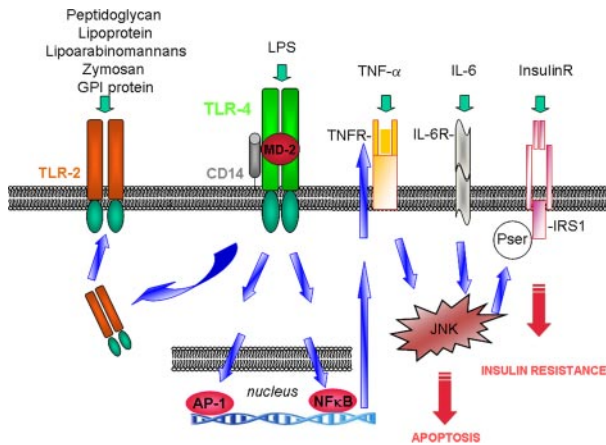


FIG. 6. Binding of LPSs with TLR4 induces translocation to the cell membrane of TLR2, which enhances inflammation and contributes to atherosclerosis. Triggering of TLR4 also activates proinflammatory pathways via activation of NF- κ B and activator protein 1 (AP-1), which, in turn, promote production and release of proinflammatory cytokines, including TNF- α and IL-6. The binding of TNF- α and IL-6 to their specific receptors prompts a signaling cascade in which JNK is pivotal, regulating serine phosphorylation of the IRS-1 and leading to apoptosis of hepatocytes. InsulinR, Insulin receptor; GPI, glycosylphosphatidylinositol; Pser, phospho-serine-containing protein; IL-6R, IL-6 receptor.

insulin receptor phosphorylates itself and several substrates, including members of the insulin receptor substrate (IRS) family, triggering downstream signaling events (88). Phosphorylation of IRS-1 serine residues reduces the ability of IRS-1 to associate with the insulin receptor and thereby inhibits downstream signaling and insulin action (89). For instance, TNF- α and IL-6 are responsible for inhibitory phosphorylation of IRS-1 (89, 90). The molecular step, which is pivotal for the integration of the metabolic and immune pathways, takes place at the level of c-Jun N-terminal kinase (JNK) (91). Inflammatory signals lead to hyperactivation of JNK, resulting in the subsequent serine phosphorylation of IRS-1 (92, 93). Different molecular steps of overlapping between immunity and insulin signaling occur at the level of the protein kinase C (93, 94); of the suppressors of cytokine signaling family, such as SOCS-1, -3, and -6 (95, 96); and of the inducible nitric oxide (iNOS) (95, 97).

In adipocytes, two different mechanisms also contribute to LPS-induced insulin resistance. First, LPS-induced activation of TLR4 in preadipocytes alters the expression of various cytokines, mainly TNF- α and IL-6 (89), which inhibit paracrinally the insulin signaling in adipocytes. Secondly, LPSs promote expression of NF- κ B (98) and activation of the MAPK pathway in adipocytes that target the expression of several genes, including insulin-dependent glucose transporter 4, adipocyte-specific fatty acid binding protein, adiponectin, stearoyl-coenzyme A desaturase, fatty acids synthase, and perilipin (8). In 3T3-L1 adipocytes, LPSs have also been found to promote expression of iNOS (98). Conversely, myoblasts seem to respond

differently to LPS-induced release of cytokines from macrophages. Indeed, conditioned medium from LPS-treated macrophages stimulated glucose uptake in cultured myoblasts with no enhanced expression of molecules of stress and inflammation. The medium contained markedly elevated levels of IL-10, which stimulates insulin action. IL-10-neutralizing antibodies blunted the positive influence of the conditioned medium (99).

B. Glucose homeostasis and uptake

Low doses of LPSs induced a biphasic change in glucose uptake in normal-weight volunteers. Insulin sensitivity was enhanced in the first few hours after the injection (100) and significantly reduced later (101). The time-course of insulin sensitivity during experimental endotoxemia and sepsis may be due to changes in the expression of iNOS (reviewed in Ref. 102). LPSs increase glucose uptake in myocytes by inducing the expression of iNOS and nitric oxide (103). Successively, exaggerated production of nitric oxide upon iNOS induction leads to insulin resistance by impairing muscle glucose uptake (104). The effect of nitric oxide on insulin action may be worsened by the enhanced LPS-induced release of TNF- α and IL-6. Moreover, overproduction of nitric oxide may worsen insulin resistance also via augmented levels of circulating fatty acids, due to hampering of LPL activity and enhancement of lipolysis (102). The time-course of insulin sensitivity during sepsis also reflects the shift from hypoglycemia to hyperglycemia, which commonly occurs during this condition (100, 105). LPS-induced hypoglycemia is associated with reduction of glucose production. Indeed, increased activity of NF- κ B pathways inhibits glucose production in cultured hepatocytes (106). Activation of NF- κ B pathways results in enhanced production of iNOS (107) and reduced expression and activity of rate-limiting enzymes in glucose production, glucose-6-phosphatase, and phosphoenolpyruvate carboxykinase (108, 109). In volunteers, exposure to LPSs leads acutely to decreased hepatic glucose output and amplified rate of glucose disappearance, measured using stable isotopes (100, 106). Conversely, doses of LPSs as low as 3 ng/kg are sufficient to induce in the 24 h after the injection a significant reduction of insulin sensitivity and an increase of circulating insulin and glucose (101). Down-regulated expression of the adiponectin receptors on the surface of circulating monocytes probably contributes to reduced insulin sensitivity (101). Release of contra-insular hormones (*i.e.*, glucagon, GH, and cortisol) after administration of low-dose LPSs can contribute to the reduction of peripheral and hepatic glucose uptake (105).

