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Review

Idiopathic nephrotic syndrome: the EBV hypothesis

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Steroid sensitive nephrotic syndrome is marked by a massive proteinuria and loss of podocytes foot processes. The mechanism of the disease remains debated but recent publications suggest a primary role of Epstein-Barr Virus (EBV). EBV replication in the peripheral blood is found in 50% of patients during the first flare of the disease. The genetic locus of steroid sensitive nephrotic syndrome was also identified as influencing antibodies directed against EBNA1. EBV is able to establish, latent benign infection in memory B cells that display phenotypes similar to antigen-selected memory B cells. Consistently, memory B cells reconstitution after rituximab infusion is a predictor of the relapse of proteinuria. We suggest that a specific anti-EBNA1 antibody internalized in the podocytes via the neonatal Fc receptor might cross-react with a major protein present in the same cell trafficking compartment. The diversion of this major podocyte protein in the urinary space and the subsequent depletion is supposed to result in podocyte damages with loss of foot processes and massive proteinuria. Immunosuppression of B cells and subsequent clearance of anti-EBNA1 antibodies would lead to a restoration of the normal level of the protein allowing recovery of proteinuria and of normal podocyte morphology.

Idiopathic nephrotic syndrome is the most prevalent glomerulopathy in children aged 1–10 y old. The disease is marked by a massive proteinuria and a loss of podocytes foot processes (**Figure 1**) (1). Both are associated in minimal change disease but can be dissociated in experimental models of massive proteinuria (2). While the urinary leakage of the major plasma proteins is 1,000–10,000-fold compared with healthy people, no detectable glomerular damage can be observed under light microscopy, enhancing the pathophysiology mystery of Idiopathic Nephrotic Syndrome for many years. A biochemical or metabolic defect that leads to episodic and potentially reversible changes in the permeability of the glomerular basement membrane was historically considered as the basic abnormality of the disease (3). Nevertheless, the hallmark of the disease is the dramatic sensitivity to prednisone observed in most patients. The seminal publication of Shalhoub in 1974 (4) along with more recent works advocated for a primary immune disease, in which the podocytes are the functional target of the immune system, leading to massive proteinuria and loss of foot process. Both changes in the sieving coefficient of proteins by the glomerular wall and in the podocyte morphology are functional alterations completely reversible within a few days under steroid therapy (5). As a matter of fact, the complete response to steroid therapy is a paradox in this disease where glomeruli are classically free from immunoglobulin deposits and inflammatory cells and has been empirically found by chance in the early 1950s (6). Further demonstrations of the efficacy of alkylating agents, cyclosporine, and mycophenolic acid (7) as well as the recurrence of proteinuria after renal transplantation have pointed out the potential link between a dysfunction of the immune system and the kidney through the mediation of a soluble glomerular permeability factor circulating in the blood during flares of massive proteinuria (8). However, direct evidence of the abnormal subset of immune cells necessarily involved in the disease is always lacking. Moreover, several attempts to identify the immune mechanism of the disease and the circulating glomerular permeability factor, which is responsible for a 1,000–10,000 fold increase of albumin clearance, systematically failed (reviewed in refs. (9–11). This led some authors to suggest that immune mechanisms have been overstated in steroid sensitive minimal change disease, and that podocyte damage may be the direct target of environmental factors via Toll-like receptors and costimulatory signal (12,13). This hypothesis of a direct action on podocytes was further strengthened by the demonstration of the role of calcineurin in the regulation of actin cytoskeleton and the effect of cyclosporine on actin organization in the podocytes (12).

