Neuroblastoma cell lineage and immunotherapy responsiveness: a complicated relationship

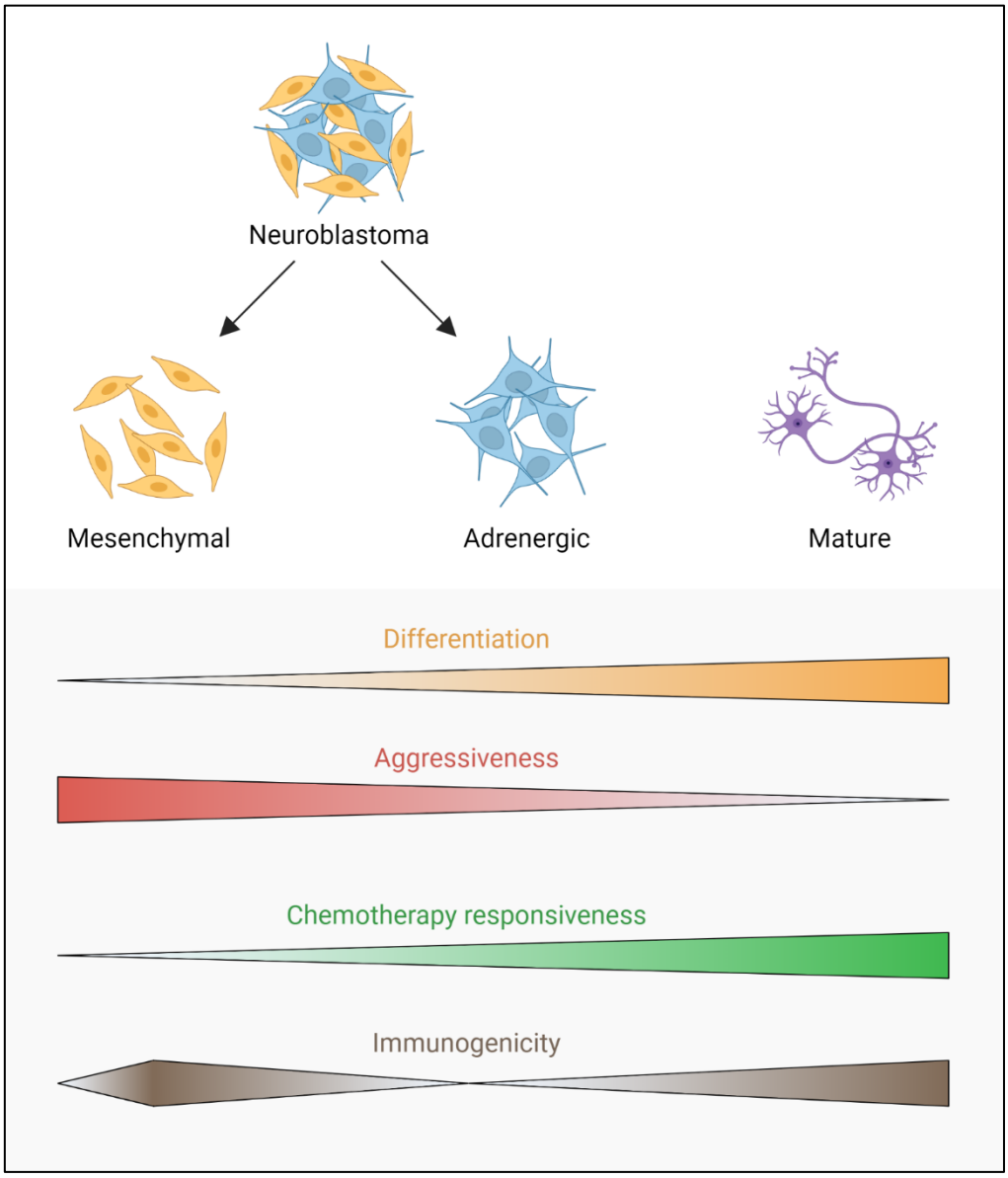

Figure 1. Graphical abstract. Created with Biorender.com.

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Writing assignment for the Graduate School of Life Sciences
23/2/2023

Plain language summary

Neuroblastoma (NBL) is a solid tumor arising from neural stem cells during development. It is the most common type of extracranial solid cancer in children. High-risk patients are difficult to treat and prognosis for survival is still a mediocre 50%. Currently, these patients undergo treatment comprising surgery, radiation, chemotherapy, stem cell transplantation, and maintenance therapy. Maintenance therapy consists of immunotherapy with anti-GD2 antibodies and isotretinoin. Recent research has shown that neuroblastoma cells can be divided into two distinct types, adrenergic-like (ADRN) and mesenchymal-like (MES) cells. ADRN cells are more differentiated than MES cells, which are more precursor-like. This review summarizes the information available on ADRN and MES identity, and the implications for therapy response and anti-tumor immunity. In addition, we describe a connection between these cell lineages and isotretinoin treatment efficacy in NBL. Finally, we speculate about future directions for treating NBL using alternative strategies.