C. Hyperinsulinemia and type 2 diabetes

LPSs stimulate insulin secretion (94), and conversely, chronic hyperinsulinemia causes decreased clearance of

LPSs (94) by inducing hypofunctionality of the Kupffer cells (110). This positive “feed forward” mechanism could be a means of first-line defense against pathogens that breach the gastrointestinal barrier. This protective mechanism may become exaggerated in the case of adiposity, hyperinsulinemia (111), high-fat feeding (57), and smoking (112). Plasma endotoxin and endotoxin neutralization capacity, an indirect measure of endotoxin exposure, increase significantly by approximately 50% after a high-fat meal or a high-fat meal with cigarettes (57) (Table 3). Hyperinsulinemia and hyperglycemia can be the indirect cause of endotoxemia because they reduce jejunal motility and gastrointestinal transit time (113), which in turn promote small intestine bacterial overgrowth (114) and leakiness of the intestinal mucosa (115). These conditions occur very frequently in patients with T2DM in whom hyperinsulinemia, high-fat consumption, and dyslipidemia may contribute synergistically to the development of endotoxemia. Individuals with T2DM have mean values of LPSs that are 76% higher than healthy controls (Table 3) (7). When treated with rosiglitazone, an agonist of the peroxisome proliferator-activated receptor (PPAR)- γ with antiinflammatory properties (116), patients with T2DM had 51% reduced fasting levels of serum insulin and 35% reduction of serum LPSs compared with untreated patients (7). Creely *et al.* (7) speculated that glitazones may silence inflammation, also indirectly favoring the clearance of LPSs via reduced insulinemia and increased bioavailability of HDL. Glitazone-induced bioavailability of HDL (117) may result in the “mopping up” of LPSs. Furthermore, elevated HDL can blunt LPS-induced release of cytokines (118).

VII. Fatty Acids and Endotoxin in the Fatty Liver Disease

Fatty acids and endotoxin can contribute synergistically to the progression of alcoholic and nonalcoholic fatty liver to steatohepatitis. NAFLD entails a spectrum of pathological changes, which range from simple accumulation of fat in the liver to fat deposition complicated by inflammation [known as nonalcoholic steatohepatitis (NASH)], fibrosis, and cirrhosis (119). In 1998, Day and James (120) theorized a two-hit model to explain the pathogenesis of NAFLD and its progression to NASH. Fatty acid deposition within hepatocytes would be the first hit. A single cytokine or complex of cytokines would represent the second hit and would promote a local condition of inflammation.

Fatty acids can directly induce inflammatory signaling in the liver, even in the absence of obesity or insulin resistance (121–123). Excess fat activates JNK and IKK in

hepatocytes. Both kinases induce hepatic insulin resistance, promote the expression of lipogenic genes (124, 125), and stimulate the expression of cytokines and cell-adhesion molecules (126–128), which in turn lead to the steatohepatitis. Importantly, saturated fatty acid-induced activation of JNK causes apoptosis of hepatocytes (“lipotoxicity”) (Fig. 6) as observed in *in vivo* and *in vitro* mouse models (127–129). Indeed, within the hepatocytes, fat enhances the hepatic expression of the death receptor Fas (130) and Fas-Fas ligand (131), thus creating an environment that favors the suicide of the hepatocytes (131, 132). Noteworthy, fatty acids enhance the hepatic expression of TLR4 and TLR2 as well as of the coreceptors CD14 and MD-2 (128), and thus the sensitivity to challenge by the TLR4 ligand LPSs (133). Signaling through these receptors activated by saturated fatty acids, by LPS, or by both can contribute to the progression from fatty liver to steatohepatitis (134). It is worth mentioning that LPSs cause liver injury in the forms of both alcoholic and nonalcoholic fatty liver disease. Acute and chronic ingestion of alcohol lead to a significant elevation of portal and systemic levels of endotoxin in mice models (135) and humans (136). Treatment with antibiotics targeting Gram-negative bacteria alleviates liver injury in alcoholic liver disease (137). Alcohol can cause endotoxemia probably through three different mechanisms (reviewed in Ref. 138). First, alcohol consumption causes changes in gut microbiota, not yet described in detail. Indeed, alcohol consumption is associated with upper gastrointestinal bacterial overgrowth (139, 140). Second, alcohol ingestion disrupts the intestinal epithelial barrier enhancing gut permeability (139). Third, ethanol consumption reduces clearance of LPSs (140) and reduces the phagocytic function of the Kupffer cells (141). Kupffer cells, the resident macrophages of the liver, are the crucial hepatic cellular target of gut-derived LPSs and orchestrate the immune response (133). Murine models of alcoholic fatty liver have shown that activation of Kupffer cells triggers MyD88-independent signaling mediated by activation of the proinflammatory proteins IKK and NF- κ B. It results in increased transcription and release of TNF- α (142–144), which in turn promotes the progression of both forms of fatty liver disease (143, 144) to steatohepatitis. By interacting with Kupffer cells, LPSs induce production of reactive oxygen species (ROS) and proinflammatory cytokines (145). Indeed, depletion of Kupffer cells by treatment with gadolinium chloride prevents alcohol-induced liver injury in animal models of alcoholic fatty liver disease by decreasing both steatosis and necroinflammation (146).