IDIOPATHIC NEPHROTIC SYNDROME, A PRIMARY B CELL DISEASE

Since Shalhoub's paper until recently, Idiopathic Nephrotic Syndrome was considered to be secondary to a T cell disorder that originates from a thymus malfunction. Five lines of evidence supported Shalhoub's hypothesis: (i) the remission of Idiopathic Nephrotic Syndrome associated with measles, (ii) the susceptibility to pneumococcal infection supposed to be in relation with a defect in T-B cooperation and secondary hypogammaglobulinemia, (iii) the rapid remission of proteinuria induced by glucocorticoids, (iv) the prolonged remission

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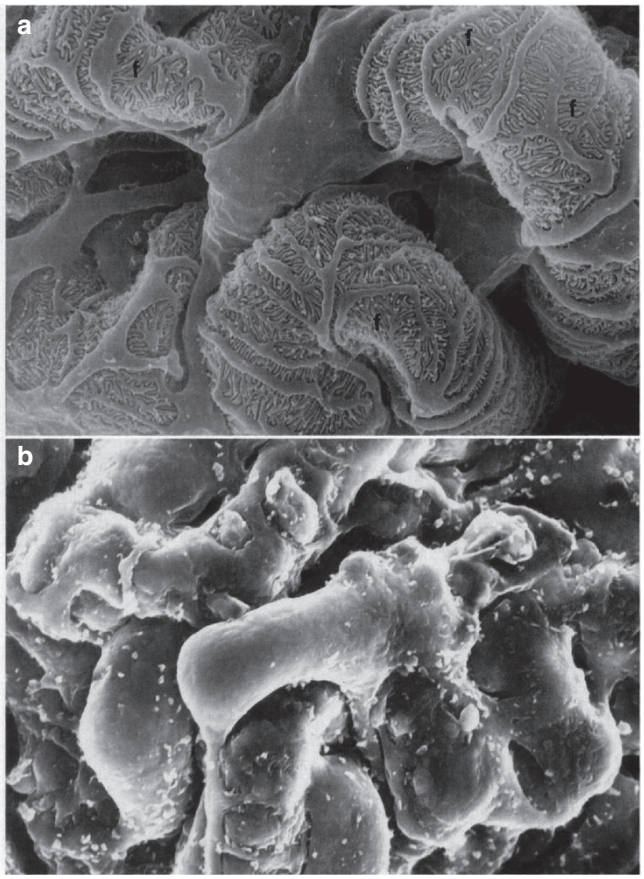

Figure 1. Loss of podocytes foot processes (foot process effacement). The most spectacular model of extensive loss of podocyte foot processes is the experimental nephrotic syndrome resulting from the intravenous injection of puromycin aminonucleoside in rats. Figures **a** and **b** are scanning electron microcopy views of rat podocytes (magnification ×3,500). The rat of Figure **a** received vehicle. The rat of Figure **b** received 180 mg/Kg of puromycin aminonucleoside in one injection and was killed at day 4 while displaying a massive proteinuria. From *Apostol et al.* (1). Reprinted with permission of publisher from ref. (1).

following cyclophosphamide therapy, and (v) the occurrence of minimal change disease in Hodgkin disease (4).

However, (i) Measles also dose-dependently impairs the proliferation of Epstein-Barr Virus (EBV)-transformed B-cell lines (14) and directly affects the synthesis of immunoglobulins, whether B cells are stimulated by antibody or by CD40L (15); (ii) The antibody response to polysaccharidic pneumococcal antigens is not under the dependency of T cells but is B cell receptor (BCR) dependent (16); (iii) Steroids are the major agent of death by neglect in the thymus (17,18) but mature B cells are also sensitive to dexamethasone-induced apoptosis (19); (iv) cyclophosphamide was historically the first treatment of multiple myeloma and has been recognized as a potent B cell and plasma cell killer (20); and (v) Hodgkin disease has long been considered as a disorder of T cell proliferation but the Reed Sternberg cells display VDJ rearrangements in the immunoglobulin locus and express numerous B cell surface markers (21).

