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Mini-Review

The immunological chimera mother/baby persists and functionally reverses after birth: immune monitoring of the milk and its role in active immunotherapy

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ABSTRACT

Very little is known about the timing for the T cell development in infants and young children that, in sharp contrast with mice, lasts until puberty. Our work suggests that immune regulation is the main efferent arm of the innate and adaptive immunity in pediatric age. Maternal immune cells are transferred with the milk to the baby indicating that the immunological microchimera plays an important protective role in the immune homeostasis of infants. Here we review the immune cells of the mother/ offspring microchimera in the uterus/placenta interface during pregnancy and the immune cells transferred to the baby by maternal milk after birth. We also discuss possible outcomes that may lead to pathology with a new light on T cell alloreactivity.

KEYWORDS: immunological microchimera, immune tolerance, immunotherapy, alloreactivity.

1. Introduction

Our laboratory explored over the past 10 years the innate and adaptive immune cells that define the immunity in infants and young children coming across the critical observation that the main efferent arm of the immune response after birth and in pediatric age is immune tolerance to restrain immune responses against the universe of airborne and commensal neoantigens.

In pediatric subjects dendritic cells (DC) have an important function beside antigen presentation to T cells: we described a population of tolerogenic myeloid dendritic cells (tmDC) phenotypically defined as CD11c+ CD11b+ CD14+ CD4+ and immunoglobulin-like transcript receptor (ILT) 4+ that is the most abundant cell type in the circulation in children [1]. TmDCs secrete the immunosuppressive lymphokine interleukin (IL)-10 when stimulated with the heavy constant region of immunoglobulins (Fc) and express high levels of the adenosine A_{2A} receptor ($A_{2A}R$), which, when activated by adenosine, inhibits the release of pro-inflammatory cytokines from most immune cells. TmDCs specifically control the differentiation of naïve T cells to a pro-inflammatory phenotype.

Moreover, during acute inflammation, the T cell clonal repertoire is mainly defined by regulatory T cells (Treg) [2]. The response to therapy in acute inflammatory conditions relays in boosting/ expanding natural Treg [3] and Treg with unique specificities [4, 5].

Here we like to point the attention on the role of maternal immune cells transferred through the milk in generating an immunological microchimera to protect the baby by directly responding to pathogens. We summarized data from the literature including our own recent results on a complete immune monitoring in maternal milk. A bulk of evidences prove the transfer to the baby of innate and adaptive immune cells that include mature myeloid DC, the most relevant and abundant cell population, natural killer (NK) cells, CD4+ CD8+ activated T cells, and NKT whose function is to respond to antigens.

The functional nature of the maternal microchimera changes dramatically after birth as during pregnancy the maternal cells in the placenta/uterus interface, reviewed below, are tolerogenic to prevent miscarriages due to alloreactivity, while after birth the milk provides an innate and adaptive immune cell repertoire that can directly respond to pathogens.

2. Immune tolerance by maternal cells in the interface placenta/uterus

Immune tolerance during pregnancy develops *via* cells of the innate and adaptive immune system that function at the interface between the placenta and uterus [6].

Maternal blood exchanges nutrients, gases, and metabolic waste products with the fetus in the decidua. Any immune defect within the immune regulation in the decidua in early or late pregnancy inevitably leads to miscarriage or pre-term birth. Low numbers and/or functional defects within these cell lineages contribute to premature delivery and several clinical complications in premature babies.