The association between small-intestine bacterial overgrowth and alterations of the gut permeability with

TABLE 3. Mean values of endotoxin in healthy persons and in patients in some studies using LAL assay

First author, year (Ref.)	No. of subjects	Anthropometrics of subjects enrolled	Brief description of the study	Endotoxin concentrations	Units of measure (LAL assay)
Danner, 1991 (245)	43	Sex, NA; age, 49 ± 2.3 yr; BMI, NA	Sepsis	440 ± 120	pg/ml
Goto, 1994 (246)	184 males, 193 females	Age, ~72 yr; BMI, ~28 kg/m ²	Elderly (24% healthy; 46% on adequate treatment for illness; 30% requiring adjustment of therapies or supplementary investigations)	Men = 6.6 ± 3.8, women = 6.9 ± 3.8	pg/ml
Lin, 1995 (247)	45 males, 36 females	Age, 59 ± 2 yr; BMI, NA	Controls	2.9 ± 0.2	pg/ml
	13 males, 12 females	Age, 58 ± 2 yr; BMI, NA	CH with ALT ≥300 IU/liter	10.1 ± 1.3	pg/ml
	30 males, 27 females	Age, 54 ± 5 yr; BMI, NA	CH	4.6 ± 0.5	pg/ml
	126 males, 112 females	Age, 63 ± 1 yr; BMI, NA	Cirrhosis (Pugh's class A)	4.9 ± 0.5	pg/ml
			Cirrhosis (Pugh's class B)	7.9 ± 1.4	pg/ml
			Cirrhosis (Pugh's class C)	10.2 ± 2.0	pg/ml
Niebauer, 1999 (248)	14	Sex, NA; age, 55 ± 16 yr; body weight, 74 ± 7 kg	Controls	0.46 ± 0.21	EU/ml
	20	Sex, NA; age, 63 ± 19 yr; NYHA class 2.6 ± 0.7; body weight 76 ± 6 kg	Edematous CHF with no edema	0.37 ± 0.23	EU/ml
	20	Sex, NA; age, 64 ± 10 yr; NYHA class, 3.3 ± 0.7; body weight, 78 ± 9 kg	Edematous CHF	0.74 ± 0.45	EU/ml
	10	Anthropometrics NA	Effect of intensified diuretic treatment	Before = 0.8 ± 0.49; after = 0.45 ± 0.21	EU/ml
Wiedermann, 1999 (179)	87 males, 140 females	Age, 60.3 ± 8.3 yr; BMI, 25.3 + 3.9 kg/m ²	No 5-yr history of incident carotid atherosclerosis	20.4 ± 22.6	pg/ml
	129 males	Age, 65.8 ± 8.1 yr; BMI, 25.1 ± 3.7 kg/m ²	5-yr history positive for carotid atherosclerosis	29.6 ± 43.2	pg/ml
Venet, 2000 (200)	43 males, 18 females	Age, 43 ± 18 yr; BMI, NA	Sepsis	310 ± 810	pg/ml
Parlesak, 2000 (136)	54	Sex, NA; age, 27–68 yr; BMI, 23.4 ± 1.1 kg/m ²	AFLD	8.3 ± 2.3	pg/ml
	17	Sex, NA; age, 28–59 yr; BMI, 22.4 ± 0.791 kg/m ²	ASH	18.4 ± 5.6	pg/ml
	18	Sex, NA; age, 36–68 yr; BMI, 22.8 ± 1.3 kg/m ²	Cirrhosis	11.4 ± 2.7	pg/ml
Wigg, 2001 (147)	23	Age, 50 ± 17 yr; BMI, 24 ± 5 kg/m ²	Controls	1.3 ± 0.2	EU/ml
	22	Age, 54 ± 16 yr; BMI, 30 ± 6 kg/m ²	NASH	1.2 ± 0.6	EU/ml
		Anthropometrics NA	NASH, no-SIBO	~1.2	EU/ml
		Anthropometrics NA	NASH+SIBO	~1.2	EU/ml
Creely, 2007 (7)	20 males, 5 females	Age, 48.1 ± 19.2 yr; BMI, 29.5 ± 4.3 kg/m ²	Controls	3.1 ± 1.7	EU/ml
	20 males, 5 females	Age, 52.2 ± 11.7 yr; BMI, 31.8 ± 4.5 kg/m ²	T2DM	Baseline = 5.5 ± 1.6; post 10 wk TZD = 4.5 ± 1.96; post-diet = 7.2 ± 1.6; post GLM = 4.6 ± 1.4	EU/ml
Erridge, 2007 (57)	12 males	Age, 32 (20–58) yr; BMI, 23 (19–31) kg/m ²	Fasting	Baseline = 8.2 (3.4–13.5)	pg/ml
			2 h after a HFD (~900 calories)	12.3 (4.7–26.3)	pg/ml
			HFD and 3 cigarettes	12.6 (5.7–24.5)	pg/ml
			3 cigarettes	10.3 (3.4–26.4)	pg/ml

(Continued)

TABLE 3. Continued

First author, year (Ref.)	No. of subjects	Anthropometrics of subjects enrolled	Brief description of the study	Endotoxin concentrations	Units of measure (LAL assay)
Amar, 2008 (210)	201	Age, ~54 yr; sex, NA; BMI, ~26 kg/m ²	Association between endoxemia and energy intake	9–39	EU/ml
Thuy, 2008 (224)	2 males, 4 females	Age, 47 ± 7 yr; BMI, 22.5 ± 1.2 kg/m ²	Controls	Not detected	EU/ml
		Age, 55 ± 4 yr; BMI, 27.8 ± 0.7 kg/m ²	NAFLD	279.5 ± 90.8	EU/ml
Miller, 2009 (9)	97 males, 96 females	Anthropometrics NA	Individuals with no features of MetS	9.2–11.3	EU/ml
		Anthropometrics NA	Patients with MetS (≥3 components)	10.6–34.9	EU/ml
Ghanim, 2009 (228)	10	Sex, NA; age, 32.4 ± 0.6 yr; BMI, 23.1 ± 0.6 kg/m ²	910 calories of a HFHC meal	Baseline = 0.39 ± 0.07; post-meal = 0.58 ± 0.10	EU/ml
	10	Sex, NA; age, 31.0 ± 1.1 yr; BMI, 22.8 ± 0.6 kg/m ²	910 calories of AHA-recommended meal rich in fiber and fruit	NA	EU/ml

