Non-inherited Maternal Antigens, Pregnancy, and Allotolerance

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Non-inherited maternal antigens (NIMA) are those protein products derived from polymorphic genes that the mothers express but not the offspring. During normal human pregnancy, a bidirectional regulation occurs in such a way that the maternal immune system tolerates the inherited paternal antigens (IPA) expressed by the fetus and the developing fetal immune system tolerates NIMA. The process by which the described bidirectional regulation is developed is related to microchimerism, due to the bidirectional traffic of cells allowed by the decidua–trophoblast interface. An extensive body of knowledge from the transplantation and pregnancy physiology fields suggests a role for microchimerism and NIMA exposure in the development of NIMA-specific alloresponse regulation, which may include transforming growth factor β (TGF-β) as well as interleukin (IL)-10 and IL-35, producing peripheral T regulatory lymphocytes. The induction of this NIMA-specific allotolerance is called the "NIMA effect." Some experimental data suggest the existence of a "split tolerance" phenomenon associated with NIMA effect, in which regulation of NIMA-specific indirect pathway is induced without tolerogenic impact on the direct pathway. In this review, the most relevant literature about the immunological phenomena underlying the NIMA effect is discussed, including the most recent proposals about the role played by antigen-acquisition and the semi-direct pathway of allorecognition. *(Biomed J 2015;38:39-51)*

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Non-inherited antigens are those protein products derived from polymorphic genes that progenitors do express but not the offspring. In this regard, there are non-inherited maternal antigens (NIMA) and non-inherited paternal antigens (NIPA).[1]

During normal human pregnancy, a bidirectional regulation occurs in such a way that the maternal immune system tolerates the inherited paternal antigens (IPA) expressed by the fetus and the developing fetal immune system tolerates NIMA.[1,2] Both NIMA and IPA define the degree of maternal–fetal mismatch. In this immunological process, the most important antigens are the surface and intracellular proteins encoded by polymorphic genes because these kinds of antigens are immunogenic enough to elicit an alloresponse that needs to be regulated in order to keep the pregnancy homeostasis.[1,3]

Considering the two properties, polymorphism and immunogenicity, the most important IPA and NIMA in physiological terms are the major histocompatibility complex (MHC) class I and II antigens, encoded by the human leukocyte antigen (HLA)-A, -B, -C, and HLA-DR, -DQ genes in man.[4] The MHC antigens are the main antigens responsible for eliciting the alloimmune response, for instance, in the context of transplantation. Such alloimmune response does not interfere in the normal pregnancy process, even when there is a high degree of HLA mismatch due to the differences between NIMA and IPA polymorphisms. It is important to notice that the role of minor HLA antigens has been described in the context of alloimmune response that occurs when donor and recipient are matched for HLA antigens; in this case, polymorphisms in normal "self" proteins may result in the generation of peptide antigens that become

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immunogenic when bound to MHC, impacting the development of fetal immune system development. As consequence, the term NIMA usually refers to all non-inherited maternal antigens, including major (HLA) and minor (non-HLA) ones, with the major ones being more immunogenic.[5,6]

The process by which the bidirectional regulation described above is developed is related to microchimerism (Mc).[7,8] This can be defined as the presence of less than 1% of allogeneic cells in a living organism, while it is possible to detect levels of Mc as low as 0.001% due to the improvements in quantitative polymerase chain reaction (qPCR) techniques.[9] During pregnancy, the trophoblast plays a permissive role allowing bidirectional trafficking of stem cells and leukocytes, giving rise to maternal microchimerism (MMc) in the fetal tissues and fetal microchimerism (FMc) in the maternal tissues.[10,11] Both kinds of Mc persist for a long time after the end of pregnancy. It has been proposed that MMc plays an essential role in inducing tolerance toward NIMA in the immune system of the offspring, whereas FMc is associated with tolerance toward IPA in the maternal immune system.[11] It should be noted, however, that whereas NIMA tolerance may be lifelong, tolerance to IPA in mothers may often be short-lived.[12] Indeed, the discovery of HLA was made possible by anti-IPA antibodies found in the serum of multiparous women.[13]

The immune response against non–self-tissues occurs essentially against allogeneic products of HLA genes, mainly those encoding for the MHC-I and MHC-II, called as consequence major histocompatibility antigens. These are highly polymorphic genes, the inheritance of which is affected by the linkage disequilibrium phenomenon, indicating a likelihood of inheritance of two alleles together at a frequency higher than expected by a random distribution. The alleles encoding for MHC-I are called HLA-A, -B, and -C, whereas those encoding MHC-II are HLA-DP, -DQ, and -DR. The linkage disequilibrium of these genes gives rise to variable degrees of mismatch between the offspring and progenitors. An example is the linkage disequilibrium of HLA-A1 with HLA-B8 and DR-17, a common European haplotype.[4]

Pathways of alloimmune response

Figure 1 illustrates the three main pathways of allorecognition that govern the immune response between mother and her offspring, as well as between any two transplant donor–recipient pairs differing in one set of MHC antigens (indicated in red and blue) while sharing one set (indicated in gray). In brief, the direct pathway of alloresponse involves the recognition of intact non-self MHC-I or MHC-II, expressed on the surface of allogeneic cells, by specific reactive clones of CD4 and CD8 T lymphocytes.

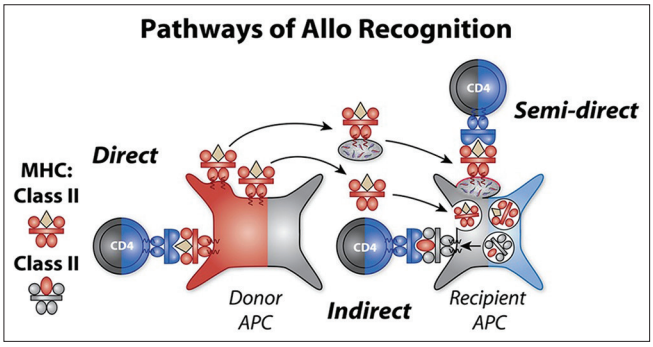

Figure 1: The three pathways of allorecognition (i.e. direct, indirect, and semi-direct) are shown in this figure. In the context of pregnancy, the role of donor cells in the fetus is played by maternal cells expressing NIMA (red). Direct pathway implies the recognition of intact allo-MHC molecules and indirect pathway implies the recognition of allopeptide–self MHC II complexes. In the semi-direct pathway, allogeneic MHC molecules (NIMA) are acquired via exosomes/trogocytosis, which contain microRNA with the capacity to reprogram the APC. IMA are shown in gray, NIMA in red, and IPA in blue.

