3rd International Immunonutrition Workshop

Session 7: Prebiotics and probiotics usefulness against pathologies
Potential role of the intestinal microbiota of the mother in neonatal immune education*

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Mucosal dendritic cells are at the heart of decision-making processes that dictate immune reactivity to intestinal microbes. They ensure tolerance to commensal bacteria and a vigorous immune response to pathogens. It has recently been demonstrated that the former involves a limited migration of bacterially loaded dendritic cells from the Peyer’s patches to the mesenteric lymph nodes. During lactation, cells from gut-associated lymphoid tissue travel to the breast via the lymphatics and peripheral blood. Here, we show that human peripheral blood mononuclear cells and breast milk cells contain bacteria and their genetic material during lactation. Furthermore, we show an increased bacterial translocation from the mouse gut during pregnancy and lactation and the presence of bacterially loaded dendritic cells in lactating breast tissue. Our observations show bacterial translocation as a unique physiological event, which is increased during pregnancy and lactation. They suggest endogenous transport of intestinally derived bacterial components within dendritic cells destined for the lactating mammary gland. They also suggest neonatal immune imprinting by milk cells containing commensal-associated molecular patterns.


Understanding of the association and interactions between the intestinal microbiota and the host immune system has advanced greatly in recent years. However, mammals are born sterile and the events shaping the establishment of a flourishing intestinal microbiota remain elusive. It is known that dendritic cells (DC), via pattern recognition receptors such as toll-like receptors, sample the microbes that continuously bombard the intestinal mucosa(1). The end result is tolerance to the normal microbiota and protection against pathogenic attack. Yet, successful, simultaneous deployment of such divergent processes requires highly sophisticated control mechanisms which are not expected of an inexperienced neonatal immune system. The very survival of the neonate after the massive bacterial insult in the birth canal is testimony to solid regulatory mechanisms which may already operate during fetal life. The subsequent assembly of specific bacterial communities, in the absence of adverse immune responses, is testimony to its long-term effects and robustness. Environmental factors, including the method of delivery and the use of antibiotics, impact the final composition of the neonatal microbiota(2). However, differences between breast-fed and formula-fed infants in intestinal

Abbreviations: DC, dendritic cells; MG, mammary gland; MLN, mesenteric lymph nodes; PBMC, peripheral blood mononuclear cells; rDNA, ribosomal DNA; TP, time point; TTGE, temporal temperature gradient-gel electrophoresis.

*This article represents the presentation given at the 3rd International Immunonutrition Workshop; the original article was previously published elsewhere(42).

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bacterial colonization and in susceptibility to intestinal disease or sepsis suggest that further education is also acquired by factors in breast milk.

More specifically, breast milk provides the suckling infant with a myriad of soluble factors such as Ig, lactoferrin and lysozyme, which influence the composition of the neonatal microbiota and play a direct role in the protection of the neonatal intestine. Another possibility is that soluble pattern recognition receptors such as CD14 and toll-like receptor 2 present in milk have an indirect effect by instructing the neonatal immune system to recognize and respond appropriately to commensals and pathogens. However, there is accumulating evidence that bacteria of maternal origin are transmitted to the infant via colostrum and milk. The majority of milk organisms arise from the mother’s skin or the infant’s mouth, but certain species are suggested to colonize the neonatal intestine. Most studies on the microbiology of human milk have addressed the transmission of pathogens or contaminating commensals in samples meant for milk banks. However, it has been suggested that breast milk is not sterile, even when collected aseptically. This raised the interesting possibility that breast milk harbours a natural bacterial inoculum. Here, we examined whether some microbial species are indeed intrinsic to human milk. Confirmation of a bacterial inoculum, albeit of low dose, prompted a subsequent investigation into its origin. Since human breast milk leucocytes arise from cells which have migrated from the intestine to the lactating mammary gland (MG) via the lymphatics and the circulation, we considered that this cellular circuitry conveys microbial components in a manner which is not deleterious to maternal health. Consequently, we examined the bacterial profile associated with the cellular compartments of human milk and maternal blood and compared this to the profile in maternal and infant faeces. This was followed by a study of bacterial colonization in various tissue compartments of virgin, pregnant and lactating mice. Taken together, our results suggest that bacterial translocation is a physiological process which is augmented in the pre- and postpartum period, one of the consequences of which is the cellular transfer of bacterial components to the suckling infant via the breast milk. The biological relevance of these phenomena to the mother and to the suckling infant remain a matter of conjecture. However, if one considers the risks associated with such an evolutionary process, it is likely that the motive goes beyond simple colonization of the neonatal intestine by small numbers of milk-borne organisms.