The LAL test is the most sensitive clinically available test for endotoxin. There are two types of LAL assay, namely, gelatin assay and chromogenic LAL assay. It seems that there is no difference in the clinical utility between gelatin LAL assay and chromogenic LAL assay. The cutoff level for positive endotoxin in most clinical studies has been 5–10 pg/ml (0.1 EU) (249). Reported in the table are mean values of endotoxin ± SD, mean (range), or range. Units are expressed as endotoxin units (EU/ml) or pg/ml. The entity EU describes the biological activity of an endotoxin. The biological activity causing pyrogenic effects depends on a variety of factors like polysaccharide chain length, aggregation status, solubility in biological fluids, bacterial source, associated substances, etc. It is taken as a rule that 1 EU corresponds to 100 pg of endotoxin. However, the use for the LAL assay of different brands in the above referred studies does not allow interstudy comparisons. LAL, Limulus amoebocyte lysate; BMI, body mass index; NA, not available; NYHA, New York Heart Association; CH, chronic hepatitis (mixed etiology); CHF, chronic heart failure; AFLD, alcoholic fatty liver diseases; ASH, alcoholic steatohepatitis; SIBO, small-intestine bacterial overgrowth; MetS, metabolic syndrome; HFD, high-fat diet; HFHC, high-fat high-carbohydrate; AHA, American Heart Association; ALT, alanine aminotransferase; GLM, glucose-lowering medications; TZD, thiazolidinediones.

NAFLD and liver damage (147) may be directly mediated by endotoxemia. Obese patients who had undergone jejunal-ileal bypass, a type of bariatric surgery, frequently developed small-intestine bacterial overgrowth and NASH. Interestingly, the symptoms of small-intestine bacterial overgrowth and histological features of steatohepatitis ameliorated after metronidazole treatment (148). A recent Italian study has demonstrated that patients with NAFLD have increased intestinal permeability, characterized by a reduction in the expression of zonula occludens 1, and that the degree of steatosis is proportional to the degree of intestinal permeability (149). The administration of probiotics modifies the intestinal flora, ameliorates the liver biochemistry in mice (150) and humans (151), induces the down-regulation of JNK and IKK β activity, and leads to the improvement of liver histology (152).

VIII. Endotoxin and Cardiovascular Disease

Chylomicrons from the gut, with embedded LPSs, do not undergo significant LPL-dependent hydrolysis in the lymphatic vessels (153) but are mainly hydrolyzed in blood vessels by endothelial lipases (154). Upon binding with TLR4 on endothelial cells and monocytes/macrophages, LPSs cause the release of proinflammatory cytokines, leading to severe endothelial dysfunction, plaque formation and rupture, oxidation of LDLs, and thrombogenesis

(155). Of note, TLRs are not constitutively expressed on endothelial cells. Mechanical and nonmechanical stimuli can promote expression of TLRs (11, 156). Stimuli include disturbed blood flow (157), oxidized LDL (158), nonlipid endogenous ligands (*i.e.*, high mobility group box 1 protein, hyaluronic acid fragments, and biglycan) (159), saturated fatty acids (160, 161), stress-induced factors (*i.e.*, heat shock proteins), advanced glycation end-products (162), as well as fibrinogen, heparin sulfate, and hyaluronate (163). Indeed, all the endothelial cells are exposed to similar levels of cholesterol, but only those cells situated at the aortic bifurcation and the lesser curvature of the aortic arch display an inflamed phenotype (164). The aortic arch represents a specific site for the development of the plaque. Here, endothelial cells present a proinflammatory endothelial phenotype that is permissive in that it allows expression of proinflammatory receptors such as TLRs. It is also causative in that it permits expression of cell surface adhesion molecules, which fosters inflammatory leukocyte accumulation in the intima (164, 165).

A. Endothelial damage and atherosclerosis

Upon interaction with TLR4 on the surface of endothelial cells, endotoxin can promote endothelial damage directly via enhanced generation of ROS (166). Nevertheless, endothelial cells challenged with endotoxin release proinflammatory, chemotactic, and adhesion molecules, which cause transmigration of monocytes (*i.e.*, the mono-

cyte chemotactic protein-1) (167–169), rolling to firm adhesion on the endothelial monolayer (170), differentiation into macrophages, and plaque formation. Among them, IL-8 is chemotactic for T lymphocyte (170), which are drawn to form the fibrous cap of atherosclerotic lesions (171). Very low levels of endotoxin (<1 ng/ml) are sufficient to promote these processes (11, 168, 169).

Endotoxin can also induce activation of β_2 -integrins, up-regulation of selectins and cellular adhesion molecules, increased phosphorylation of the platelet/endothelial cell adhesion molecule-1, release of platelet-activating factor, and expression of its receptor by endothelial cells (172).

Monocytes and macrophages challenged with LPSs contribute endothelial damage and plaque formation as well. One fascinating conjecture is that statins may be effective in reducing cardiovascular risk by their ability to attenuate the effects of endotoxin in endothelial cells and macrophages (173).