This type of alloreactivity has long been thought to result from widespread cross-reactivity of "self + X" (where X is a viral or bacterial peptide)-specific T cells with a given allo-MHC due to T cell receptor (TCR) interaction outside the peptide binding groove, regardless of the specific peptide present in the allo-MHC.[14] However, this view of the peptide-independence of direct pathway allorecognition has been challenged recently.[15,16] The key point is that in direct allorecognition, the host T cell [Figure 1] binds to the intact donor allo-MHC/peptide complex.

On the other hand, in the indirect pathway [Figure 1], as originally described by Lechler[17] and Benichou[18] in the early 1990s, the self (or syngeneic) antigen presenting cells (APCs) phagocytose whole allogeneic cells engulf and process soluble MHC molecules released by the donor cell, and subsequently express specific allogeneic processed peptides in self MHC-II molecules. Then, host CD4 T cell clones recognize the allopeptide–self-MHC-II complex, eliciting a specific immune response. It has been proposed that indirect pathway is more important in the context of chronic graft rejection and alloantibody formation, whereas the direct pathway predominates in the early acute cellular rejection phase of alloresponses.[19] In the context of pregnancy, immigrant maternal cells play the role of the graft in the fetus and vice versa.[20,21] It is important to note that all maternal APCs coexpress NIMA and IMA MHC molecules, and thus could present peptides from either NIMA or IMA proteins on class I or class II molecules. The still debated question is once the population of maternal cells is reduced to very low levels in the adult, how can the functions of direct and indirect allorecognition of maternal antigens be maintained?

In the last decade, a new "semi-direct" pathway was described in which allogeneic cells deliver exosomes con-

taining allo-MHC molecules, which are then acquired by a specific subset of host dendritic cells (DCs) [Figure 1]. This process gives rise to a subset of host DCs expressing allo-MHC molecules on its surface, allowing its interaction with "direct-like" T cells. This experiment was originally done to solve the so-called "4 cell paradox."[21,22] This paradox consists of the fact that indirect T cells may interact only with syngeneic APCs, whereas the direct T cells can interact only with allogeneic cells. According to this idea, there is no possibility for a single APC to influence (activate or regulate) both direct and indirect T cells. The semi-direct pathway suggests the existence of syngeneic APCs expressing both self-MHC and acquired allogeneic MHC molecules, which would then reduce the required number of interacting cells from four to three, with a single APC now capable of interacting with both direct and indirect T cells, thus solving the paradox.[21]

The role of semi-direct pathway had been described as an amplifier of antiviral immune response[23] and as a primer of direct pathway in alloimmune response.[24] Nevertheless, recent data from our lab suggest that semi-direct pathway may play a role in inducing tolerance toward NIMA.[25]

Globally, the allospecific immune effector [Th1, Th17, and cytotoxic T lymphocyte (CTL) response] response must be controlled in order to establish tolerance to an allogeneic graft. In the same way, allospecific tolerance is needed during the normal development of pregnancy. This phenomenon during pregnancy does not imply a global maternal or fetal immunosuppression, but a specific regulation of the pathways of immune response toward NIMA on the fetal side and IPA on the maternal side.[26-28]

One aspect of allorecognition which is not covered in Figure 1 is the phenomenon of cross-presentation, whereby alloantigens released from one cell are processed and presented on class I molecules for recognition by CD8 T cells. Cross-presentation must occur in the area of minor antigen recognition, allowing the induction of minor-antigen–specific, class I–restricted CD8 suppressor T cells in both mother and offspring.[29] It has recently been shown that for HY minor antigens, baby girls may be exposed to HY antigens of older brothers by a transmaternal route, either by cross-presentation on class I to CD8 or by indirect presentation by class II to CD4 regulatory T cells (Tregs).[30]

Transplacental cell traffic

Since the initial proposal by Starzl *et al.*[31] about the role of Mc in the induction of allospecific tolerance, there has been a growing body of evidence suggesting that MMc is responsible for the development of tolerance toward NIMA in the fetal immune system. Since MMc implies the existence of maternal cells in offspring tissues, it implies *per se* the exposure of fetal leukocytes to NIMA.

In eutherian mammals, a variable degree of interaction and exposure exists between maternal and fetal tissues, specifically between decidua (maternal side) and trophoblast (fetal side). For example, humans, non-human primates, and mice exhibit hemochorial placentation, with the maternal blood directly contacting fetal placental tissue.[28,32] On the other hand, ruminants like sheep and cows have epitheliochorial placentation wherein maternal–fetal exchange occurs in small units termed placentomas comprising maternal caruncles and fetal cotyledons. The chances of transplacental cell traffic between mother and fetus are theoretically lower in epitheliochorial placentation than in hemochorial placentation.[33,34] However, immunologic tolerance owes its discovery by Ray Owen[35,36] to the phenomenon of placental fusion, blood exchange, and resulting mixed (50:50) chimerism between dizygotic cattle twins, made possible by epitheliochorial placentation.

Decidua is constituted by decidual stromal cells, which are differentiated endometrial stromal cells under the sustained influence of estradiol and progesterone. On the other hand, the placental trophoblast is derived from a subset of cells from the blastocyst. The function of the trophoblast is the invasion of uterine wall (decidua). Invasive trophoblastic cells originate from the trophectoderm, the external cell layer of the blastocyst. The placenta develops subsequently to reach a mature stage made of two layers of fetal trophoblast and one layer of maternal decidua. In this trophoblast–decidua unit, the main allogeneic maternal–fetus interaction occurs between decidual stromal cells and extravillous trophoblast cells. After the development of placental vessels and remodeling of uterine spiral arteries, biochemical exchange of nutrients occurs at the maternal–fetal interface, including those of toxins and hormones, while excluding macroscopic exchange of blood.[28] Nevertheless, the trophoblast plays a permissive role allowing bidirectional traffic of maternal and fetal cells. Specifically, the interface between mother and fetus allowing the passage of cells in either direction is double in mouse and human hemochorial placenta, from the fetal tissues/blood through the maternal decidua at the site of implantation, and through the layer of syncytiotrophoblast into the maternal blood lacunae bathing it. The magnitude of such an interface is variable depending on species, being, for instance, more prominent in humans, compared with mice, in which the trophoblast invasion into uterine vasculatures is negligible.[32,34] This may have an impact on the Mc phenomenon, and should be considered if murine models are used in research. Figure 2 shows a scheme of MMc phenomenon, in which maternal cells traffic through the placenta and seed into different fetal organs (mainly bone marrow and heart), allowing the exposure of fetal immune system to NIMA expressed by microchimeric maternal cells. In eutherian mammals with a