Methods

Human milk, blood and faecal samples

Breast milk was collected from healthy lactating mothers who delivered at term. Foremilk was discarded, the breast cleaned with antiseptic soap, rinsed with sterile distilled water and dried using sterile gauze before aseptic collection using an electric breast pump. As a control, a swab of the areola was taken before milk collection. Samples of whole milk were plated on Man, Rogosa and Sharpe medium containing cysteine, on Eugon Tomato, Drigalski, Shaedler Neo Vanco or on blood agar (Bio-Mérieux, Marcy l’Etoile, France) and incubated aerobically or anaerobically at 37°C. Cells were collected from the remaining milk by centrifugation and exposed to sterile PBS containing 1% gentamycin to kill extracellular bacteria. Cells were aliquoted and used to make cytopreparations, frozen in RPMI media (Life Technologies, Basel, Switzerland) containing 10% dimethylsulphoxide in fetal calf serum for flow cytometric analysis or lysed using cold sterile distilled water and passed through a sterile needle for plating on bacterial culture media.

Venous blood (10 ml) was collected from eight lactating women at different times postpartum or from five age-matched, non-gravidus, non-lactating women. The blood was centrifuged over Ficoll-Hypaque media (Sigma-Aldrich, St. Louis, MO, USA), washed and then processed as for milk cells. Maternal and infant faecal samples were collected in sterile tubes, aliquoted and stored frozen at −80°C until required. Protocols were approved by an internal ethical committee and the Swiss authorities.

Flow cytometry

Myeloid and lymphoid DC in the peripheral blood mononuclear cells (PBMC) fraction were examined using the FACS Calibur and DC-KIT (Becton Dickinson, Basel, Switzerland). This kit contains fluorescein isothiocyanate-conjugated lineage cocktail with antibodies to CD3, CD14, CD16, CD19, CD20 and CD56; peridinin chlorophyll-protein-conjugated anti-human leucocyte antigen-DR, allophycocyanin-conjugated anti-CD11c and phycoerythrin-conjugated anti-CD123. In separate tubes, cells were labelled with fluorescein isothiocyanate-anti-CD11c and PE-anti-CD14 (Becton Dickinson) according to the manufacturer’s instructions before analysis using the FACS Calibur.

Temporal temperature gradient gel electrophoresis

Total DNA was extracted from 200 mg faecal samples and from milk and blood samples as described previously for faeces and biopsies, respectively, except that for milk and blood, DNA precipitations were done overnight and the pellets centrifuged for 1 h at 4°C.

Isolated DNA were then used as templates to amplify the V6–V8 regions of 16S ribosomal DNA (rDNA), using primers U968-GC-F and L1392-R. Several dilutions of template DNA were tested if the presence of PCR inhibitors was suspected, in order to optimize PCR yield and limit possible bias. The PCR amplification and temporal temperature gradient-gel electrophoresis (TTGE) were performed as before and Gel Compar II software (Applied Maths) used to compare TTGE profiles. A marker consisting of a PCR amplicon mix of seven cloned rDNA from different bacterial species was used to normalize the profiles.

DNA from milk, maternal blood cells and infant faecal samples at week 4 postpartum were amplified by PCR with primers U350-F and L1392-R. Ligation and cloning in pGEM-T Vector system I (Promega) were performed as described previously. Forty-eight clones per library were sequenced using the primers M13F and M13R. An equal portion of the small subunit rDNA (Escherichia coli...
position 350 to 1392) was used for the sequence analysis. These sequences were checked manually and contig were made using BioEdit software. These sequences were submitted to Genbank for preliminary analysis using the Blast programmes (Blast and Megablast) of the Ribosomal Database Project in order to identify putative, close phylogenetic relatives. The sequences were tested for chimera structure using the Ribosomal Database Project analysis service Check Chimera and during manual inspection of alignment. The sequences were aligned to their nearest neighbour with the automated alignment tool of the ARB programme package and the arb database (Technical University of Munich) of January 2004. Mother and infant sequences were compared using the Blast2sequences programme of the National Center for Biotechnology Information.