Nowadays, the increasing therapeutic experience as well as basic investigations on Idiopathic Nephrotic Syndrome brings additional arguments for a primary B cell disease. The beneficial effect of Rituximab is certainly the most outstanding evidence supporting this hypothesis. Rituximab is a monoclonal antibody raised against CD20, a specific surface marker of B cells that is no longer expressed by the plasma cells. For more than 10 y, retrospective studies gathering hundreds of patients have suggested that B cell depletion resulting from Rituximab injection was a powerful mean to prevent the relapses of proteinuria in patients with steroid dependent nephrotic syndrome (22). More recently, two prospective randomized trials have formally evidenced that rituximab and the subsequent B cell depletion were a powerful mean to maintain a remission in those patients (23,24). Additional clinical observations and experimental facts imply B cells in the disease. High dosage of prednisolone impairs proliferation and differentiation of B cells as well as IgG production (25,26). Calcineurin inhibitors (cyclosporine and tacrolimus) widely used in Idiopathic Nephrotic Syndrome to avoid steroid intoxication also modify the balance of apoptosis, differentiation, and proliferation in B cells (27). Mycophenolate, another steroids-sparing agent in Idiopathic Nephrotic Syndrome blocks the proliferation of either T or B lymphocytes and impairs antibody production (28,29). B cell but not T cell leukemia are associated with massive proteinuria and minimal change disease (30,31). CD23, the low-affinity IgE receptor that regulates IgE synthesis and is a marker of B cell activation is increased in B cell of patients with relapse of proteinuria (32). Atopy has also been reported for a long time in childhood nephrotic syndrome, although a formal evidence of the association is still expected (33,34). The absolute count of peripheral B cells is decreased in nephrotic patients sampled during remission compared with controls (35,36). By contrast, the level of memory B cell, and especially those of switched memory B cells, is predictive of a relapse's occurrence after B cell depletion by Rituximab in steroid dependent Idiopathic Nephrotic Syndrome (37).

FROM B CELLS TO PROTEINURIA VIA IMMUNOGLOBULINS

While B cells are neither normal resident nor present in the patients' glomeruli, the natural link between the B cells on one hand and the proteinuria as well as the loss of podocyte foot processes in the other hand, should logically be a circulating antibody. Indeed, the possibility of a circulating and soluble glomerular permeability factor has been mainly based on the transfer of the disease to neonates and renal graft. Neonates born from women with an active disease display massive but transient proteinuria during the first days of life suggesting a transplacental transport of the soluble factor (38). Patients with steroid resistant forms that receive a renal graft for end stage renal failure frequently develop early recurrence of massive proteinuria and nephrotic syndrome although renal grafts come from nonproteinuric donors (8). Other special clinical observations also include the dramatic recovery of massive proteinuria in a non-nephrotic recipient receiving a renal graft from a cadaveric donor who died from a thrombotic event during a relapse of massive proteinuria (39,40) or the recurrence of a massive proteinuria on a renal graft in a nephrotic patient

that secondarily fully recovered in 48 h after being regrafted in a non-nephrotic recipient (41). Consistently with the hypothesis of a circulating antibody, immuno-adsorption of plasma immunoglobulins is able to control the massive proteinuria in patients experiencing a recurrence of the disease after a renal transplantation (42,43). Additional arguments for the involvement of immunoglobulins plasma level have been gathered from steroid sensitive patients. Changes in immunoglobulins have been recognized for a long time not only in relapse, when urinary wasting of proteins affects the level of immunoglobulins, but also in remission (44). Immunoglobulin G remains significantly decreased in remission compared with non-nephrotic controls even several years following the last relapse, suggesting a constitutional impairment of immunoglobulin G metabolism (44). Half of patients with minimal change disease display a mesangial staining with fluorescent anti-immunoglobulins antibodies, the most prevalent being antiIgM (45). In addition, IgM autoantibodies directed against actin, the major component of the foot process cytoskeleton in podocytes, have been evidenced in the plasma of some nephrotic patients but not in a set of patients with genetic nephrotic syndromes (46). The total fraction of plasma IgM extracted from patients with antiactin antibodies was able to induce a proteinuria in rats while those from nonproteinuric controls did not (46).

