POINT OF VIEW

New insights into SMA pathogenesis: immune dysfunction and neuroinflammation

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Abstract

Spinal muscular atrophy (SMA) is a neuromuscular disorder characterized by motor neuron degeneration, although defects in multiple cell types and tissues have also been implicated. Three independent laboratories recently identified immune organ defects in SMA. We therefore propose a novel pathogenic mechanism contributory to SMA, resulting in higher susceptibility to infection and exacerbated disease progression caused by neuroinflammation. Overall, compromised immune function could significantly affect survival and quality of life of SMA patients. We highlight the recent findings in immune organ defects, their potential consequences on patients, our understanding of neuroinflammation in SMA, and new research hypotheses in SMA pathogenesis.

Spinal muscular atrophy as a multi-organ disorder

Spinal muscular atrophy (SMA) is a devastating neuromuscular disorder clinically characterized by paralysis and muscle weakness. It affects 1 in 6000 to 10,000 live births, making it one of the most deadly genetic disorders in infants.[1] In 1995, the genetic basis of SMA was shown to be due to mutations or deletions of the *Survival Motor Neuron 1* (*SMN1*) gene.[2] The encoded protein, termed SMN, is ubiquitous and complete knock out is embryonic lethal.[3] Consistent with this, SMA is not a disease of complete absence of the SMN protein but rather due to the low basal levels of SMN produced by a second nearly identical copy of *SMN1*, termed the *SMN2* gene.[4]

The preferential susceptibility of motor neurons to low levels of SMN is still poorly understood. However, in the last 5 years, a number of studies have highlighted abnormalities in other cell types in SMA (reviewed in[5,6]). In addition, studies have highlighted the benefit of systemic delivery of therapeutic compounds when compared to CNS restricted delivery on the phenotypic amelioration in preclinical studies.[7,8] Altogether, SMA is emerging as a multi-organ disorder rather than simply a motor neuron disease per se.

Lymphoid organ defects are a consistent feature in different SMA mouse models

This past year has marked a major development into the contribution of immune organ defects to SMA pathogenesis. Indeed, our group along with two others has independently published a whole array of abnormalities in

immune organs, such as the spleen and thymus, in different SMA mouse models (Fig. 1).[9–11] The first striking abnormality in the lymphoid organs consists of a considerable reduction in spleen size, which was observed in four SMA mouse models of different severity.[9–11] Interestingly, the reduction in spleen size was noted before any motor impairment, with the milder $Smn^{2B/-}$ model showing the most drastic splenic atrophy.[11] Similarly, the spleen architecture was disrupted in three out of four SMA mouse models. Reduction of red pulp area, loss of clear white and red pulp borders and loss of B-cell follicles were described.[9–11] Increased fibrotic tissue and abnormal accumulation of smooth muscle cells were also noted.[9,11] Interestingly, the most severe mouse model, termed $Smn^{-/-};SMN2$, did not show any structural abnormalities.[11] This might be due to its limited lifespan, which may not permit the full phenotype to develop. These defects might be particularly clinically relevant since SMA type 1 patient necropsies showed an array of splenic abnormalities such as accessory spleens, congested red pulp and increased numbers of precursors.[9]

The reasons behind the small spleen sizes in the SMA mouse models are not clear. Proliferation of cells in the spleen appeared abnormal only at late stages in the

Taiwanese SMA model mice, while it remained unchanged in the $Smn\Delta7$ mice.[9,10] Investigation of cell death revealed very little change in the Taiwanese model.[9] Vasculature abnormalities were reported in other organs and could have been the initial trigger.[12,13] Immunostaining of blood vessels markers revealed very little change in the spleens of Taiwanese and $Smn\Delta7$ mice.[9,10] However, the $Smn^{2B/-}$ spleens displayed increased necrosis on gross morphological observation, which may suggest abnormal blood flow.[11] In a similar manner, smooth muscle cell clumping observed in these spleens may be due to abnormal vasculature.[11.]