**Bacteria localization in human milk and blood cells**

After fixation in absolute ethanol, cytopreparations of human milk and blood cells were incubated for 5 min with 100 mg/ml acridine orange, washed extensively, and rehydrated using PBS. Non-specific staining was blocked using 10% fetal calf serum in PBS before labelling with fluorescein isothiocyanate-labelled hamster anti-mouse CD11c or the control isotype hamster IgG1 (Becton Dickinson) according to the manufacturer’s instructions.

**Statistical analysis**

The proportion of animals with bacteria in different tissues was analysed by the Fisher’s exact test. Differences in the median percentage of DC in human blood were compared using the Mann–Whitney test. TTGE profiles were compared using Gel Compar II software (Applied Maths, Kortrijk and Belgium). Similarity coefficients (Pearson correlation method) were then calculated for each pair of profiles, yielding a similarity matrix. A dendrogram was constructed from this matrix by using an Unweighted Pair Group Method with Arithmetic Mean algorithm.

**Results**

**Bacterial signatures common to the neonatal microbiota and maternal blood and milk cells**

To minimize contamination of expressed milk with skin bacteria or organisms residing in the superficial ducts of the breast, the foremilk was rejected and the breast cleaned with antiseptic soap prior to milk collection. No bacteria were grown from swabs of the nipple areola. Expressed milk contained a total concentration of $<10^3$ cfu/ml bacteria composed of *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Peptostreptococcus*, *Staphylococcus*, *Corynebacterium* and/or an occasional *Escherichia* species.

Since endogenous intracellular trafficking of bacterial components from the intestine to the lactating breast would implicate the lymphatic and systemic circulation, we next used TTGE to examine the bacterial rDNA content in human milk cells and maternal PBMC and faeces during lactation. Furthermore, we examined corresponding infant faecal samples to address a possible transmission of maternal bacteria via milk. The advantages of TTGE analysis lie in its sensitivity and its capacity to detect non-culturable bacteria. Thus, a more extensive number of bacteria can be catalogued.

Maternal faecal samples gave classic TTGE profiles which were specific for each individual and of greater biodiversity than those of infant faeces (Fig. 1). Although breast milk cells harboured a less complex microbiota than in maternal faeces, TTGE revealed a greater biodiversity than previously observed by plating of the milk. Thus, non-culturable bacteria in the DNA from dead bacteria may also be present intracellularly. Fig. 1 shows one mother–infant couple analysed over weeks 1–4 postpartum. Similar TTGE profiles were observed for seven other mother–infant pairs (data not shown). Interestingly, some bacterial signatures (indicated by arrows) were common to the infant faeces and to several samples of maternal origin (Fig. 1). Upon excision from the TTGE gel and sequencing, the lowest of these bands, especially intense in infant faeces and comigrating in maternal faeces and blood, was identified as *Bifidobacterium longum* on the basis of 369 nucleotides. Sequencing of the other common bands
identified DNA from *Streptococcus salivarius* and *Rhodococcus erythropolis*.

Next, PCR products were used to prepare rDNA libraries from the milk cells. Besides the species previously identified by plating and by sequencing of the excised TTGE gel bands, sequencing of milk cell clones revealed the presence of *Bacteroides*, Clostridia, *Eubacterium* and Enterobacteria among a total of 15 genera typical of ileal and colonic microbiota. The most represented bacterial genera in infant faeces were less diverse and essentially lactose degrading, lactic acid producing bacteria together with *Staphylococcus* species. Out of 23 sequences corresponding to bifidobacteria, 17 were related to *B. longum*, five to *Bifidobacterium bifidum* and 1 to *Bifidobacterium infantis*. Two identical rDNA sequences (99% identity out of 1117 base pairs), corresponding to *Streptococcus thermophilus* and *Staphylococcus epidermidis*, were identified in the milk cell clones and in the infant’s faeces (data not shown).