B. LPS-induced apoptosis

In the presence of proinflammatory and prooxidative stimuli, programmed cell death of endothelial cells represents a defense mechanism. The role of apoptosis in the pathogenesis of atherosclerosis has been clearly defined (174). Upon binding with CD14, LPSs cause down-regulation of CD14 expression on the surface of monocytes, which promotes cell survival (175). Concurrently, LPSs down-regulate expression of the molecule on the surface of endothelial cells, thereby inducing endothelial apoptosis (174). Protection against apoptosis in monocytes is due to an induction of antiapoptotic factors via NF- κ B activation (176, 177). This phenomenon is fundamental to host protection against pathogens because macrophages act as phagocytes to remove moribund cells and apoptotic bodies (178). The multifunctional CD14 receptor induces survival and antagonizes apoptosis, representing a recognition receptor of macrophages, which enables interaction with apoptotic cells (156).

C. Endotoxin as marker of cardiovascular risk

The Bruneck study in 1999 showed for the first time evidence in support of a clinical association between levels of LPSs and cardiovascular risk (179). Levels of plasma LPSs were measured at the baseline in a random sample of 516 men and women from an Italian cohort. End-points of the survey were incident (early) atherosclerosis in the carotid arteries, as assessed by high-resolution duplex ultrasound, and incident cardiovascular disease. Levels of endotoxin ranged from 6.0 to 209 pg/ml (Table 3). Subjects with levels exceeding 50 pg/ml faced a 3-fold risk of incident atherosclerosis. Notably, smokers with low endotoxin levels and nonsmokers did not differ in risk factors,

whereas smokers with high endotoxin levels almost invariably developed new lesions. Similar results were obtained for incident cardiovascular disease (179).

Very recently, the Wandsworth Heart and Stroke Study (180), a British multiethnic study, reported graded increase in levels of LPS from black Africans to whites and to South Asians. This increase was consistent with ethnic differences in risk factors for cardiovascular disease (180), with an increase in components of the metabolic syndrome across all ethnic groups (9) and with the 10-yr cardiovascular risk, estimated using the Framingham equation (181). LPS concentration was higher in men than in women, as sex hormones modulate intestinal permeability (145). The positive association between increased endotoxin levels and prevalence of abnormalities of the metabolic syndrome suggests the potential utility of measuring endotoxin as markers of low-grade inflammation and related increased cardiovascular risk in the clinical practice. This estimation would provide information not only about “traditional risk factors,” but also about insulin resistance and organ damage at the sites of liver and endothelium. Of course, large longitudinal studies are needed to evaluate the sensitivity and specificity of endotoxin in the prediction of the disease.

IX. TLR-Triggered Immunity in Obesity

LPSs not cleared by hepatocytes trigger activity of TLR4 by binding receptors on the surface of Kupffer cells in the liver, of preadipocytes, and of adipocytes, inducing up-regulation of soluble CD14 (182) and expression of TLR2 (7). Hence, preadipocytes and mature adipocytes express both TLR2 and TLR4. Preadipocytes, adipocytes, and macrophages within the visceral adipose tissue seem to respond synergistically to proinflammatory insults. The enormous potential, in terms of proinflammatory response and buffering of nutrients, of visceral adipose tissue compared with fat from extravisceral sources has been attributed to its capacity to be more readily mobilized in stress situations and to expand. Visceral fat produces three times as many proinflammatory cytokines per gram of tissue (183), and its size varies from a few milliliters up to 6 liters (184). Because of these characteristics, visceral fat might be paramount in determining the degree of proinflammatory response powered by triggering TLRs.

A recent study by the group of Samuel Klein (185), however, drives us to reconsider the role of visceral adipose tissue in the pathogenesis of the cardiovascular risk. The study provides strong evidence that increased visceral adipose tissue does not directly cause the metabolic abnormalities associated with obesity (mainly dyslipidemia), but the commonly observed association between in-

creased visceral adipose tissue (186) is due to the correlation between visceral adipose tissue and intrahepatic fat content of triglycerides. The alteration in CD36 content, which regulates tissue uptake of free fatty acids from plasma (187) in adipose tissue, muscle, and probably liver, might keep up the association between ectopic fat distribution and obesity-related metabolic disease. Thus, whereas intrahepatic fat content seems to sustain dyslipidemia directly through the enhanced production of very low density lipoproteins and triglycerides, it cannot be excluded that the amount of visceral adipose tissue determines more significantly the systemic proinflammatory response via activation of TLRs than intrahepatic fat content does.

A. The adipocyte as immune modulator

Macrophages and adipocytes colocalize in the adipose tissue, both participating in the innate immune response: macrophages in their role as immune cells by killing pathogens and secreting inflammatory cytokines and chemokines; adipocytes by releasing lipids that modulate inflammation or participate in neutralization of pathogens. Under normal conditions, adipocytes store lipids and regulate metabolic homeostasis, and macrophages participate in the inflammatory response. In certain conditions (*e.g.*, overnutrition and obesity), preadipocytes act as immune cells, exhibit phagocytic and antimicrobial properties, and differentiate to macrophages (188, 189). Adipocytes, primarily preadipocytes (188), and macrophages express the same cytokines (*i.e.*, TNF- α , IL-6), proteins such as metalloproteinases, fatty acid binding proteins, nuclear hormone receptors including PPARs, and many other factors (188, 190–192). Fatty acid binding proteins modulate lipid storage in adipocytes and cholesterol accumulation in macrophages. The pathways of nuclear hormone receptors, which oppose inflammation and promote both cholesterol efflux from macrophages and lipid storage in adipocytes, are a paradigm of the integration between immune and metabolic functions. The evidence that, in obesity, the tissue becomes inflamed via infiltration of adipose tissue by macrophages and as a result of adipocytes themselves becoming producers of inflammatory cytokines, is also a paradigm of this integration (183, 193).