The thymus was examined in $Smn^{2B/-}$ and the $Smn^{-/-};$ $SMN2$ mice, although no significant differences in size were observed.[11] Nonetheless, the histological structure in the thymus showed cortex thinning and increased apoptotic bodies in these two mouse models of SMA.[11] As the thymus is primarily responsible for T-cell maturation, it was proposed that T-cells might be stalled in maturation, leading to smaller spleens. T-cell development was abnormal at late stages of the disease, but relatively normal at presymptomatic stages.[11] This infers that the defects in the thymus are either a consequence of abnormalities in the spleen and the periphery, or a totally independent process.

Importantly, these processes appeared mediated by SMN. Firstly, SMN levels in wild type spleen and thymus were strikingly high, at par or higher than in spinal cord and considerably higher than in skeletal muscles.[11] Secondly, ubiquitous introduction of one copy of $SMN2$ in the $Smn^{2B/-}$ mice rescued splenic and thymic phenotype.[11] In a similar manner, intracerebroventricular injection of antisense oligonucleotides also abrogated the splenic defects in the $Smn\Delta7$ spleens.[10] The latter findings highlight the potential importance of autonomic nerve fibers in triggering lymphoid organ defects.

Potential functional consequences of lymphoid organ defects in SMA

Primary lymphoid organs include the bone marrow and the thymus. These organs are involved in immune cell maturation. Secondary lymphoid organs include the spleen, the lymph nodes and mucosal-associated lymphoid tissues (MALT) (Peyer's patches, adenoids, tonsils and others - reviewed in[14]). These organs act as sentinels against foreign bodies and antigens that could be harmful to our bodies. The spleen has a wider array of functions, including filtrating senescent red blood cells out of the circulation, iron homeostasis, and acting as a reservoir for platelets, red blood cells and white blood cells (Fig. 2).[15] Defects in any one of these organs could lead to great functional consequences as detailed below.
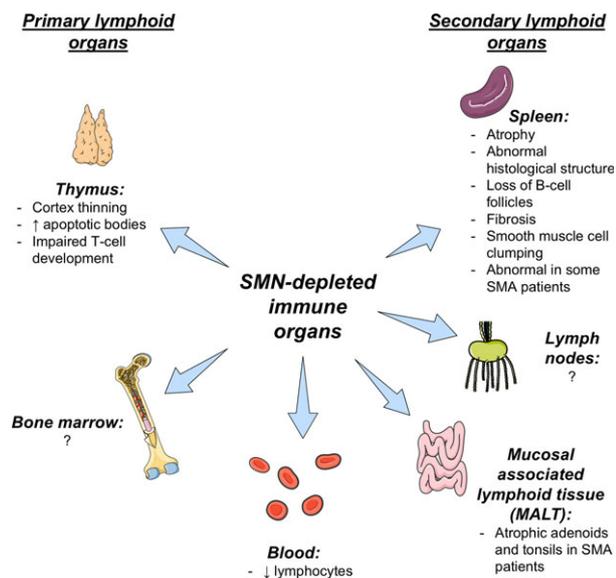

Figure 1. A summary of immune organ defects described from studies on SMA model mice. Spleens have the most drastic changes, although cortex thinning has also been described in the thymus. Very limited information is available on the status of the bone marrow, lymph nodes, and mucosal associated lymphoid tissues MALTs in SMA. The schematic art pieces used in this figure were provided by Servier Medical art (http://servier.com/Powerpoint-image-bank). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.
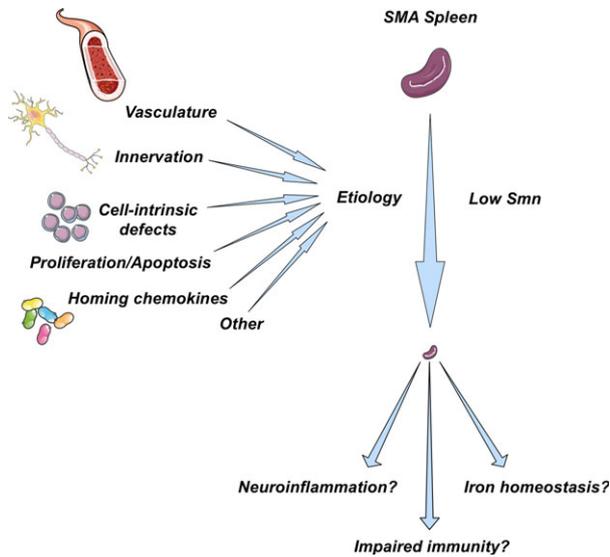
Figure 2. Possible mechanisms leading to small spleens in SMA mice and its consequences. Vasculature, innervation, cell-intrinsic defects, proliferation/apoptosis and homing chemokines could all lead to small spleen size. The consequences of small spleen size include neuroinflammation, impaired immunity, and iron homeostasis defects. The schematic art pieces used in this figure were provided by Servier Medical art (http://servier.com/Powerpoint-image-bank). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