EBV REPLICATION AND IDIOPATHIC NEPHROTIC SYNDROME

A prospective, multicentric, and population based case–control study led in Paris area (12 million inhabitants) included incident patients aged from 6 mo to 15 y old with newly diagnosed Idiopathic Nephrotic Syndrome (the Nephrovir cohort), and controls matched for gender, age, and period of sample. The rate of EBV DNA detection was greater in total blood sampled from cases prior steroid therapy compared with controls, with no difference in the viral load (<100 copies/μg of DNA in most cases and controls). The difference remains significant after multivariate analysis accounting for race. Consistently, IgM anti-VCA antibodies were also higher in cases than in controls. These results suggest that the first flare of Idiopathic Nephrotic Syndrome is associated with EBV replication due to a recent primary infection or a reactivation, in half of patients (47). Of note, Idiopathic Nephrotic Syndrome is a rare but classical complication of Infectious Mononucleosis, a condition associated with very high EBV DNA loads (48,49), meaning that EBV replication is not the only trigger of Idiopathic Nephrotic Syndrome flares. Specificities linked to the triggering of Idiopathic Nephrotic Syndrome may be related to EBV polymorphisms, especially in the EBNA-1 protein sequence and also to a specific antibody response to EBV epitopes that may be related to human histocompatibility leukocyte antigen (HLA) polymorphisms.

Although the association between EBV replication and steroid sensitive nephrotic syndrome onset from the Nephrovir study should be interpreted with caution, a second paper indirectly supporting a role for EBV was released a few months later. Hypothesizing that coding variation may underlie the risk of steroid sensitive nephrotic syndrome, a large consortium conducted an exome array association study of the disease (50). Four common single nucleotide polymorphisms in HLA-DQA1 and HLA-DQB1 located in 6p21.32 were significantly associated with steroid sensitive nephrotic syndrome. Two of these single nucleotide polymorphisms, the missense variants C34Y and F41S in HLA-DQA1, were replicated in an independent cohort of children of white European ancestry with steroid sensitive nephrotic syndrome with a *P*-value of 10^{-17}. Seventy four other diseases have been found associated with the 6p21.32 locus, including several autoimmune diseases, atopy, lymphomas, and leukemias, nasopharyngeal cancer, chronic hepatitis, and miscellaneous conditions (http://www.ebi.ac.uk/gwas/). Interestingly, IgA nephropathy, lupus nephritis, and membranous nephropathy that are inflammatory nephropathies closely linked to auto-antibodies have been also matched in 6p21.32 (50). It turns out that the locus 6p21.32 is also linked to the ability to make up anti-EBNA-1 antibodies (51,52).

EBNA1: THE KEY FOR A STABLE VIRAL INFECTION IN MEMORY B CELLS

EBV is a B cell transforming virus able to establish a benign, lifelong, and latent infection in the resting memory B cells of 90% of the human population worldwide (53). Unlike human immunodeficiency virus, EBV load remains stable at a low level in healthy individuals. EBV uses the normal pathways of B cell biology in the germinal center of tonsils and adenoids employing the sequential expression of four virus-encoded latency transcription programs. Those programs, known as growth (latency 3), default (latency 2), EBNA1-only (latency 1), and latency (latency 0), respectively, drive infected resting naive B cells to become proliferating blasts, to participate in germinal center reactions, and finally to enter the resting memory B cell compartment (53). This sequence involves the expression of two latent membrane proteins, LMP1 and LMP2a. The latter mimicks the action of CD40 and the former induces an antiapoptotic signal via Bcl-2 family members, respectively. By providing prosurvival signal, EBV could rescue pathogenic clones from the proapoptotic environment of the germinal center, allowing them to differentiate into memory B cells. This signal is sufficient to allow survival of BCR-negative B cells, that may enter germinal centers, undergo somatic hypermutation, and persist in peripheral lymphoid tissues. EBNA1 is the only EBV protein required to replicate and segregate the EBV episomal genomes when memory B cells occasionally enter in a cell cycle division, resulting in the maintenance of the EBV genome at a stable copy number in the immune system (the EBNA1-only program) (53,54). As a consequence, EBV remains present in a subtly unique niche of the memory B cell compartment that melts into the normal immune landscape (55). Resting memory B cells latently infected with EBV can return to tonsils and adenoids, receive signals that initiate terminal differentiation in plasma cells that enter in a lytic cycle, leading to viruses' release. The released viruses can initiate a new round of naive B cell infection or infect the epithelium. The overall result is the survival of EBV in a pool of long-life