B. LPSs, preadipocytes, and adipocytes

Adipose tissue is composed of approximately 50–70% adipocytes, 20–40% stromal vascular cells (which include preadipocytes, fibroblasts, and undifferentiated mesenchymal cells), and 1–30% of infiltrated macrophages (194). LPSs, at the nanograms per milliliter level (195), induce release of proinflammatory molecules from

macrophages and preadipocytes (8, 196), which in turn triggers insulin resistance in mature adipocytes (8). Triggering of insulin resistance in adipocytes occurs via activation of NF- κ B and MAPK signaling, reduced PPAR- γ activity, and insulin responsiveness in adipocytes. Moreover, LPSs inhibit the expression of adiponectin on preadipocytes, and addition of LPSs to cultures containing almost exclusively adipocytes did not adversely affect insulin-stimulated glucose uptake or adiponectin gene expression (8). Chung *et al.* (8) speculated that LPS-mediated impairment of insulin responsiveness and PPAR- γ activity in adipocytes is due to changes in the affinity of PPAR- γ for its ligand.

Furthermore, human preadipocytes robustly express monocyte chemoattractant protein-1 when challenged with endotoxin. Therefore, preadipocytes may initiate monocyte recruitment to adipocytes in white adipose tissue under inflammatory conditions, resulting in differentiation and anchoring of macrophages to adipocytes (197).

C. LPSs enhance expression of TLR2

Induction of TLR2 expression may be partially responsible for the higher proinflammatory responsiveness of preadipocytes to LPSs (8). Although TLR4 is constitutively expressed in both preadipocytes and adipocytes, expression of TLR2 is induced by LPSs, TNF- α , and CD14 (195). In keeping with this notion, Lin *et al.* (195) found that upon LPS binding with TLR4, TLR2 expression was acutely induced on the cell surface of adipocytes. Newly expressed TLR2 amplifies proinflammatory signals (Fig. 5). Because it seems that TLR2 shifts to a higher molecular weight, it is probably recruited by TLR4 to form a complex. Alternatively, TLR4 activation may induce the formation of intracellular effectors, which then complex with TLR2 (195). Peak levels for LPS-triggered TLR2 induction are reached within 1 h and subsequently decrease, suggesting the existence of a mechanism for negative feedback inhibition (164).

D. Modulation of TLR activity by fatty acids

Recent evidence supports the concept that fatty acids, in detail lauric and palmitic acid, can promote insulin resistance, weight gain, and associated low-grade inflammation also through targeting TLR2 and TLR4 (reviewed in Ref. 83). Saturated fatty acids have been proved to activate TLR4 in different cellular models [*i.e.*, cells transfected with TLR plasmids (160, 198), macrophages (160, 198, 199), 3T3-L1 cells (160, 161), muscle (201), and endothelial cells (202)] and in murine models of TLR4 knockout mice, TLR2-TLR4 knockout mice, and TLR4 mutant mice (161). However, a recent study (203) suggests that the effect of saturated fatty acids on TLR acti-

vation might be due to LPSs or lipopeptides contained in BSA preparations. Erridge and Samani (203) observed that saturated fatty acids alone did not activate NF- κ B in 293-cells transfected with TLR2, TLR4, or TLR5 expression plasmids. Conversely, when fatty acids were conjugated with fatty acid-free BSA, they activated NF- κ B in those cells transfected with TLR2 or TLR4/MD-2. Therefore, the authors concluded that the activation of NF- κ B by the fatty acid-BSA preparation is attributable to other contaminants in BSA preparation and not to a specific effect of saturated fatty acids. In this regard, Lee *et al.* (83) argue, in a recent review on fatty acids and modulation of PRR-mediated inflammation, that the cell model used by Erridge and Samani (203) was not a good model to verify the stimulatory effects of saturated fatty acids on TLR activation. The reason is that these fatty acids activate neither TLR2 nor TLR5 alone, but they activate TLR2 and TLR6 or TLR1 heterodimers (198). Furthermore, in a couple of studies, the content of LPSs in BSA was estimated, and it was not sufficient to activate TLR response (83).

Fatty acids seem to induce activation of TLR4 through the formation of lipid rafts, which are membrane microdomains where receptors, coreceptors, adaptors, and downstream signaling molecules colocalize (83). Although saturated fatty acids activate, polyunsaturated fatty acids inhibit dimerization and recruitment of TLR4 into lipid rafts in a ROS-dependent manner (199). The different role of saturated and unsaturated fatty acids in the modulation of TLR activity is in agreement with the huge body of literature demonstrating the detrimental role of the former and the positive effects of the latter on insulin metabolism and low-grade inflammation (reviewed in Ref. 204). For instance, during diabetes, augmented levels of circulating fatty acids from increased lipolysis can enhance the propensity to activation of TLRs in the different tissues. Of note, high blood glucose and oxidized LDLs, which are common metabolic abnormalities in diabetic patients, enhance expression and activation of TLR4 as well (205, 206). Thus, fatty acids play a pivotal role in the link between nutrition and immunity.