Immunity

It is likely that the multiple abnormalities observed in the lymphoid organs of the SMA mice result in immune impairment. The structure of the spleen is crucial for its function.[16] For example, the incoming blood goes through the marginal zone, where many macrophages and B-cells act to filter the blood for foreign antigens.[16] In this manner, they trigger an innate immune response directly but also an adaptive immune response given their close proximity to the white pulp region, where T-cells and B-cells are located.[16] Moreover, the spleen contains unique subsets of specialized macrophages that have better recognition for certain pathogens.[17–20] Indeed, this places the spleen as a major player in the clearance of encapsulated bacteria like *Mycobacterium tuberculosis* and *Streptococcus pneumoniae*, but also of *Staphylococcus aureus*, and potentially of viruses.[16–20] Moreover, asplenic patients are immunodeficient, are recommended to be on a more stringent immunization schedule, and more aggressive clinical management is initiated at an earlier stage when facing fever or infection.[21,22]

In SMA mouse models, the decreased spleen size and the disrupted splenic structure is likely to result in impaired immune functions against pathogens. Additionally, loss of

B-cell follicles, diminished circulating lymphocytes, and abnormal T-cell maturation are in keeping with this possibility. The fact that SMA patients also harbor various splenic abnormalities,[9] highlights that this phenomenon is also relevant to human patients. It is interesting to note that early reports described atrophic Waldeyer's ring and cervical lymph nodes in addition to impaired cell-mediated immunity, as assessed by lymphocyte transformation and skin test, in *clinically* diagnosed SMA patients.[23,24] Accordingly, many SMA patients had nonmucocutaneous candidiasis, which usually only occurs in immunodeficient individuals.[25] Moreover, pulmonary infection, especially pneumonia, appears to be a common feature of SMA patients.[26–30] Decreased respiratory efficiency and stasis of secretions can increase risk of infections.[29,31] For this reason, superimposed immunodeficiency has not previously been considered as contributory to the disease etiology but may indeed play a major role in the presentation of chronic pulmonary infection in SMA patients. Nonetheless, evidence in human SMA patients remains sparse at the moment and more research is warranted on this aspect to better understand whether murine defects are reflective of true human pathology.

Many areas of the immune system remain unexplored in the context of SMA. The lymph nodes, bone marrow and MALTs are lymphoid tissues that have yet to be investigated (Fig. 1). Of importance, lymph nodes are distributed all over the body and abnormalities in this tissue could lead to impaired antigen screening, impacting immunity as a whole. Similarly, the bone marrow is responsible for B-cell and myeloid cell (monocytes, erythrocytes, neutrophils and others) development[32] and alterations in maturation of these cells could also be detrimental. Indeed, B-cell follicle loss in the spleen may be related to bone marrow deficiency.[9] Given that T-cell maturation defects were identified, it would not be surprising to uncover similar features in the bone marrow. Furthermore, the blood circulating immune cells were not deeply studied either. Future endeavors should aim at expanding our knowledge on the role of other organs of the immune system in SMA pathogenesis, and decipher whether SMA mice and patients can correctly mount an immune response to a variety of pathogens that include both bacteria and viruses. Of course, retrospective clinical studies should also aim at discovering over-represented pathogens causing infections in the SMA patient population and their likelihood of infections in comparison to healthy individuals to ensure enhanced care is provided if needed. The recent efforts identifying the immune organ defects have offered limited mechanistic insights. The mechanism leading to the most prominent defect, the small spleens, remained to be determined. Possible explanations include abnormal vasculature, denervation, and

cell-intrinsic defects, which have all been previously identified in other organs (Fig. 2).[13,33,34] However, other possibilities also include lack of proliferation or increased apoptosis, which seem unlikely based on current observations,[9,10] and abnormal expression of homing chemokines.[16]