Decidual natural killer cells (dNK): These cells differ from the peripheral NK (pNK) and are key in the development of the decidual vessels *via* interferon (IFN)-γ production [7-9]. dNK represent 70% of the immune cells of the decidua and functional defects in this cell population correlate with miscarriage or premature birth. dNK cells are defined by specific surface markers ($CD56^{high}$, CD16-) that are in common with a small population of pNK cells (9-10%) but with different transcriptional profiles [7]. 90% of pNK are in fact $CD56^{low}$, CD16+. Functionally, dNK interacts with the HLA-C allele expressed on the trophoblast (the only histocompatibility complex expressed in the trophoblast) that binds the killer-cell immunoglobulin-like receptor (KIR) on the dNK cell surface. Collectively, the literature clearly suggests that the interactions between dNK cells and the trophoblast are essential for the success of pregnancy. dNK cells have to be regulated to prevent fetal damage by an excess of inflammatory lymphokines. Two main mechanisms control the pro-inflammatory effects of dNK: the cognate interactions with HLA-G and E (minor HLA alleles) expressed by the extra villus trophoblast and interleukin (IL)-10 secreted by the decidual stromal cells. IL-10 is a suppressive lymphokine that down-regulates inflammation and induces dNK to switch functional phenotype to start secreting IL-10 rather than IFNγ. In fact, unlike other NK cells, dNK can secrete IL-10 when in proximity to tolerogenic dendritic cells (DC-10, see below) and Treg that reach the decidua *via* the cord blood from the periphery [10].

A possible imbalance within the two immune regulatory compartments (tolerogenic DC and Treg) in the decidua may jeopardize the functional control of dNK toward IL-10-secreting cells. Imbalanced function of IFNγ-producing dNK could be a contributing cause of prematurity.

Decidual macrophages: Represent 20% of the decidual immune cells. Their most relevant feature is to provide immune regulation *via* IL-10 secretion during the first trimester [6]. Decidual macrophages can be identified with very specific markers as CD209 (DC-SIGN) that binds to the adhesion molecule ICAM-3, $CD11c^{low}$, the scavenger receptor CD163, the complement component C1q, fibronectin and matrix metalloprotease-9. Functionally these decidual macrophages are tolerogenic cells meaning they secrete the suppressive lymphokine IL-10 and they express HLA-G that engages NK receptors to downregulate NK functions and ILT4, a transcription factor that promotes IL-10 secretion. Decidual macrophages are very similar to M2 regulatory macrophages relevant in the heart, other vascular compartments and lungs but the transcriptional program of decidual macrophages is unique. Their interaction with dNK occurs *via* CD209 and ICAM-3, expressed on dNK.

DC-10: DC capture antigens and transit to the lymph nodes where they activate naive T cells and are essential for T cell priming [11]. In the mother-fetus interface HLA-G+ ILT4+ DC, named DC-10, are very important in orchestrating the early interaction with the trophoblast by creating a tolerogenic environment essential to prevent miscarriages and early delivery [12, 13]. These DC differ phenotypically from the tmDC that we described in infants and young children that do not express HLA-G and express CD4 [1].

HLA-G expression on DC leads to the polarization of T cell lineages toward a regulatory phenotype *via* the ILT4/HLA-G pathway [13, 14]. This is another difference with the pediatric tmDC that control naive T cell differentiation toward a pro-inflammatory phenotype but do not polarize Treg [1].

Treg: Treg limit inflammation and restore immune tolerance to self and allo-antigens in humans, thus playing a critical role in allergy, autoimmunity, and transplantation [15-17]. It is believed that maternal Treg play a critical role in the immune homeostasis at the mother-fetus interface but they have never been phenotypically and functionally characterized. The clinical validation for a role of Treg in the uterus/placenta interface is the evidence that miscarriages frequently occur in women with autoimmune diseases. Two main Treg lineages have been described [18, 19]: 1) natural (n)Treg that are derived from the thymus during fetal life and recognize *self* peptides [20, 21]; 2) peripherally-induced (p)Treg that recognize *not-self*, and arise from naïve T cells under appropriate conditions (i.e. transforming growth factor (TGF)-β) [22, 23] and repeated antigenic stimulation [24-26]. The specificities and lineages of Treg in the human placenta have never been studied.

3. Maternal milk as cell-based immunotherapy and its role in the response to pathogens

We have a good understanding of the role of milk as a vehicle of passive immunity in veterinary medicine [27-29], but too little has been addressed so far in humans. Below is a summary of the most relevant findings and our own immune monitoring.