Interestingly, PBMC of lactating mothers contained a restricted variety of bacterial rDNA sequences (Fig. 1). Bacterial signals were also present in blood cells of non-gravidus, non-lactating women, but the complexity of bacterial signatures was greater in PBMC from breast-feeding mothers (data not shown). Profiles for control women were similar, while those of lactating women were specific for each individual.

Acridine orange staining of milk and blood cytopreparations identified bacterial bodies in association with mononuclear cells (Fig. 2).

To further define endogenous cellular transport of bacterial components, we next examined intestinal uptake of bacteria in conventional non-pregnant, pregnant and lactating mice.

In contrast to the confined bacterial translocation to MLN observed in control animals, heightened translocation to MLN in the perinatal period was followed by bacterial colonization of the breast in the immediate postpartum period (Fig. 3). While 10% control animals (C) had positive MLN cultures, 70% pregnant animals had detectable levels of bacteria in their MLN, 5–6 d before delivery (time point (TP) 1). Within 24 h postpartum, fewer animals had positive MLN cultures, but 80% of the mice had viable bacteria in the mammary tissue. Although
this value decreased to 50% by 3–4 d postpartum, it was still significantly different from that of control mice (P<0.005). From the present study, one cannot deduce whether subsequent waves of bacteria translocate at other time points later during lactation. Bacterial colonization of the MG coincided with an increased number of positive blood cultures and occasional translocation to the spleen and liver (Table 1). In contrast to pathological conditions where translocating micro-organisms are mainly Gram negative, penetrating organisms in pregnant and lactating mice included *Streptococcus*, *Lactobacillus* and *Bifidobacterium* species whose numbers gradually subsided over time (data not shown).

During lactation, bacteria were visualized histologically in the lamina propria of the small bowel, in the sub-epithelial dome and interfollicular regions of the Peyer’s patch and associated with cells in the glandular tissue of the MG (data not shown). Double labelling using antibodies to CD11c and bacterial lipoteichoic acid showed co-localization of micro-organisms and CD11c<sup>+</sup> cells suggesting that bacterial cells were inside the DC (data not shown).

The Peyer’s patches of pregnant and lactating mice were enlarged compared to those of controls, had a more prominent sub-epithelial dome and more dilated lymphatic vessels (Fig. 4). Furthermore, CD11c<sup>+</sup> cells were localized predominantly in the sub-epithelial dome and interfollicular regions of the Peyer’s patches. In the patches of pregnant and lactating animals, mononuclear cells were observed within the draining lymphatics (Fig. 4).

**Myeloid and lymphoid dendritic cells are diminished in the circulation of lactating women**

Breast milk has a high proportion of phagocytes which readily ingest microbes but are ineffective at killing (17). Recent work demonstrating that CD14<sup>+</sup> milk mononuclear cells express HLA-DR, CD86 and CD83 as well as DC-specific intercellular adhesion molecule 3-grabbing non-integrin, a C-type lectin considered to be exclusively expressed on DC, suggests that these cells are partially differentiated DC (18). Since milk DC-like cells may be derived from the maternal circulation and are potential vehicles of intestinally derived microbial components, we next evaluated the distribution of DC phenotypes in the PBMC of lactating and non-lactating women using flow cytometry. The percentages of CD123<sup>+</sup> lymphoid DC and CD11c<sup>+</sup> myeloid DC were determined after gating on lin<sup>−</sup> HLA-DR<sup>+</sup> PBMC (data not shown). In addition, the numbers of CD14<sup>+</sup>CD11c<sup>+</sup>, potential DC precursors were studied. We found that the frequencies of differentiated lymphoid DC (lin<sup>−</sup>CD14<sup>+</sup>-HLA-DR<sup>+</sup>CD11c<sup>+</sup>-CD123<sup>+</sup>) and myeloid DC, (lin<sup>−</sup>CD14<sup>−</sup>HLA-DR<sup>−</sup>CD11c<sup>−</sup>CD123<sup>−</sup>) phenotypes and CD14<sup>+</sup>CD11c<sup>+</sup> cells in the circulation of lactating women are lower than those of controls. Of note, both CD14<sup>−</sup>CD11c<sup>+</sup> and CD14<sup>−</sup>CD11c<sup>+</sup> cells are present in the circulation of control and lactating women and while there is a tendency towards a higher proportion of the CD14<sup>−</sup>CD11c<sup>−</sup> intermediate DC-like phenotype during the first week of lactation, the results were not significantly different from controls.