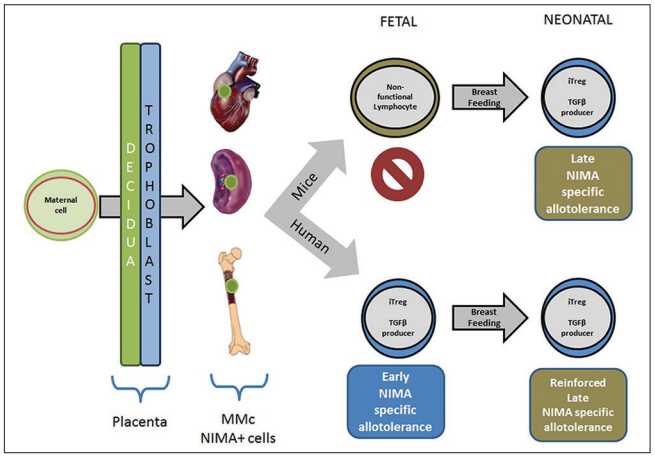

Figure 2: Schematic model for MMc development and tolerogenic impact. Maternal cells traffic through placenta (decidua–trophoblast interface) and seed in different organs. Those microchimeric cells induce NIMA-specific pTregs, producers of TGF-β. In humans, the induction of Tregs begins in fetal life, whereas in other eutherian mammals with poor fetal lymphopoiesis (like mice), the Tregs are developed after delivery in a manner dependent on breast feeding.

fully developed lymphopoiesis during fetal life, like humans, the allospecific Tregs are induced during fetal life, giving rise to an early NIMA-specific regulation, later reinforced by nursing. On the other hand, in eutherian mammals with poor fetal lymphopoiesis (like mice, which are lymphopenic until the first week after birth), the development of NIMA-specific Tregs occurs after delivery, and breast feeding plays a critical role in the establishment of persistent MMc and development of tolerance.

The immunological phenomena involved in placental implantation, which include the expression of non-classical HLA, and chemokine receptors (like CXCR3, CXCR4, CXCR6) in trophoblast and decidual cells, are complex and out of the scope of this review.[28]

Notwithstanding, this traffic phenomenon is not enough to explain the development of persistent MMc and FMc, because it implies the seeding of allogeneic cells in specific organs and the development of tolerance to NIMA and IPA, respectively, in order to create an immunologic niche where such allogeneic cells can survive.[8,9] It is proposed that induction of peripheral tolerance allowing Mc to take hold is the first step in the development of global allospecific tolerance with an impact on pregnancy physiology, as well as transplantation outcomes.[37]

It is important to note that breast feeding seems to play a significant role in the establishment of long-term MMc. In murine models, it has been shown that breast feeding is essential for the existence of life-long evidence of MMc; otherwise its magnitude tends to decay.[38] In human studies, mother-to-offspring renal transplant survival was found to benefit from a nursing history of the child.[39] It is still unclear if maternal cells in breast milk, or soluble HLA molecules are responsible for that boosting phenomenon, or the role played by gastrointestinal mucosae.[40] Recently, it has been found that monocyte-derived gastrointestinal macrophages (CX3CR1+) might be responsible for such effects by processing external antigens and transferring allopeptides to tolerogenic CD103 + DCs via gap junctions.[41]

Mc and tolerance

Starzl *et al.* analyzed the existence of Mc in female recipients of livers from male donors using fluorescence *in situ* hybridization (FISH) of Y chromosome. They found male Mc in blood, skin, and lymph nodes up to 12 years after transplant in recipients with a functional graft.[31]

Burlingham *et al.*[42] demonstrated a causal relationship between Mc and CTL unresponsiveness in a tolerant kidney transplant patient. Tolerance implies the acceptance of a functional allogeneic graft without any immunosuppressive (IS) pharmacological treatment. Peripheral blood mononuclear cells (PBMCs) were obtained from a patient 7 years post-transplant, who had been off all IS drugs for 5 years. His PBMCs were found by PCR to contain an Mc equivalent to 1 donor cell per 10^4-10^5 cells. An *in vitro* CTL assay demonstrated negligible anti-donor CTL response in primary culture; however, strong anti-donor CTL responses were recovered in secondary culture after adding recombinant IL-2. Interestingly, the anti-donor response was anti-NIMA specific since the donor was patient's mother. The anti-NIMA CTL response was again abrogated in secondary culture after adding infrequent donor cells positive for mismatched HLA, freshly isolated from patient PBMCs. This study demonstrated in a mechanistic manner that Mc induces allospecific regulation of the direct pathway of CTL response.[42] Is important to mention that at that time, the semi-direct pathway was not described yet; therefore, it was possible that in this "add-back" experiment, not only Mc cells but also antigen-acquiring APCs were associated with the abrogation of anti-NIMA response. In this case, it was clear that either Mc derived from the maternal kidney graft, pre-existing MMc, or both was associated with CTL functional unresponsiveness.

Several other studies have demonstrated a correlation between allotolerance and MMc. Most have been observational and not mechanistic studies. In this regard, a linear relationship between tissue distribution of MMc and regulation to NIMA had been described.[43,44] While this finding in mice represents a major step forward in establishing an Mc–tolerance connection, nevertheless this does not necessarily imply a direct causal relationship between the two.

In a multicenter retrospective study analyzing post-transplant kidney patients, Burlingham *et al.*[45] demonstrated that patients receiving a haploidentical HLA graft from sibling in whom the mismatch corresponded to a NIMA

show a significantly greater 10-year graft survival than those in which the mismatch was a NIPA. Overall, the only difference between the two groups that could account for the difference in outcomes was the exposure to mismatched HLA antigens during fetal/nursing period and beyond.[45]

The described studies opened the gates to the hypothesis that MMc is the vehicle that leads to fetal and continuous adult exposure to NIMA, which, by a mechanism still unclear, induced allospecific tolerance to maternal antigens. Its implications, initially described in the transplantation field, have been applied to the immunological regulation allowing the pregnancy itself.