4. Stem cells

Microchimerism mom-baby and the transfer of maternal stem cells *via* lactation is very well documented [30-32]. These cells persist during the course of life [33] but their differentiation and function in different tissues has still to be understood.

5. Dendritic cells, pro-inflammatory pathogen-specific T cells, NK and NKT

T cell transfer, including CD4+ T helper (Th)1 cells and CD8+ cytotoxic T cells with human colostrum and milk has been well documented [34-37]. The function of these T cells is likely to respond to pathogens as elegantly shown with the CD8+ T cell transfer specific for the human immunodeficiency virus (HIV) [38]. The protective function of maternal T cells is suggested by the demonstration that CD8+ T cells transferred through the milk migrate to the Peyer's patches, and activate very rapidly protecting the baby against the constant risk of oral/mucosal infections [39].

In murine models, maternal T cells reach the thymus and the spleen with the scope to help the development of the T cell repertoire in the offspring [40]. This is possible also in humans but difficult to be directly demonstrated.

However, it remains unclear how maternal T cells can function *in vivo* in the absence of autologous antigen presenting cells (APC). HLA complexes have been found inside exosomes in the milk [41, 42] but it is unlikely that the exosomes alone can productively re-stimulate milk-transferred T cells for their survival in the absence of APC and their co-stimulatory molecules to activate T cells. In Figures 1 and 2 we show our own data proving a very significant presence of mature and activated myeloid DC in maternal milk two weeks after delivery together with NK cells, NKT cells and activated pro-inflammatory CD4+ CD8+ T cells. The T cell transfer together with the mDC transfer suggests that probably T cells survive and re-expand getting activated by antigens presented by autologous APC.

An interesting observation is that maternal milk has very few tmDC and lacks Treg, supporting the idea that the baby's immunity is mostly dedicated to immune tolerance while the mom's immune cells transferred to the baby with the milk are responding to antigens, functionally reverting from the tolerogenic cells that define the placenta/ uterus interface.

6. Discussion

A bulk of evidence indicates that maternal cells are the first line of defense against danger in babies. The baby tolerates mom's cells (immune regulation by tmDC and Treg, the largest cell types in circulation in pediatric subjects), but the homing and fate of maternal immune cells transferred with the milk and possible outcomes that may jeopardize the health of the baby remain incompletely understood.