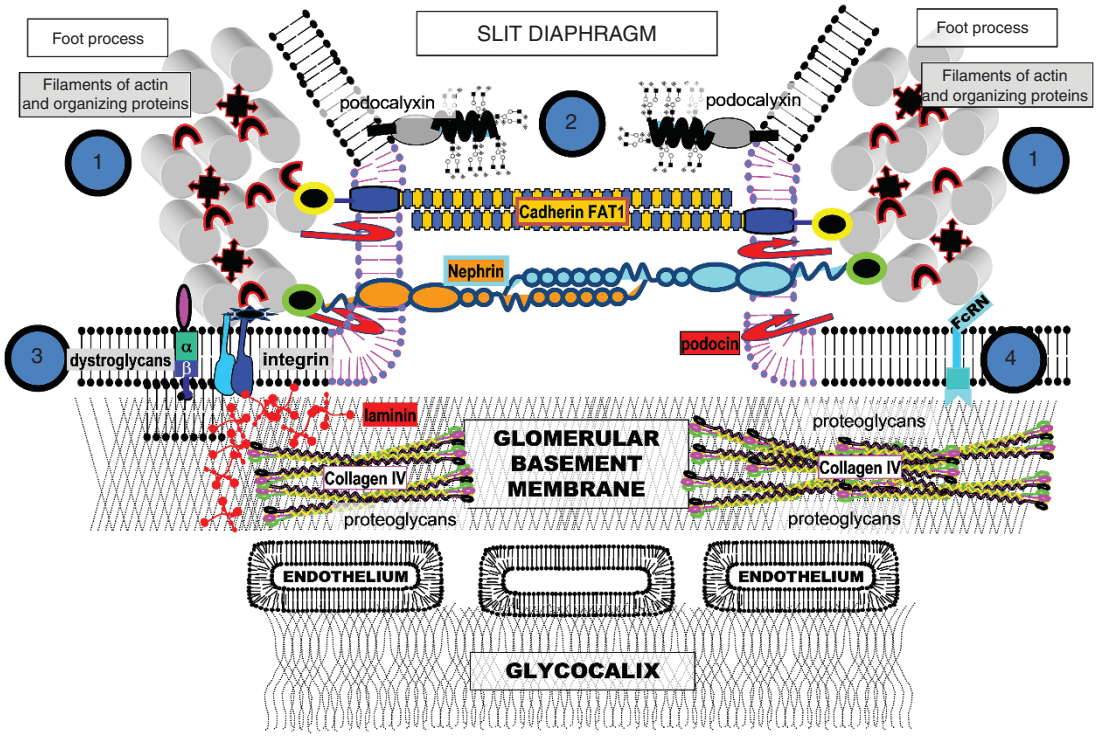

Figure 2. Simplified molecular structure of the glomerular wall. Foot processes rely on the organization of the underlying actin cytoskeleton that is similar to those of the axons ①. The free space between two processes is classically named the slit diaphragm ②. The resistance of this structure is brought about by two molecular systems of adhesion that cross the slit diaphragm and strongly stick the foot processes in vis a vis: the nephrin and the cadherin FAT ②. In addition, the foot processes are also stuck on the laminine of the glomerular basement membrane by two other systems of adhesion: the integrin and the dystroglycans ③. The synergies between these adhesion systems prevent any dislocation of the endothelium and the capillary structure. Despite the efficiency of the glycocalyx to retain immunoglobulins and albumin, a small amount of plasma proteins reach the podocyte layer, are internalized in the foot process by the neonatal Fc receptor ④ and transferred by trancytosis in the urinary chamber, beyond the slit diaphragm. See text for details and references.

quiescent memory B cells where it can persist latently all lifelong in immunocompetent host (53).