In 2002, van Rood *et al.* found similar results as Burlingham, analyzing the incidence of graft-versus-host diseases (GVHDs) after bone barrow transplantation, in cases in which the donor was a parent or a haploidentical sibling. In that study, the grafts were non–T-cell depleted and the recipients were on remission of acute myeloid leukemia, acute lymphocytic leukemia, or chronic myeloid leukemia. Among the sibling transplantations, the incidence of GVHD was lower in the NIMA-mismatched group compared with the NIPA-mismatched group. On the other hand, the incidence of GVHD was lower in the mother-to-child transplantations compared to father-to-child ones. Nevertheless, the NIMA beneficial effect was more evident in NIMA-mismatched sibling transplantations than mother-to-child transplantations in terms of treatment-related mortality.[46]

Stern *et al.*[47] analyzed the outcome of bone marrow transplantation with lymphocyte-depleted hematopoietic stem cells (HSCs) in leukemic patients after remission (both myeloid and lymphoid acute leukemia). In that context, the event-free survival time was greater in patients receiving HSC transplantation from the mother, and as consequence being the HLA mismatch a NIMA, compared with patients receiving a NIPA mismatch transplant.[47] Van Rood *et al.* found in a retrospective study on cord blood transplants that anti-IPA effect mediates an anti-leukemic effect with negligible increase of GVHD incidence.[7]

NIMA effect – A "split" tolerance

Most of the literature about the effect of exposure to NIMA during fetal/neonatal life pertains to the field of transplantation because its relationship with allospecific tolerance and graft survival, including solid grafts (kidney, heart) and hematopoetic stem cell grafts, has been found.[3,7,37,45,47]

The first clear description of NIMA effect was made by Ray Owen *et al.* in 1954, analyzing the humoral anti-Rh response in pregnant women. They found the existence of a subset of Rh-negative women who did not develop anti-Rh antibodies during pregnancy of an Rh-positive fetus. This "tolerant" condition was strongly associated with the Rh status of the mother of the pregnant woman. In that way, the Rh-negative pregnant woman with an Rh-positive mother has an increased probability to be tolerant, suggesting the tolerogenic impact of non-inherited Rh antigens (NIMA).[48] Later on, in 1988, Claas *et al.* analyzed the anti-HLA antibody responses in chronic renal failure patients. The humoral allosensitization toward HLA as a consequence of pregnancy, graft rejection, and blood transfusions is a common issue that makes it difficult to find a compatible kidney donor. They found that as much as 50% of those patients did not develop antibodies against NIMA; no such protection was afforded to the NIPA in the same cohort of highly sensitized individuals.[49]

Another important work was an observational study by Zhang *et al*. in 1991. In that study, wherein the frequency of CTLs was analyzed, it was found that not only self-HLA antigens determine the CTL repertoire. In that study, up to 50% of homozygous twin pairs showed disparity of CTL allorepertoire, suggesting that environmental factors influence T cell selection and regulation. It was then proposed that NIMA exposure might be one such environmental factor.[50] However, in that study, neither the mechanism of such phenomenon nor its association with MMc was addressed.

Using *in vitro* CTL assays, Moretta *et al.*[51] demonstrated slight differences of the alloimmune response toward NIPA versus NIMA in cord blood cells. In the former, the clonal expansion gave rise to CD3+/CD8bright cells (flow cytometry), compatible with classical cytotoxic CD8 lymphocytes. On the other hand, NIMA stimulation induced expansion of CD3neg/CD8dim, most likely corresponding to natural killer (NK) cells, which are known to be able to induce alloresponse but not GVHD. These findings suggest the existence of CTL (direct pathway) regulation toward NIMA at birth.[51]

Nevertheless, several other studies using CTL assay have consistently demonstrated the lack of regulation of direct pathway in adults associated with exposure to NIMA during fetal/nursing period.[52-54] In fact, Akiyama *et al.*,[52] using a TCR transgenic murine model, demonstrated that NIMA-specific allotolerance is developed despite the existence of functional alloreactive CD8 T cells (anti-NIMA direct pathway). NIMA tolerance in this model depended entirely on indirect pathway Tregs. These results, along with the recent evidence of regulation of indirect pathway response to NIMA MHC and minor H antigens in adult human subjects,[29,55] suggest the existence of a "split tolerance" phenomenon associated with NIMA effect, in which regulation to NIMA-specific indirect pathway is induced without tolerogenic impact on the direct pathway.[56]

The main murine model used to study MMc is the so-called "NIMA^{d} model," in which homozygous H2^{b}

offspring are obtained from breeding a heterozygous $H2^{b/d}$ female with a homozygous $H2^{b}$ male. In this model, first described by Zhang and Miller,[57] offspring are exposed to $H2^{d}$ antigens (NIMA) during fetal life and nursing. With an inverse breeding strategy called "$NIPA^{d}$ model" (male $H2^{b/d}$ and female $H2^{d}$), an ideal control group is obtained, since $H2^{b}$ offspring share minor antigens with NIMA offspring, but they are not exposed to NIMA during pregnancy/nursing period, since $H2^{d}$ is NIPA and not a NIMA. In fact, Dutta *et al.* demonstrated using this model the importance of nursing in establishing a long-term MMc, by exchanging $H2^{b/d}$ mothers with $H2^{b}$ females during nursing period and analyzing the timeline of MMc. It was found that NIMA exposure during nursing is needed for a persistent MMc, whereas eliminating such exposure leads to a lower and transient level of MMc.[38,43,57]

Using the $NIMA^{d}$ model, Dutta *et al.*[47] and Molitor-Dart *et al.* demonstrated the rate of tolerance to full haplo-mismatch heterotopic heart graft in $NIMA^{d}$ offspring to be 47%, with the graft being homozygous for $H2^{d}$ (NIMA). The tolerogenic effect was not found if the graft was homozygous for a third-party $H2^{k}$ antigen, proving that the tolerogenic effect was NIMA-specific. On the other hand, the tolerance rate using $NIPA^{d}$ offspring as recipients was 0%.[9,58] They also found existence of MMc in different tissues (heart, liver, blood) belonging mainly to CD11c and CD11b subsets (monocyte/macrophages and DCs).[9] In the same studies, they found the existence of a MHC-II subset of cells dimly expressing H2-K^{d} in NIMA recipients.[9,58] Interestingly, such expression was transient in NIMA rejecters, but persisted lifelong in tolerant ones. Furthermore, in another work analyzing the effect of NIMA exposure in tolerance versus sensitization, Molitor-Dart *et al.*[40] found a subset of allogeneic H2-K^{d} dim positive APC in NIMA-tolerant but not in NIMA-sensitized recipient mouse strains, suggesting again the possible role of alloantigen (NIMA) acquisition and semi-direct pathway in the NIMA phenomenon associated with MMc and tolerance.[58] We will address the importance of the phenomenon of antigen acquisition in NIMA tolerance below (see section "Taking MMc, NIMA exposure, and antigen acquisition together").