Iron homeostasis

The spleen is also involved in blood filtration and iron homeostasis.[16] Strikingly, macrophages involved in iron recycling are depleted in SMA.[10] However, splenic iron metabolism was not investigated in the latter study. Interestingly, impaired liver development, iron overload and embryonic lethality were the main features of *Smn* conditional knockout restricted to the liver.[35] Recently, iron homeostasis defects were also observed in the Taiwanese mouse model of SMA.[36] Whether the spleen is causative of iron dysregulation, and potential cross-talk between the liver and the spleen exists to regulate the pool of iron remains to be determined.

Status of neuroinflammation in ALS and SMA

Neuroinflammation is a well established characteristic of neurodegenerative disorders.[37] This process is mainly mediated by astrocyte, microglia and T-cells.[38] Defective immune organs, and more particularly a potentially defective T-cell compartment, might signal defective inflammatory response in SMA, an aspect that has so far not been studied in the field (Fig. 3). Indeed, after over 20 years of innovative research, the events triggering motor neuron death in SMA remain elusive. It is interesting to note that lymphoid organs, and more specifically the spleen, may actively participate in the process of neurodegeneration.[39] ALS, an adult onset motor neuron disorder, has received attention from the SMA community for their possible common molecular ties in disease pathogenesis.[40] Whether or not SMA and ALS actually have similar underlying molecular mechanism, neuroinflammation is an important part of disease pathogenesis in ALS, and the lack of research in this aspect in SMA warrants further investigation.

What have we learned from the ALS field?

Neuroinflammation research in ALS has gained considerable ground since reports described microglial and astrocytic activation features, as well as presence of lymphocytes in necropsies of ALS patients.[41,42] These findings have been confirmed numerous times both in humans and mutant SOD1 (mSOD1) mouse models.[43–45]
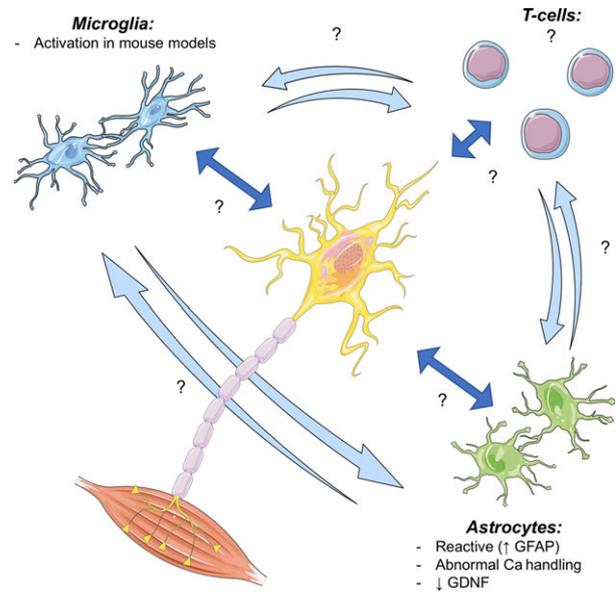

Figure 3. Current knowledge of neuroinflammation in SMA and its main contributors. Most of the work so far has focused on astrocytes with very limited information on microglia and T-cells. Their interaction in the CNS milieu has also not been addressed in the SMA context. The schematic art pieces used in this figure were provided by Servier Medical art (http://servier.com/Powerpoint-image-bank). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