Evidences of EBV involvement as a major environmental factor in the generation of autoimmune diseases are accumulating, although a strong demonstration is still lacking (56). Autoimmune diseases are complex and multifactorial, as genetic, epigenetic, and environmental risk factors are involved. Autoimmune diseases often display with periods of waning disease activity and intermittent flares. This fits well in theory to a latent virus infection, which occasionally switches to lytic cycle, and EBV infection has for long been consistently suspected to be involved (57). The experience of acute EBV infection appears to increase the risk of developing multiple sclerosis by 20-fold, and EBV carriage has also been shown to be an independent risk factor for lupus (58,59). Of note, EBV appears to be particularly strongly associated to juvenile forms of these diseases. In addition, the persistence of EBV has been evidenced in the salivary glands of patients of Sjogren syndrome, in the synovial fluid and the bone marrow of patients with rheumatoid arthritis, in the thymus of myasthenia gravis and in the fibroblasts of patients with sclerodermia (60–62). Yet from a basic point of view, there is apparently no major arguments to support a role for EBV latency in autoimmune diseases while EBV effectively persists within self-reactive and polyreactive B cells but neither favors the survival of pathogenic autoreactive B cells nor the overexpression of self-reactive and especially polyreactive antibodies (57). Interestingly, anti-EBNA1 antibodies have been shown to cross react with peptidic sequences of myelin basic protein in multiple sclerosis, with the spliceosome Ro and Sm protein in lupus, and with collagen as well as keratin in rheumatoid arthritis (56). In addition, mice expressing the complete EBNA1 protein can develop antibodies to double stranded DNA (63). EBNA1 is a protein of 641 residues composed of an irregular copolymer of the amino acids glycine and alanine. EBNA1 displays conserved domains preventing its degradation by the proteasome as well as conferring the DNA binding ability. Beside conserved domain, EBNA1 is highly polymorphic with numerous variants in the protein sequence. Although, EBNA1 is an immunogenic viral protein, <50% of the general population is able to develop an immune response that leads to the production of antibodies specifically directed against EBNA1 (52). A significant association of anti-EBNA1 antibody levels was found with 6p21.32 in Mexican American family members. The top two independent loci in this region were HLA-DRB1 and HLA-DQB1. This finding was specific to EBV and not to 12 other pathogens that were examined. This work clearly links the immune response against EBV to the polymorphisms of MHC class II (52), that are also linked to Idiopathic Nephrotic Syndrome.

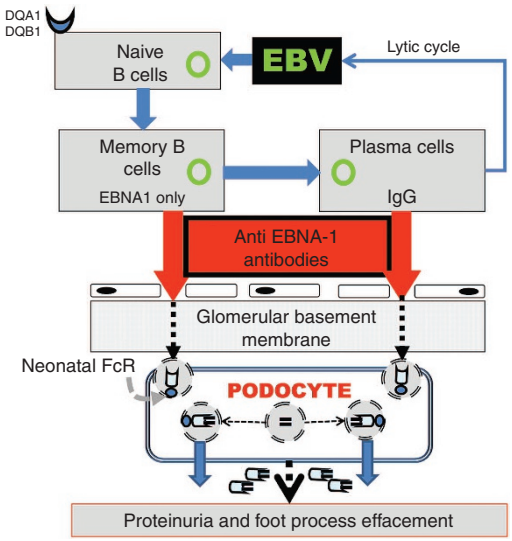

Figure 3. The EBV model in Idiopathic Nephrotic Syndrome. EBV is able to establish a lifelong, latent benign infection in memory B cells that display similar phenotype to antigen-selected memory B cells but express the latency 0 or EBNA1 only program. The ability to produce anti-EBNA1 antibodies is determined by class II Major Histocompatibility Complex DQA and DQB. The physiological internalization of anti-EBNA1 antibodies in the podocytes via the neonatal Fc receptor might lead to a cross-reaction with a podocyte protein present in the same trafficking compartment, mistakenly directing this protein toward the urinary space. See text for details and references.