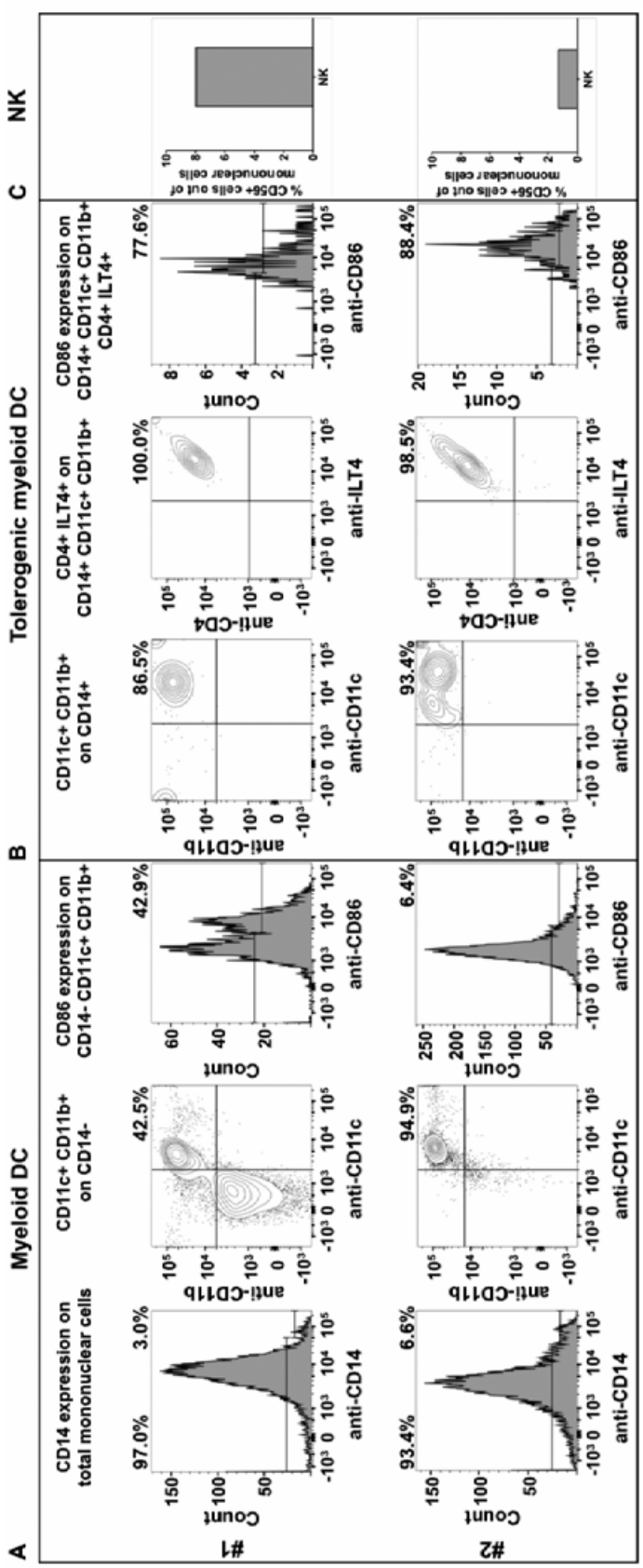

Figure 1. Immune monitoring of the innate immune cells in the milk. Milk samples (11 ml) were collected in a sterile heparin tube from two healthy moms that volunteered to provide us with milk. Mononuclear cells were separated by Ficoll gradient for immunophenotyping by flow cytometry. A combination of monoclonal antibodies was used to define monocytes, macrophages, myeloid dendritic cells, tolerogenic dendritic cells and NK cells: anti-human CD11c-APC (mouse IgG1κ, clone B-ly6), anti-human CD11b-APC/Cy7 (mouse IgG1κ, clone ICRF44), anti-human CD14-PE/Cy7 (mouse IgG2aκ, clone M5E2), anti-human CD86-FITC (mouse IgG1κ, clone FUN-1), and anti-human CD4-AF700 (mouse IgG1κ, clone RPA-T4) from BD Bioscience; anti-human ILT-4-Percp-eFluor710 (mouse IgG2aκ, clone 42D1), and anti-human CD56-PE/Cy7 (mouse IgG1k, clone CMSSB) from eBioscience. Data were acquired with FACSAria II (BD Bioscience) and analyzed using FlowJo software (BD Bioscience). **(A)** Enumeration and characterization of myeloid DC (CD14- CD11c+ CD11b+) and their CD86 expression. Very few CD14- CD11c-CD11b+ macrophages were detectable. **(B)** Analysis of myeloid markers on CD14+ innate cells. CD14+, CD11c-, Cd11b- monocytes were undetectable. The large majority of the CD14+ cells were CD11c+ CD11b+ CD4+ ILT4+ tmDC that were not abundant in the two milk samples. **(C)** Enumeration of CD56+ NK cells. CD56+ cells were detectable in the two milk samples although not in a high percent.