The same group, using a similar approach, analyzed the regulation to NIMA using trans-vivo Delayed Type Hypersensitivity (tv-DTH). In brief, in that assay, splenocytes from a NIMA mouse were injected in the footpad of a syngeneic mouse along a recall antigen (tetanus toxoid), and 24 h later, the footpad swelling was measured. In another footpad, the splenocytes were injected with recall antigen plus antigens obtained from sonication of maternal splenocytes. The existence of a lower swelling in the latter case detected linked suppression due to regulatory (anti-inflammatory) response of Tregs present in the injected cells with specificity to NIMA. In that study, they demonstrated that allospecific regulation determined by tv-DTH predicted tolerance to a fully HLA-mismatch heart allograft expressing NIMA.[37]

An important paper by Mold *et al.* analyzed the NIMA effect in the context of pregnancy and found that such exposure leads to development of fetal tolerance *in utero* toward maternal antigens, suggesting its importance in the bidirectional regulation needed for existence of maternal–fetal unit in a physiological manner. According to their results, MMc gives rise to a population of maternal cells seeded in the lymph nodes and induces there the development of peripheral T regulatory cells (pTregs), which express an immunophenotype CD4 + CD25 + FoxP3+. They found that those cells seem to be producers of transforming growth factor β (TGF-β), an immune-regulatory cytokine that suppresses the anti-NIMA response of fetal T effector cells. Interestingly, they did not find thymic Tregs (tTregs) overproduction on analyzing fetal and neonatal thymuses. They concluded that MMc leads to the induction of Tregs in peripheral tissues, mainly in lymph nodes, but not to thymic training of NIMA-specific tTregs.[26,59] NIMA exposure was not limited to professional APCs; MMc was found in multiple lineages in cord blood including both CD4 and CD8 T cells, B cells, monocytes, and NK cells. It is, therefore, possible that some of these cells lacking co-stimulation might also anergize NIMA-specific CD4 and CD8 T cells.[26]

Along the same lines, Molitor-Dart *et al.*, using an *in vivo* Mixed leukocyte reaction (MLR) approach, injecting splenocytes from $NIMA^{d}$-exposed, $H2^{b/b}$ mice into $H2^{b/d}$ recipients, demonstrated lack of proliferation compared with $NIPA^{d}$ adoptively transferred splenocytes. Furthermore, a higher proportion of transferred TGF-β/LAP + cells was found, without significant difference in FoxP3 + cells compared with the NIPA control, suggesting the existence of a regulator phenomenon associated with induced Th3–like regulatory lymphocytes that were FoxP3 negative.[60]

The discrepant results between Mold *et al.* and Molitor-Dart *et al.* in terms of pTregs FoxP3 expression suggest that multiple regulatory T cell types are involved in controlling response to NIMA. Indeed, we have recently found that IL-35 producing Tregs, as well as TGF-β producers are equally important in NIMA-specific tolerance in humans and non-human primates.[61] On the other hand, Mold's research was performed using *in vitro* MLR assays in which direct pathway is dominant, whereas Molitor-Dart *et al.* worked with sonicated cells as the antigen source for tvDTH assay, which evaluates indirect pathway. Interestingly, Mold *et al.* were unable to detect direct pathway immunoregulation in adults by removing CD4 + CD25 + Foxp3 + Treg cells from the MLR, whereas in human tv-DTH analysis of indirect pathway, NIMA-specific regulation was consistently strong. While detailed analysis of the indirect pathway NIMA-specific Tregs in humans has not been done yet, the key T regulatory subset in a case of tolerance to a kidney al-

lograft was found to be CD4 + CD25low TGF-β/LAP + and Foxp3 negative.[62]

Araki *et al.* found indirect evidence that minor histocompatibility antigens (miHA) influence NIMA effect, using an MHC-mismatched/miHA-matched model, demonstrating lack of tolerogenic NIMA effect, and evidence of a sensitizing effect, if miHA were matched between mother and offspring. On the other hand, if both miHA and MHC were NIMA, they found a strong tolerogenic effect of NIMA exposure. They also found a correlation between MMc and the proportion of allospecific CD4 + CD25 + T cells in mice. Furthermore, high levels of MMc and Tregs were associated with the attenuation of GVHD in an MHC-mismatched bone marrow transplant model. They also could predict such tolerant status and distinguish it from sensitized status using a pre-transplant Mixed leukocyte reaction-Enzime-linked immunoSpot (MLR-ELISPOT) assay to determine the amount of interferon gamma (IFN-γ) producing cells (most likely, classic Th1 effector cells). A low response predicted high MMc-specific Treg activity and tolerance.[5,6]

Other aspects of Mc and NIMA effect

An association between certain HLA haplotypes and autoimmune diseases has been statistically demonstrated. The clearest example is the association of HLA-B27 with ankylosing spondylitis, an autoimmune disease affecting preferentially axial joints, without any clear specific humoral response. Up to 95% of patients with this disease are HLA-B27 positive, making this haplotype a major diagnostic criterion.[63]

HLA polymorphisms, mainly in class II, can increase the risk for other autoimmune diseases, for example, HLA-DR15 for Goodpasture's disease, HLA-DR3 for Grave's disease, HLA-DR3/DR4 for diabetes mellitus type 1, and HLA-DR4 for rheumatoid arthritis.[63,64]

It has been proposed that the reason for such an association is the higher molecular affinity of certain HLA products with self-peptides derived from the affected tissues, playing a role in the initiation of autoimmune response. Nevertheless, other underlying mechanisms associated like lack of tTregs and pTregs, as well as lack of thymic deletion of autoreactive T effectors cells are also associated with the development of these pathologies.[4]

Interestingly, the NIMA effect also seems to be associated with the risk of autoimmunity. For instance, the status of HLA-DR4 as a NIMA is associated with the development of rheumatoid arthritis. It means that offspring not expressing such polymorphism, but exposed to maternal HLA-DR4 during pregnancy and nursing also have an increased risk to develop this autoimmune disease.[64] Interestingly, Guthrie *et al.* found that HLA-DR4 NIMA exposure was associated with younger-onset rheumatoid arthritis, but not with older-onset rheumatoid arthritis. This increased risk of autoimmunity might be considered a deleterious consequence of the NIMA effect, contrasting with the tolerogenic impact described above in relation to pregnancy and allotransplantation.[65]