PODOCYTES AND ANTIBODIES

The glomerular wall is a filter that drains water, salts, and small molecules from the blood, leaving the major proteins and the cells within the blood compartment. Podocytes provide the glomerular wall with two main characteristics essential to any filter: to maintain the integrity of the filtering wall despite a high filtration pressure, which is here half the aortic pressure in the capillary lumen and to prevent any clogging of the pores. Accordingly, podocytes display a specific morphology marked by a highly organized arborization that results in tiny foot processes that mesh letting a free space between them. Foot processes rely on the organization of the underlying actin cytoskeleton that is similar to those of the axons (**Figure 2**). The free space between two processes is permeable to water and solutes and is classically named the slit diaphragm. The resistance of this structure is ensured by two molecular systems of adhesion (**Figure 2**) that cross the slit diaphragm and strongly stick the foot processes together: the nephrin and the cadherin FAT (64). Moreover, the slit diaphragm is maintained wide-open via the surface expression of the podocalyxin that is negatively charged by numerous sialic residues able to repulse the two surfaces that limit the slit diaphragm (65). In addition, the foot processes are also stuck on the glomerular basement membrane by two other systems of adhesion (**Figure 2**): the integrin $\alpha 3\beta 1$ that is linked to the actin cytoskeleton through the complex talin-paxillin-vinculin and the dystroglycans that are linked to the basement membrane by the utrophin (66). The synergies between these adhesion systems prevent any dislocation of the capillary. From a functional point of view, most of the plasma proteins are retained at the level of the endothelial glycocalyx and in the lamina rara interna of the glomerular basement membrane (67). Nevertheless, because the podocytes form the final barrier to glomerular filtration, they possess an active transport system to clear the small amount of proteins that reaches the external face of the glomerular basement membrane; otherwise accumulated proteins would clog the slit diaphragm. Accordingly, the neonatal Fc receptor, which is able to take either in charge albumin or immunoglobulins, the major proteins in the blood flow, is expressed by the podocyte and are especially located on the foot processes (68). As already demonstrated in the placenta, lungs, breast, and intestinal epithelial cells, the neonatal Fc receptor binds to those proteins after endocytosis and acidification, and releases its cargo in the urinary space at physiological pH (68). Consistently, mice lacking the neonatal Fc receptor accumulate immunoglobulins in the glomerular basement membrane and the subpodocyte space between the podocyte and the glomerular basement membrane (69). Of note, there is a paradox between the small amount of proteins that comes in the subpodocyte space and the massive proteinuria in case of podocyte damage (70). The same paradox can also be raised for congenital nephrotic syndrome where the defect of nephrin, a protein only expressed in the slit diaphragm, leads to severe antenatal and neonatal nephrotic syndrome.

THE EBV MODEL IN IDIOPATHIC NEPHROTIC SYNDROME

Based on all the previous findings reported above, we hypothesized a possible integrated mechanism for Idiopathic Nephrotic Syndrome (**Figure 3**). It is likely that anti-EBNA1 antibodies that reach the glomerular basement membrane are internalized into the podocyte via the neonatal Fc receptor as any other antibody, regardless to its specificity. Thereafter it is supposed that anti-EBNA1 antibodies cross react with a major podocyte protein that is present in the same trafficking compartment, directing by mistake this protein toward the urinary space. Massive proteinuria and loss of foot processes might consequently result from the accidental depletion of a major podocyte protein. Since the ability of the podocytes to produce the intact protein remains normal, the clearance of anti-EBNA1 in the blood circulation due to the suppression of B cells by steroids or other immunosuppressive treatments would dramatically restore the normal level of the protein leading to the full recovery of the massive proteinuria and of the foot processes organization. The long lasting remissions obtained with cyclophosphamide and rituximab that are CD20-depleting drugs in 30–40% of patients with steroid dependency are also consistent with a central role of EBV latency in B cells. This mechanism might fully explain the unique status of Idiopathic Nephrotic Syndrome as a cold autoimmune disease without any inflammatory features, where high dosage of steroids are able to restore a normal urinary excretion of protein (a few mg/day) in a very short delay. To date, no cross reacting anti-EBNA1 antibodies targeting a podocyte protein have been evidenced in patients with steroid sensitive nephrotic syndrome, however this possibility have not been explored yet and should be investigated.

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