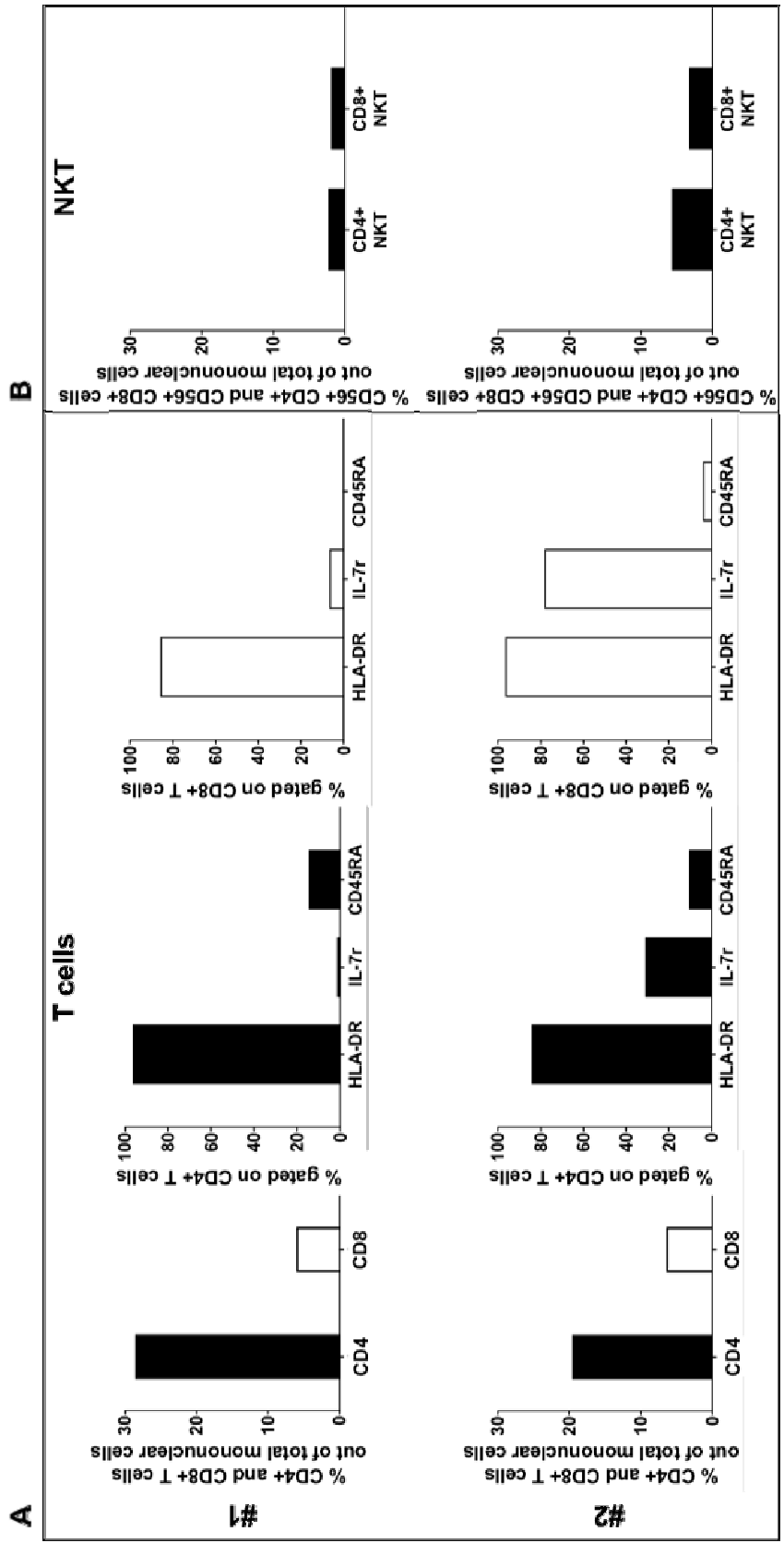

Figure 2. Immune monitoring of the adaptive immune cells in the milk. In the same milk samples studied for the enumeration of innate cells, we looked at T cells and their functional phenotype. A combination of monoclonal antibodies was used to define CD4+ T helper cells, CD8+ cytotoxic T cells, their DR expression as a marker of recent activation, interleukin (IL)-7 expression as a marker of expansion and development of memory, and CD45RA as a marker for naive T cells not antigen experienced: anti-human CD4-PerCp/Cy5.5 (mouse IgG1κ, clone RPA-T4, eBioscience), anti-human CD8-AF700 (mouse IgG1κ, clone RPA-T8, BD Bioscience), anti-human HLA-DR-APC/H7 (mouse IgG2aκ, clone G46-6, eBioscience), anti-human IL-7r-FITC (mouse IgG1κ, clone eBioRDR5, eBioscience), anti-human CD45RA-APC (mouse IgG2bκ, clone HI100, eBioscience), and anti-human CD56-PE/Cy7 (mouse IgG1k, clone CMSSB, eBioscience) were used for FACS staining. The data were acquired with FACSAria II (BD Bioscience) and analyzed using FlowJo software (BD Bioscience). **(A)** Analysis of activated and naive CD4+ and CD8+ T cells. T cells were abundant in the two milk samples and activated. Naive CD45RA+ were measurable only within the CD4+ T cell population but within the CD8+ T cell population. CD4+ $CD25^{high}$ Treg were not detectable. **(B)** Enumeration of CD4+CD56+ and CD8+CD56+ NKT cells. NKT cells were numerous and previously undescribed in maternal milk.

We suggest a paradigm that may explain mysterious pediatric acute and chronic immune-mediated inflammatory pathologies, giving a new prospective to maternal milk in pediatric diseases.

7. GVSH-like disease and surrogate moms

The baby tolerates the immunological chimera with the mom that last for a long time, probably until puberty and, in the case of the stem cells, for the rest of life.

An open question is the extent of tolerance for the baby's allo antigens by maternal immune cells outside the uterus. It is likely to envision situations where the baby's exposure to immunological danger such as acute viral, bacterial or fungal infections, allergies and its own self-inflammatory autoimmune process, could generate an inflammatory homing in the secondary lymphoid organs, in particular tonsils, adenoid and Peyer patches, where the maternal cells do not tolerate the chimera reverting toward graft versus host (GVSH)-like alloreactive T cell responses.