On the other hand, patients not inheriting HLA-DRB1 containing the "DERAA" sequence in the third hypervariable region (HLA-DRB1 *0103, *0402, *1102, *1103, *1302) have a decreased hazard ratio to develop rheumatoid arthritis in life, suggesting that exposure to that particular NIMA is a protective factor. It can be proposed that such a phenomenon is associated with development of pTregs, notwithstanding that it has not been demonstrated yet.[66]

Another interesting finding by Nijagal *et al.* is that pediatric patients with biliary atresia receiving a liver graft from mother have better outcome than those receiving paternal grafts. This beneficial effect was not found in patients receiving liver transplant but with a different underlying disease than biliary atresia.[67,68] They pointed out that MMc and NIMA exposure might be increased in patients with biliary atresia, playing beneficial tolerogenic effect in the context of transplantation and also probably having a pathogenic impact in development of this disease.

Mother's response to IPA: Genesis of the NIMA paradox?

The tolerogenic impact of FMc by inducing IPA-specific pTregs in mother, allowing the tolerance of fetus, appears to have been a necessary step in the evolution of viviparous mammals.[69] In fact, an increased and persistent proportion of allospecific Foxp3 + Tregs is found not only during murine pregnancy, but also after delivery, apparently having a tolerogenic effect in further pregnancies.[70] On the other hand, lower levels of Tregs and imbalance with T effector cells are observed in patients developing preeclampsia.[1,71]

Notwithstanding, there is also convincing data showing that FMc leading to IPA exposure in mothers generates sensitization, and eventually an anti-IPA T effector response,[72] with the final impact persisting long after the first pregnancy. This brings us to the so-called "NIMA paradox"[2]: The observation that a NIMA-mismatched kidney graft leads to better outcome in siblings than NIPA-mismatched ones[45] seems to contradict the finding that NIMA-mismatched grafts from maternal donors show worse outcome than NIPA-mismatched paternal grafts. Van Rood proposed that a possible explanation is that maternal anti-HLA IPA sensitization might overwhelm the tolerogenic NIMA effect.[3] This speculation was substantiated in a prospective study in a living-related renal transplant population 12 years later. It was discovered that pre-transplant regulation to NIMA only benefited the transplant between mother and daughter or mother and son pairs when regulation was reciprocated

on the maternal (anti-IPA) side. This rarely happened in the case of healthy maternal kidney donors.[55] The benefits of bidirectional regulation, and the costs of unidirectional regulation, are not yet universally acknowledged, as the role of donor-derived T cells in allografts has not yet been intensively studied. However, in cord blood transplantation, the effects of donor T cells in graft-versus-host and graft-versus-leukemia are widely accepted. Van Rood *et al.*, in a recent retrospective clinical study, found than anti-IPA maternal sensitization is an explanation for anti-leukemic effect in patients receiving an IPA-mismatched cord blood HSC transplant to replenish the bone marrow after chemotherapeutic-induced relapse of acute myeloid and lymphoid leukemia. This anti-leukemic effect, associated with anti-IPA maternal sensitization, enhances the survival of the leukemia patient.[7]

Fetal immune system

Studies addressing the impact of MMc and NIMA exposure in fetal immune system consistently demonstrate development of TGF-β producing pTregs and tolerance to maternal antigens associated with regulation of anti-NIMA T cell response.[58]

This consistent finding about the effects of tolerogenic NIMA on the fetal side demonstrates a natural tendency of fetal immune system to behave in a pro-regulatory manner. This may explain why *in utero*, both direct and indirect pathways are regulated by Tregs, whereas in the adulthood, only the indirect pathway is regulated.

The layered immune system hypothesis (proposed by Herzenberg, 1989), supported and revisited by Mold *et al.*,[73,74] suggests that fetal immune system is not just immature, unresponsive due to lack of development. In contrast, it proposes that fetal immune system is functionally different than post-delivery one and even derived from different HSC progenitors. Some evidence about a relatively constant Vγ/Vδ TCR re-arrangement in thymocytes, which progressively switches, supports this theory.[74] The final proposal is that fetal immune system is functionally pro-regulatory and prone to development of allospecific Tregs due to active properties, and not as a consequence of immaturity. According to this theory, after delivery, this system is progressively substituted for another pro-reactive system, derived from newly arising precursors, that is, another "layer" of cells appears.[74] This might be associated with the difference between hematopoietic organs during fetal life (liver) and the predominant bone marrow hematopoiesis during adult life.

It must be said that this theory is not entirely accepted and might be only partially accurate. The already known plasticity of lymphocytes raises doubts about the existence of such rigid and functional constant "layers." Instead, the particular fetal environment, the strong MMc exposure to NIMA, and the mentioned plasticity might generate the particular regulatory behavior of fetal immune system.

Further studies are needed to elucidate this issue, but the bottomline is that fetal immune system shows a clear pro-tolerogenic behavior, with a tendency to be prone to development of allospecific Tregs, responsible for the tolerant NIMA effect.

Taking MMc, NIMA exposure, and antigen acquisition together

Until now, in this review, we had exposed a representative sample of experimental and observational data about the impact of NIMA and MMc on tolerance to maternal antigens, both in the context of transplantation and pregnancy. A logical explanation would be that immunological tolerance to NIMA plays a similar role in pregnancy and transplantation, with the development of NIMA-specific pTregs being the final effector in the phenomenon and TGF-β being the most likely molecule mediating it [Figure 2]. It should be noted, however, that recent studies in our lab implicate IL-35 strongly in NIMA-specific regulation in humans and non-human primates.

The relationships between MMc (amount and number of organs seeded) and tolerance, as well as between NIMA exposure and allospecific regulation with tolerance to NIMA have been demonstrated in murine models. The current concept proposes that MMc leads to an exposure of fetal immune system to NIMA antigens and, as a consequence, a regulatory status is achieved due to the induction of Tregs.[37,38,42,44,45,60] Nevertheless, the immunological and mechanistic link between Mc and tolerance is still missing.