Microglia came under thorough scrutiny for their possible involvement in disease pathogenesis. Indeed, it appears that the timing of their activation and proliferation occur in the rapidly progressing phase of motor neuron degeneration.[46] Interestingly, diminution of mSOD1 restricted to microglia leads to slower disease progression and extends survival considerably.[47] In a similar manner, wild type bone marrow transfer in either lethally irradiated mSOD1 or mSOD1; PU.1$^{-/-}$ mice leads to slower progression and increased survival.[48,49] These changes were suggested to be conferred by wild type microglial cells.[48,49] However, these reports did not take into account that donor wild type lymphoid cells, like T-cells, may also contribute to the beneficial effect.[48,49] In vitro, mSOD1 microglia release more pro-inflammatory cytokines like IL-1β and TNFα, superoxide, but less neuroprotective IGF1.[49–51] Moreover, when cocultured with motor neurons, microglia could cause cell death.[49,51] While much of the information points toward significant microglial contribution to disease, other reports have argued otherwise.[52] Similarly, minocycline, a tetracycline derivative that has anti-inflammatory properties in addition to off-target inhibition of microglial activation, showed promise in preclinical models of ALS but failed in clinical trial.[53,54]

T-cell presence is usually minimal in the CNS.[38] However, under pathological circumstances, they can infiltrate the spinal cord.[55] In the mSOD1 mouse model, T-cells infiltrate the spinal cord at a time point associated with microglial activation, highlighting the potential synergy of these cells in disease in a mouse model of ALS.[56–58] In this context, T-cells appear particularly protective.[56,57] Importantly, hybridizing RAG2$^{-/-}$ onto the mSOD1 mice, creating ALS without any T-cells or B-cells, lead to severe worsening of disease progression.[57] Similar results are also obtained with double mutant CD4$^{-/-}$ mSOD1 mice.[57] Mechanistically, CD4 T-cells appear to mediate microglial activation and their absence resulted in increased cytotoxic markers and diminished neuroprotective markers.[57] Of interest, adoptive transfer of activated regulatory T-cells or effector T-cells from wild type mice into mSOD1 recipients leads to delayed motor symptoms and extended survival.[59] Moreover, regulatory T-cells are associated with slow progressing phases in mSOD1 mice and are negatively correlated with rapid progression in ALS patients.[58,60] Nonetheless, it appears that mSOD1 T-cells are not functionally impaired.[57,58,61] Contrastingly, B-cells were not found to be present in the spinal cords of ALS mouse models and complete ablation of B-cells did not change the disease phenotype.[56,57,62]

Astrocytic activation is also present in multiple regions of the spinal cord and brain in ALS patients and mouse models,[44,46,63] and is associated with motor neuron loss.[44,46,64] Importantly, reduction of mSOD1 restricted to astrocytes slowed disease progression, in a similar manner as with microglial restricted mSOD1 reduction, likely attributed to inhibition of microglial activation.[64] In fact, conditioned media from mSOD1 astrocytes is toxic to motor neurons in vitro.[65] In vivo, astrocytes may mediate a neurotoxic environment because of their lowered expression of EAAT2 (or GLT-1 in mice), a glutamate transporter able to clear glutamate surplus in the synaptic space.[66,67] Interestingly, T-cells also seem to have some effect on astrocytic function and proper expression of GLT1.[57] The full array of changes in astrocytes is beyond the scope of this manuscript but this has recently been reviewed.[68] It is interesting to note that the kinetics of action of astrocytes, microglia and T-cells and their interactions are orchestrated with the rate of disease progression in ALS. For example, Th-2 T-cells and M2 microglia molecular anti-inflammatory profiles are associated with slow progressing phase of ALS course, which eventually convert to a predominantly Th-1 T-cell and M1 microglial proinflammatory response mediating the rapidly progressing phase[58] (reviewed in[69]).