**Discussion**

After discarding the foremilk, we found that aseptically collected human breast milk contained a low total concentration of up to 10<sup>3</sup> cfu/ml microbes comprising *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Peptostreptococcus*, *Staphylococcus* and/or *Corynebacterium*, with an occasional *Escherichia* species. In spite of every precaution, some of these isolates may still arise from contamination. One might argue that bacterial contaminants in milk may be biologically relevant to the breast-fed infant. Indeed, *Staphylococcus* species are common constituents of the early neonatal microbiota (2). However, a bacterial presence in all samples suggests that a discrete microbe may naturally exist in breast milk. Consistent with this, we made the observation that the bacteria were associated with breast milk cells examined microscopically (Fig. 2).

Human breast milk contains an appreciable leucocyte population, the lymphocytes of which originate from the gut-associated lymphoid tissue and bronchial lymphoid

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**Table 1. Bacterial translocation to extra-intestinal tissues during pregnancy and lactation.** The proportion of animals with viable bacteria in the spleen, liver and blood was examined after plating on blood agar. Positive samples had a low number of micro-organisms (blood: 10<sup>1</sup>–10<sup>2</sup> cfu/ml; spleen and liver: 10<sup>2</sup>–10<sup>3</sup> cfu/g). Bacteria isolates were characterized by macroscopic and microscopic morphology, Gram’s staining, culture characteristics and using Fluorescence in situ hybridisation technology (42) and specific probes (Bif 1412, MWG Biotech). Time points TP1, TP2 and TP3 are the same as for Fig. 3.

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<th>Group</th>
<th>Blood</th>
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<td>Pregnant</td>
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<td><em>Bifidobacterium</em> spp.</td>
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<td><em>Staphylococcus/Micrococcus</em> spp.</td>
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<td><em>Streptococcus</em> spp.</td>
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tissue of the mother (19) and the mononuclear phagocytes from peripheral blood (20). Here, we propose that the latter also arise in the gut-associated lymphoid tissue, capture components of the luminal microbiota prior to their departure and transfer them to the suckling infant via breast milk. Since it is generally accepted that tissue macrophages are resident, non-migrating cells, we considered DC-like cells as the most likely vehicles for trafficking within the common mucosal system.

In contrast to the two or three viable bacterial genera observed by plating of individual milk samples, TTGE analysis of human milk cells revealed a wide diversity of bacterial signatures, corresponding to the dominant autochthonous ileal and colonic commensal micro-organisms. This confirms the expected uptake of bacteria at these tissue sites and suggests that non-culturable bacteria or the DNA from dead bacteria may also be present intracellularly.

Migration of bacteria, or bacterial components, within intestinally derived leucocytes to the lactating breast is supported by the observation that some of the rDNA bands on TTGE gels are common to the faecal, blood and milk samples of each mother examined. Furthermore, some of these bands are also found in infant faeces (Fig. 1) and may represent microbes transferred to the suckling infant via the milk.

To our surprise, PBMC contained a restricted variety of bacterial rDNA sequences which was more extensive in the blood of breast-feeding mothers. However, no viable bacteria were isolated from these cells. The reason for this discrepancy is not known. One could speculate that the few, bacterially laden cells may be diluted in the circulation. However, it is also possible that certain blood-borne bacteria may be quiescent or dead due to intracellular antimicrobial effects.

Awareness that some bacteria may nevertheless be environmental contaminants and that PCR amplification may have inadvertently led to false positives, we confirmed microscopically whether bacteria were associated with cells in the milk or maternal blood. Unlike sepsis where translocating bacteria are associated with polymorhonuclear cells (15), intact bacterial bodies were observed in association with a limited number (<0·1%) of milk and blood mononuclear cells (Fig. 2). However, it is not excluded that the components from dead organisms may be associated with milk and blood polymorphonuclear cells.