In this regard, the persistence of syngeneic APCs dimly expressing allogeneic MHC-I (NIMA) in tolerance, compared with its transiency in NIMA^{d}-exposed rejecters, gives rise to the possibility that semi-direct pathway (associated with antigen acquisition) plays the role of an amplifier phenomenon of NIMA effect, allowing such a small proportion of maternal cells to elicit a global immunological training, with a profound tolerogenic impact.[25,44,60] This phenomenon is observed in about half of the NIMA^{d}-exposed mice. It is possible that a certain threshold and/or quality of MMc are needed to induce the tolerogenic antigen acquisition process. On the other hand, in NIMA^{d}-exposed but non-tolerant mice, the processing of allopeptides (originating from rare MMc sources) by plasmacytoid DCs might generate functional indirect alloresponse. Indeed, we have recently found evidence of indirect pathway allopresentation not only in NIMA^{d}-exposed but non-tolerant mice, but also in "NIPA" mice from the third or fourth litters that were exposed to paternal "d" antigens via transmaternal trafficking, that is, of cells from elder H2$^{b/d}$ siblings.[75]

The vast majority of pregnancies undergo a successful progression despite (or perhaps because of[76]) HLA mis-

match between mother and fetus. Nevertheless, in the mouse, just 50% of offspring seems to benefit from NIMA effect in the context of transplantation.[37,44] It might be proposed that constant transplacental traffic of maternal cells leads to a persistent tolerogenic effect on fetal immune system, but not in all cases it leads to a sustained NIMA effect. This might be associated not only with the quantity of MMc, but also with its quality, meaning that some subsets of maternal cells are the ones capable to seed persistently in fetal tissues even after the end of the pregnancy/nursing period. It might be proposed that not only stem cells, but also APCs are needed as maternal cells to induce a tolerogenic MMc.

In this regard, Herrera *et al.* showed that only a specific subset of DCs (APCs) have the capability to acquire allogeneic antigens in a way independent of cell-to-cell interaction, and then are most likely mediated by exosomes. Furthermore, only a subset of APCs as well as endothelial cells seems to be the source of exosomes.[22]

Exosomes are nanovesicles with a diameter ranging between 50 and 200 nm and are produced in endoplasmic reticulum. In this way, reticulum-derived microvesicles containing exosomes are released into the cytoplasm. After the fusion of such microvesicles with the cellular plasmic, the exosomes are released into the extracellular media. Exosomes have specific surface markers like CD9 and CD63. Furthermore, MHC molecules are also included on its surface during its formation, apparently in an ubiquitin-dependent manner.[77] Another important characteristic is the content of microRNAs. This particular kind of RNA, typically ranging in length between 18 and 25 nucleotides, has the capability to regulate the translational process of specific genes in an epigenetic way, by interfering with the formation of messenger RNA–ribosome complex. This phenomenon is gene-specific due to the variable sequence of miRNA, allowing the complementary interaction with specific messenger RNA before its translation.[77,78] As a consequence, the acquisition of specific exosomes by APCs seems to lead to reprogramming of the antigen-acquiring cells,[79] meaning that its physiological impact is not restricted to the expression of surface allo-MHC molecules and also implying epigenetic regulator phenomena. Figure 3 shows the surface punctate pattern of acquired NIMA on myeloid DCs, which is compatible with exosome-mediated acquisition phenomenon.

The semi-direct pathway had been described as a mechanism of amplification of antiviral response, as well as a critical step in priming direct response.[24] MicroRNA may induce different reprogramming effects, not necessarily anti-inflammatory, depending on its specific sequence. Nevertheless, the evidence of alloantigen acquisition (AAq) and the potential role of microRNA in reprogramming antigen-acquiring APCs lead to the possibility that in the context of MMc, AAq plays an amplifier role of tolerogenic

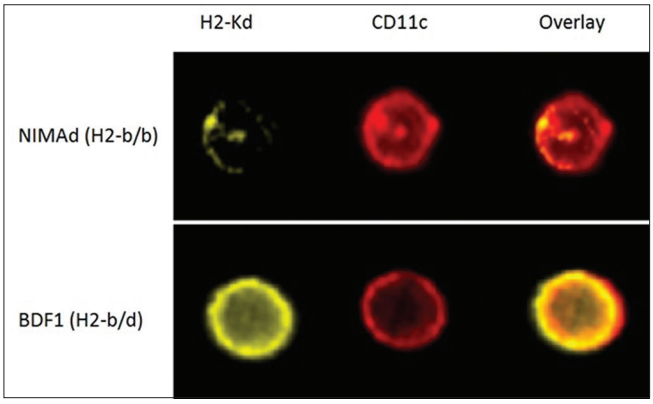

Figure 3: In the upper row, the expression of allogeneic MHC-I (H2-K^d) by H2^b myeloid dendritic cells is shown (green), demonstrating a punctate pattern compatible with antigen acquisition via exosomes. These patches are co-localized with CD11c surface expression (red) resulting in a yellow color on overlay. In the lower row can be seen the even distribution of H2-K^d surface antigen in heterozygous H2$^{b/d}$ (BDF1). The negative control B6 showed CD11c+, but not H2-K^d positive staining of mDCs. Microscopy obtained using ImageStream® ×40.

effect by enhancing the induction of pTregs, or even more, inducing anergy in alloreactive T cell clones. Some preliminary data in our lab point in that direction.[25]

Proposed model

Considering the existing body of knowledge and some recent results obtained in our lab, we propose a model in which antigen acquisition leading to semi-direct pathway is the missing link between MMc and NIMA-specific tolerance, which not only impacts transplantation outcomes but also leads to the tolerogenic effect needed for pregnancy immune homeostasis.

In this model, maternal cells' traffic (mainly constituted by maternal HSCs and APCs) reaches a certain threshold, and also involves specific leukocyte subsets, like DCs. Then an extensive MMc is generated, implying seeding in several organs. These maternal cells generate exosomes, containing NIMA on their surface and specific microRNA retained inside. The acquisition of MMc-derived exosomes by myeloid DCs not only generates a subpopulation expressing allogeneic maternal MHC molecules, but also induces a re-programming of those cells, including overexpression of *PDL1* and *CD86* [Figure 4A and B]. Such functional status change may convert immunogenic DCs into tolerogenic DCs, which are then capable not only to induce pTreg development but also to induce anergy via abortive activation in allospecific effector T cells. A split tolerance phenomenon is proposed, in which acquired allo-MHC molecules (NIMA) generate a functional semi-direct pathway, but the indirect pathway involving those tolerogenic mDCs induces anergy (T cells abortive activation) [Figure 4A]. Such