Examples of T cell-mediated diseases with unknown pathogenesis that affect young children are Kawasaki disease (KD) and acute vasculitis of the coronary arteries defined by CD8+ T cells that infiltrate the arterial walls [43] associated with a lymphokine storm that resemble GVSH, and juvenile idiopathic arthritis (JIA), where human leucocyte antigen (HLA) associations suggest "non-HLA restricted" T cell responses [44]. This interpretation of a pathogenic pro-inflammatory role of maternal cells needs further validation but also opens new views on the role of alloreactive T cell responses in physiopathology not confined to transplantation.

8. Tonsils and adenoid for the homing of the maternal immune cells

The summary of the results presented here raises the question of the homing for the maternal cells transferred with the milk and their fate. The viability of immune cells passing through the stomach where they encounter low pH is a matter of great debate. A rational possibility that deserves to be explored is that immune cells reach tonsils and adenoids in proximity to the palate through blood vessels. The function of the adenoids and their role in immunity is highly debated [45]. Future studies will address if tonsils and adenoids are the reservoir for maternal immune cells. Adeno tonsillar hypertrophy is very common in children that received solid organ transplantation, further suggesting that tonsils and adenoids are involved in allogeneic T cell responses [46].

9. Conclusions

The data here presented and the summary of the work from several laboratories imply the imperative need for some changes in the current practice to improve the health of the baby. The most important would be to avoid cell death by storing maternal milk at 4 °C rather than freezing and preserve cell viability.

When lactation is not sufficient to feed the baby, non-maternal sources of human milk including milk from surrogate moms (very much used in the wealthier population in the past) should be depleted of cells *via* centrifugation to avoid allogeneic-specific T cell responses (mix lymphocytes reactions-like) by the baby's T cells, that may cause inflammation in the tonsils and adenoids.

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CONFLICT OF INTEREST STATEMENT

The authors do not have conflict of interest.

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