E. The Adonis phenotype

Loss-of-function mutations in the TLR4 gene seem to confer a particular phenotype to mice, which is characterized by stronger bones and reduced fat mass (the “Adonis” phenotype) (207). Mice carrying a single point mutation or a complete deletion of the TLR4 gene (C3H/HeJ, C.C3H-TLR4^{lpsd}, and C57Bl/10ScNCr mice) present with increased bone area, mineral content, and density, and decreased fat mass than wild-type controls. Furthermore, these animals are protected completely or partially against obesity and metabolic abnormalities due to high-fat diet (5, 160, 208, 209) or LPS infusion (5). Male C3H/HeJ

mice fed a high-fat diet for 8 wk were protected against diet-induced obesity and showed decreased adiposity and increased insulin sensitivity, increased oxygen consumption, decreased respiratory exchange ratio, and enhanced insulin-signaling capacity in adipose tissue, muscle, and liver compared with control mice. Isolated soleus muscle from wild-type and mutant mice was perfused for 4 h with palmitate (100 μ M/liter). Insulin-stimulated glucose uptake and glycogen synthesis were reduced by 40–50% in wild-type animals. Stearic and lauric acid treatments reduced glucose uptake by approximately 40 and 20%, respectively. Accordingly, a down-regulation of insulin-induced phosphorylation of IRS-1 in tyrosine and Akt in isolated soleus muscle was seen. These findings were not observed in mutant mice, which had blunted responses (209). Hampered insulin signaling in muscle and reduced insulin-induced glucose metabolism was observed in wild-type animals and not in TLR4 knockout mice also after a 5-h lipid infusion (160). In this study, Shi *et al.* (160) also investigated the role of TLR4 in energy balance in response to a high-fat diet. Interestingly, they observed that female but not male TLR4 knockout mice fed a high-fat diet had increased body weight, which was associated with increased food intake as compared with controls. This may also suggest a role for TLR4 in the regulation of appetite. Nevertheless, these fat females were partially protected against high-fat diet-induced insulin resistance (160). Moreover, TLR4 mutant or deficient mice also present with blunted alcohol-induced liver injury despite elevated endotoxin levels (142).

X. Gene Polymorphisms in Humans

It might be valuable to verify in humans whether certain polymorphisms in the TLR4/CD14 machinery carry on a sort of Adonis phenotype, or at least explain why some obese individuals are healthier than others despite a similar degree of obesity. Unfortunately, these data are still scarce and inconsistent (175–179). Persons who carry the single nucleotide polymorphism (C→T) in position –260 of the CD14 promoter have increased transcriptional activity, enhanced activity of the molecule, and higher concentrations of soluble CD14 (211). This polymorphism has been associated, but inconsistently, with risk of myocardial infarction (critically discussed in Ref. 156) and with a milder proinflammatory phenotype in diabetic patients (212).

Asp299Gly, a common variant of the TLR4 gene, attenuates receptor signaling and diminishes inflammatory response to Gram-negative pathogens (213). It has been found to be either protective (214–217) or neutral against cardiovascular events. Indeed, patients carrying this poly-

morphism presented reduced prevalence of angiographic coronary artery disease and low levels of C-reactive protein (214). In the Bruneck study (179), patients who carried the same polymorphism had less intimal-medial thickening, were at lower risk for development of newly diagnosed carotid disease over a 5-yr follow-up, and had reduced levels of C-reactive protein, IL-6, fibrinogen, and vascular cell adhesion molecule-1 than noncarriers (215). In a group of French individuals, 183 patients with acute coronary syndrome and 216 controls, the 299Gly allele was associated with a reduced risk of acute coronary events independent of standard risk factors (216). However, in a cohort of 655 men with angiographically documented coronary artery disease, the Regression Growth Evaluation Statin Study trial failed to find significant differences in incident death and myocardial infarction between carriers and noncarriers. Carriers benefited more from treatment with pravastatin for secondary risk reduction than did noncarriers. Nevertheless, the number of events was too small to infer any significant association (217). The Southampton Atherosclerosis Study also failed to find any significant association between the polymorphism and coronary artery disease or coronary risk factors (218).

The interaction between genetic background and environment may explain the inconsistent epidemiological results achieved so far concerning the association between gene polymorphisms and cardiovascular disease. Yet again, LPSs may influence the risk in individuals genetically prone to an exaggerated immune response, and particularly in people with a westernized lifestyle. Boekholdt *et al.* (219) have proposed a three-step model to explain the association of TLR4 gene polymorphism with reduced cardiovascular risk. The extent and risk of plaque rupture depend primarily upon the number of inflammatory triggers activating TLR4, and on the efficacy of TLR4 removal. Secondly, they depend upon the amount of TLR4 present in the vessel wall, and thirdly, upon the efficacy of TLR4 in mounting a local inflammatory response.

XI. Perspectives in Therapeutics

The alimentary microbiota and the TLR4 emerge as potential therapeutic targets against obesity and associated morbidities. Hence, novel therapeutic strategies targeting to modulate the gut microbiota, the microbiota-host mutualistic interaction, and the LPS-TLR4 machinery may be put forward in the future and their efficacy evaluated in longitudinal studies.

A. Modulation of the gut microbiota and microbiota-host mutualism

Antibiotics, prebiotics, and probiotics can provoke the manipulation of the gut microbiota.

In mice models of insulin resistance (*ob/ob* and diet-induced obesity mice), gut microbiota was modulated via antibiotic administration. A combination of norfloxacin and ampicillin maximally suppressed the numbers of cecal aerobic and anaerobic bacteria in insulin-resistant mice. After a 2-wk antibiotic intervention, a significant improvement in fasting glycemia and oral glucose tolerance was observed in *ob/ob* mice, and it was independent of food intake or adiposity. Reduced liver triglycerides and increased liver glycogen correlated with improved glucose tolerance in the treated mice. Concomitant reduction of plasma endotoxin and increase of adiponectin further supported the antidiabetic effects of the antibiotic treatment in *ob/ob* mice (220). Antibiotic treatment (*e.g.*, polymyxin B and neomycin) of TLR4 mutant mice fed with fructose markedly reduced fructose-induced hepatic lipid accumulation and ameliorated endotoxemia (221).