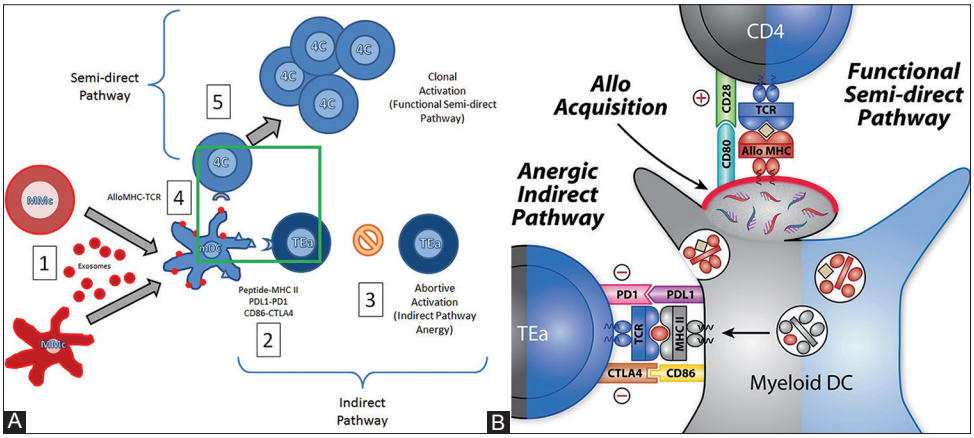

Figure 4: (A) Proposed model linking MMc, AAq, and tolerance. mDCs acquire allogeneic antigens (red) via exosomes.[1] AAq + mDCs show tolerogenic phenotype:[2] upregulation of *PDL1* and *CD86*. They induce abortive activation of NIMA-specific indirect pathway T cell clones via PDL1–PD1and CD86–CTLA4 interaction.[3] Tolerance is not uniform, but "split" since *PDL1* and *CD86* are excluded from the exosomes and also from the patches of acquired alloantigen. [4] Semi-direct alloresponse proceeds normally.[5] TCR transgenic mouse strain expressing Vα2 and Vβ6 (TEa) cells are IA^d peptide-IA^b restricted indirect pathway CD4 T cells, whereas 4C are IAd specific direct pathway CD4 T cells. (B) Details of the proposed mechanism underlying split tolerance [green square in Figure 4a]. In red is shown a patch of antigen acquisition expressing allo-MHC molecules (NIMA), but not *PDL1* and *CD86*. These allogeneic MHC molecules generate a functional semi-direct pathway (4C cells). The same antigen-acquiring myeloid dendritic cell overexpresses *PDL1* and *CD86* in the regions where indirect allopeptide presentation takes place, and then induces abortive activation (anergy) of indirect pathway CD4 T cells (TEa) via PDL1–PD1 and CD86-CTLA4 interaction.

differential behavior of indirect and semi-direct pathways has been recently demonstrated *in vitro* by Breman *et al*., proving the functionality of the latter.[80] In our model, the reason of such split tolerance might be that *PDL1* and *CD86* are excluded from exosomes and then from the patches of allo-MHC acquisition, whereas they are overexpressed in the areas where peptide–MHC II complexes are exposed in the mDC surface [Figure 4B]. This uneven distribution of *PDL1*, as well as its lack of co-localization with allo-MHC molecules has been observed on fluorescence microscopy studies in our lab.[25]

Conclusions

NIMA are those protein products encoded by polymorphic genes expressed by mother, but not by fetus. An important impact of NIMA on immune system is due to minor and majors antigens, the latter having more profound impact due to their higher immunogenicity. NIMA and IPA define the degree of HLA mismatch between mother and fetus.

Even without a macroscopic exchange of blood between mother and fetus, the placenta allows the bidirectional traffic of cells, mainly stem cells and leukocytes. This phenomenon gives rise to MMc and FMc, implying seeding of allogeneic cells in different organs, like liver, lungs, bone marrow, and heart.

MMc is the vehicle allowing the exposure of fetal, pediatric, and adult immune cells to NIMA. An extensive body of knowledge from the transplantation and pregnancy physiology fields suggests a role of MMc and NIMA exposure in the development of NIMA-specific alloresponse regulation, which may include TGF-β as well as IL-10 and IL-35[61] producing pTregs. During and after pregnancy, FMc seems to be associated with a similar effect in the maternal immune system toward IPA.[70] However, FMc more frequently sensitizes the maternal host; while the mother retains fetal cells, FMc appears to be restricted to bone marrow stem cells which express low levels of MHC antigens.[72]

Bidirectional regulation is needed for the allogeneic fetal–maternal interaction in the placental unit without mutual rejection. Such regulation allows physiological homeostasis during pregnancy. In fact, lack of maternal regulation toward IPA is associated with preeclampsia.[71] The NIMA tolerogenic effect seems to be essential in reaching such bidirectional maternal–fetal tolerance.

A still unsolved issue is how such a small proportion of maternal cells involved in MMc may elicit such a profound impact in fetal immune system. We propose that semi-direct pathway, associated with the acquisition of allogeneic MHC molecules and specific microRNA by fetal myeloid DCs (via exosomes delivered by maternal cells or via trogocytosis), plays a critical role in the amplification of MMc, enhancing NIMA exposure and inducing regulatory DC and Treg development. Nevertheless, considering that NIMA tolerogenic effect is restricted to 50% of NIMA-exposed offspring, it is possible that certain characteristics of MMc (like quantity and immunophenotype of chimeric cells) are needed to achieve NIMA-specific regulation after the neonatal period. Finally, split tolerance toward NIMA, restraining the indirect allorecognition

pathway[55] without affecting direct pathway CTL,[53,54] is developed by nearly all human offspring studied to date. (One caveat here is the relative dearth of information on NIMA effects in African, African-American, and Hispanic families; research in these communities is now underway.) The more pronounced NIMA impact seen in humans and non-human primates[61] might be a consequence of the well-developed and pro-regulatory function of their fetal immune system before delivery, unlike eutherian mammals like mice with poorly developed lymphopoiesis until after birth. This leads one to hypothesize a very strong influence of a NIMA effect in human immunity, with implications in pregnancy physiology as well as in the transplantation field. Indeed, the full clinical history of a maternal kidney transplant, in which the ultimate loss of tolerance to NIMA involved the disappearance of direct pathway T cell clonotypes from blood and graft,[81] along with the emergence of indirect pathway T cell response[82] strongly supports the conclusion that NIMA tolerance is indeed split tolerance. The semi-direct pathway [Figures 3 and 4] may provide the mechanistic explanation for the latter.

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