During lactation, bacteria were visualized beneath the epithelium of both the Peyer’s patch dome and the intestinal...
lamina propria of mice. This suggests M-cell-mediated uptake towards DC in the Peyer’s patches, direct sampling of luminal bacteria by dendrites of lamina propria DC or a low-level, physiological leakiness of the epithelium 

In any event, an extremely limited number of bacteria are thought to cross the intestinal epithelium, evade uptake and killing by intestinal macrophages, but remain viable after phagocytosis by DC 

Bacterially loaded DC are then thought to migrate to the MLN where they initiate protective immune responses 

These migrating DC phagocytose bacteria well but are relatively ineffective at killing internalized organisms 

The more prominent Peyer’s patches observed in pregnant and lactating animals, with localization of CD1c+ at the sites of noticeable mononuclear cell exit through lymphatic vessels (Fig. 4), provides indirect support of mononuclear cell-mediated transport of intestinally derived microbes to the lactating MG.

The presence of a modest population of enteric-like bacteria in association with a milk cell compartment suggests that this inoculum might serve an alternative purpose to that traditionally associated with bacterial implantation in the neonatal intestine. These organisms may indeed eventually colonize neonatal tissues, but we speculate that they educate the neonatal immune system in the recognition of commensal-associated molecular patterns of bacteria.

It has been previously shown that human milk contains soluble pattern recognition receptors for bacterial molecular motifs and that these may mediate a differential response to Gram-negative and Gram-positive organisms 

Uptake of maternal leucocytes into neonatal tissues occurs during gestation and lactation 

Perhaps prolonged penetration of inconspicuous bacterial molecular patterns via maternal DC, induces tolerogenic responses that are analogous to those for self-antigens. The TNF-family molecule receptor activator of NF-κB ligand (osteoprotegerin ligand, TNF-RANKL) and its receptor activator of NF-κB are key regulators of bone remodelling and regulate T-cell/DC differentiation and mobilization in serum samples of three out of eight mothers (range 13.9–71.7 pg/ml, data not shown).

We detected a decrease of DC subsets in the blood of lactating women. These findings are in agreement with previously reported results that showed reduced numbers of circulating DC subsets in the third trimester of pregnancy 

These decreased DC numbers may reflect cellular trafficking towards the breast or intestine. Indeed, we detected fetal liver tyrosine kinase-3 ligand, a stimulator of DC differentiation and mobilization, in serum samples of three out of eight mothers (range 13.9–71.7 pg/ml, data not shown).

Our observations not only imply a novel form of mother-infant communication, but they also highlight a potentially new mechanism of immune regulation in healthy individuals. As shown previously, the blood of normal healthy subjects contains bacterial components. Some DNA may arise from human or microbial contamination. However, the greater number of bacterial DNA signatures in the PBMC of healthy, lactating women suggests that certain bacterial species are inherent to circulating cells. It is tempting to speculate that this represents an evolutionary strategy of immune surveillance and that such bacterial imprinting maintains tolerance to specific bacterial species and alerts distant anatomical sites of changes in local lymphoid tissues. Such a mechanism may also provide a colonizing advantage to bacteria of the mother’s intestinal microbiota at a time when the low bacterial diversity in the neonatal intestine is permissive to colonization.

**Acknowledgements**

A. D.-H., J. B., P. S., I. S.-R. and E. J. S. are Nestlé employees. P. F. P., J. D., M. L. and F. L. declare no conflict of interest. P. F. P. performed animal studies, microbiology, analysis of bacteria-associated human cells and flow cytometry; J. D., M. L. and F. L. performed TTGE analysis and cloning of bacterial DNA; J. B. contributed to the animal studies and cytokine analysis in human blood; P. S. and I. S. R. prepared and analysed samples for flow cytometry and immunohistochemistry; E. J. S. and A. D. H. generated the original hypothesis, prepared and analysed human samples, contributed to the animal studies and cytokine analysis in human blood; A. D. H. and E. J. S. wrote the manuscript. All the authors discussed the results and commented on the manuscript. Much of the work described in this paper has been published elsewhere.
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