Like SMA, peripheral immune alterations are also present in ALS. Spleens become smaller and their histological architecture is mildly disrupted at symptomatic age in mSOD1 mice.[59] Interestingly, increased cell death and diminished T-cell proliferation were also described.[59] Moreover, increased activated circulating macrophages, abnormal numbers of CD4 T-cells, antibodies and circulating immune complex have been described in blood of ALS patients, but the results have been inconsistent.[70,71] Interestingly, the development of C9orf72 null mouse models, after identification of a mutated version of this gene in a significant proportion of ALS patients, did not lead to overt motor impairment.[72,73] Instead, an array of peripheral immune organ abnormalities arose, including splenomegaly, lymphadenopathy and eventually an autoimmune-like phenotype.[72,73] Nonetheless, macrophages and microglia appeared particularly susceptible, showing lysosomal dysfunction and a proinflammatory state.[73] Strikingly, transcriptomic analysis of spinal cord reveals similar changes in pathways involved in immune function both in mice and C9orf72 ALS patients, likely leading to neuroinflammation.[73]

Current status of our understanding of neuroinflammation in SMA

In the context of SMA, neuroinflammation has never been thoroughly investigated (Fig. 3). In contrast to ALS, microglia and T-cells have been mainly overlooked while some reports have highlighted the contributions of astrocytes to disease pathogenesis. More specifically, increased astrogliosis was observed in necropsies of patients[74–77] and in the *Smn*Δ7 mouse model at both presymptomatic and symptomatic stages.[78] SMA patient induced pluripotent stem cell-derived astrocytes revealed abnormal calcium regulation, decreased glial cell-derived neurotrophic factor (GDNF) production but normal GLT1 expression.[78] There is no doubt that astrocyte intrinsic abnormalities contribute to SMA pathogenesis, however their link to neuroinflammation and motor neuron death has not been determined.[79] SMN restoration restricted to the astrocyte compartment significantly increased lifespan and motor behavior, but did not improve motor neuron survival.[75] More related to the context of neuroinflammation, pro-inflammatory cytokines could be elevated in SMA patients and mouse models.[11,75] Microglial activation has been observed in the *Smn*Δ7 mouse model but not in the more severe *Smn*$^{-/-}$;*SMN2* mice.[76,77,80] To our knowledge, there is no information on the contribution of microglia to neuroinflammation and to SMA pathogenesis (Fig. 3). The involvement of T-cells in spinal cord has never been studied and their involvement in neuroinflammation in the context of SMA remains unknown.

Despite the paucity of information, it is very likely that neuroinflammation has a significant contribution to SMA disease onset or progression. At the moment, the very few

reports on astrocytes are focused on cell-autonomous dysfunction and its relation to motor neurons while emphasis on its overall relationship with other glial cells in the potential neuroinflammatory CNS milieu is overlooked (Fig. 3). Given the importance of microglia on ALS progression, an in-depth analysis of the significance of microglia in SMA is warranted. Importantly, functional studies investigating the status of microglial activation (M1 - proinflammatory or M2 - anti-inflammatory) over time may give us insight on pathways that can be modulated to halt or reduce progression.[38] With the recent reports concerning peripheral immune organ abnormalities and T-cell maturation dysfunction in SMA mouse models, it is possible that intrinsic T-cell alterations result in an abnormal neuroinflammatory response and exacerbation of disease. Investigation of the status of T-cells in the spinal cord and whether they exhibit a protective or cytotoxic function in SMA will be of interest.

New perspectives for SMA

The defective peripheral immune organs in SMA preclinical models raise several important questions that will need to be addressed. How do immune organs become affected by SMN depletion? What are the functional consequences of defective immune organs, more particularly on immunity and neuroinflammation? Do patients show similar immune system abnormalities? With the most recent FDA approval of nusinersen (Spinraza), which represents the first approved drug for the treatment of SMA, it will be instrumental to continue to understand disease etiology. Indeed, while the results of the nusinersen clinical trial looked promising, the treated patients still lag far behind healthy individuals on motor functions.[30] Thus, it is critical to understand and target various pathways involved in SMA pathogenesis that may not currently be covered with by Spinraza (restricted to the CNS), such as the peripheral immune organs. Establishing the pathogenic status of peripheral immune system as well as neuroinflammation in the context of SMA will be of critical importance in this context.

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Author Contribution

MOD and RK wrote manuscript.

Conflict of Interest

None.

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