The rationale for the use of prebiotics (oligosaccharides such as the galacto-oligosaccharides; the inulin derivatives including the fructo-oligosaccharides; and soluble fibers) is based upon their capacity to stimulate the growth of beneficial bacteria (*i.e.*, Bifidobacteria and Lactobacilli) in the gut; to generate fermentation products such SCFAs with antiinflammatory effects through the binding of SCFAs to SCFA receptors on leukocytes (222); to reduce the appetite; and to mimic the pathogen binding sites that coat the surface of gastrointestinal epithelial cells and thereby may inhibit enteric pathogen adhesion and infection (reviewed in Ref. 223).

Prebiotics in combination with probiotics have been found to ameliorate liver injury in different experimental models of liver disease (reviewed in Ref. 134), including alcohol-induced liver disease (225). Conversely, the consumption of probiotics seems not to lead to any sustained compositional change in the gut microbiota and not to affect body weight (226). Nevertheless, treatment with VSL#3 (a combination of *Streptococcus thermophilus*, *Bifidobacterium*, and *Lactobacillus* species) was associated with the amelioration of the liver injury in patients with chronic liver disease (227), in experimental NASH (152, 229), and in LPS-induced liver failure (230). Use of pre- and probiotics yields encouraging results, mostly for the treatment of fatty liver disease in experimental models, but data on human obesity and cardiovascular disease are inconsistent. As far as the use of the probiotics is concerned, there are important strain-to-strain differences that can determine their effects (226). Studies are needed to address

the efficacy of the probiotics, the combination of them or with the prebiotics or the polyunsaturated fatty acids.

B. Modulation of the LPS-TLR4 interaction

Lipid A mimetics that bind to the TLR4-MD complex in a large internal pocket in MD-2 can inhibit TLR4 activation (MD-inhibitors) (231). These molecules blocked induction of LPS-induced cytokine release and symptoms after endotoxin injection in healthy volunteers (232–234). *In vitro*, they inhibited IL-6 and macrophage inflammatory protein-1 α production after LPS stimulation.

A second class of TLR4 antagonists are represented by drugs that inhibit LPS-TLR4 interaction at the intracellular domain of TLR4 (235). These molecules prevented the serum increase of a wide range of cytokines in mice injected with LPSs and protected animals from LPS-induced lethality (236). These drugs are currently being tested in phase III clinical trials in patients with septic shock (237). To the best of our knowledge, the TLR4 and MD-2 inhibitors have not been tested against obesity-associated metabolic disturbances.

XII. Conclusion

The symbiosis of microbiota and the host enriches the diversity and functionality of human metabolism. Originally, it was probably a useful means in time of famine of enhancing the ability of the host to extract calories from nutrients and to store them as fat. Today, this mechanism can be disadvantageous for human beings with a modern lifestyle mainly characterized by an imbalance between calorie intake and energy expenditure. Long-term metagenomic observations of the gut microbial community in individuals with different diseases and in different experimental settings may be helpful to understand better the contribution of gut diversity to both health and disease of the host. The aim of the ongoing International Microbiome Project (238) is to answer, at least partially, some of these open questions. A huge effort will be needed to translate preliminary data on the association between changes in microbiota and obesity into a means of defining categories of increased risk for this disorder.

Studies aimed at defining cutoff values for LPSs and/or polymorphisms in the genotype of the CD14/TLR4 machinery associated with an increased risk for cardiovascular events should be encouraged, too. At present, no data are available to verify whether measurement of LPSs and typing of LPSs based on O-antigen, core, and/or Lipid A could be useful for morbidity and mortality risk stratification in individuals with other stigmata of the metabolic syndrome. If these studies materialize and the data become available, a new definition of the metabolic syn-

drome would become necessary, including categories of high-risk microbiota, high-risk genotypes, and endotoxemia. Hence, it can be hypothesized that metabolic endotoxemia becomes, like the metabolic syndrome, not a disease entity *per se*, but a condition of increased cardiovascular risk.

This emerging relationship among gut microbiota, LPSs and the innate immune system has led to a shift in the interest of researchers studying the pathogenesis of cardiovascular disease from a scenario exclusively involving adipose tissue and muscle to one in which the main players are immune and nonimmune cells from the adipose tissue and liver. Many of the signals coming from this network converge in the portal system.

Although we have not mentioned the role of adaptive immunity, the current concept of immunity supports the existence of a continuum of immune cell populations and the interplay of diverse cells of both the innate and adaptive immune responses in host defense and the regulation of metabolic processes (238).

Future progress in this field of investigation might lead to novel therapeutic modalities (*e.g.*, probiotics and prebiotics, or immunomodulators) to reduce the impact of the western lifestyle on cardiovascular risk.

XII. Summary

This manuscript summarizes the huge body of recent literature dealing with the role of gut microbiota in modulating the host's energy metabolism. Via different mechanisms, gut microbiota can also modulate an individual's cardiovascular risk. Chronic exposure of the host to Gram-derived LPSs has been associated with the onset of insulin resistance, weight gain, and low-grade inflammation (a condition referred to as "metabolic endotoxemia"). Via binding with TLR4, LPSs can systemically trigger the proinflammatory response of the innate immune system. In doing this, LPSs act synergistically with saturated fatty acids.

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