Abstract

Neuroblastoma (NBL) is a heterogeneous solid tumor that arises from neural crest cells and accounts for 10% of pediatric cancer-related deaths. Immunotherapy, consisting of the anti-GD2 antibody dinutuximab and 13-cis-retinoic acid (13-cis-RA), is applied in high-risk NBL patients as maintenance therapy. Yet, overall survival of high-risk NBL is still only 50%. Recent studies have shown that NBL cells generally exist in one of two distinct epigenetic phenotypes: more differentiated adrenergic-like (ADRN) cells and neural crest cell-resembling mesenchymal-like (MES) cells, which have been found to respond differently to several stages of the NBL treatment regimen. This review gives insight into these inter-lineage differences in susceptibility and response to chemotherapy, immunotherapy and retinoic acid (RA) therapy, and discusses its implications for the effectivity of the current NBL therapy regimen. Lastly, we provide a recommendation on future directions for the NBL regimen.

Introduction

Neuroblastoma (NBL) is a solid tumor that arises from neural crest cells (NCCs) of the developing autonomic nervous system. Because of its epigenetic and biological heterogeneity, determining an effective therapy against NBL has been challenging. NBL accounts for 10% of cancer-related deaths in the pediatric population and overall survival (OS) of high-risk NBL still stagnates at 50%[1,2], showing that there is a desperate need for more effective strategies.

NBL treatment is multimodal and intense. Diagnosed patients are classified into risk groups based on risk factors such as patient age, tumor size and localization, histological classification, and (epi)genomic alterations, of which MYCN amplification is the most prominent[3]. The treatment regimen currently consists of surgery, radiotherapy, and chemotherapy followed by autologous stem cell transplantation (ASCT). Immunotherapy is applied as maintenance therapy in high-risk patients to eradicate residual disease and prevent relapse[3]. In this stage, a combination of dinutuximab, an anti-GD2 antibody, and isotretinoin, or 13-cis retinoic acid (13-cis-RA), are used as the standard treatment and have already shown great potential[4,5]. But the limited number of patients available for research makes it challenging to test therapies on a structural basis at a large scale, resulting in some treatments being tested mostly empirically.

In recent years, two distinct epigenetic phenotypes of neuroblastoma cells were described: cells resembling a lineage-committed, adrenergic-like identity (ADRN) and cells with a mesenchymal-like identity (MES), resembling neural crest cell precursors[6]. Both cell states have been shown to be able to spontaneously transdifferentiate through epigenetic reprogramming in a process called adrenergic-to-mesenchymal transition (AMT)[6,7]. Additionally, these cell types have demonstrated different responses to various agents in the treatment protocol[6].

This review provides an overview of the properties of these two lineage identities and their susceptibility to different components of the NBL therapy regimen. We describe the current knowledge available the response of ADRN and MES cells to chemotherapy, immunotherapy and to combinational therapy with retinoic acid (RA). Finally, we discuss the possible problems with the existing treatment protocol and provide a recommendation on future directions for the NBL regimen.

The MES and ADRN cell lineage respond differently to treatment

NB consists of cells with an adrenergic and a mesenchymal identity

In 2017, van Groningen et al. and Boeva et al. independently described for the first time two NBL cell types with a distinct super-enhancer (SE) landscape and SE-associated transcription factor (TF) network[6,8]. TF networks form the core regulatory circuit (CRC), containing autoregulatory feed-forward loops that regulate gene expression and establish cell identity. The ADRN CRC is the best established of the two and contains PHOX2B, DBH, HAND2, ISL1, GATA3, TBX2 and ASCL1 TFs, among others[6,8,9]. These genes are involved in adrenergic differentiation and correlate with the neuronal sympathetic phenotype of a spherical semi-attached conformation *in vitro* and low motility[6,10]. The MES CRC cell lineage was characterized by expression of mesenchymal marker genes such as PRRX1, FOSL1/2, NOTCH, VIM, FN1, and SNAI2[6,8]. MES-type cells closely resemble undifferentiated NCCs, grow attached *in vitro*, and have a higher migrational potential[6,11]. Concordantly, gene expression patterns of MES and ADRN cells have been shown to parallel immature Schwann precursor cells and lineage-committed sympathoblasts, respectively[12]. In primary tumors, additional ADRN subtypes have been described, namely MYCN-amplified, MYCN non-amplified high-risk, and MYCN non-amplified low-risk[13].

ADRN and MES NBL cells are capable of bidirectional plasticity

To investigate cell lineage identity in NBL *in vitro*, the SH-N-SH cell line is frequently selected due to its derivation of the ADRN-phenotyped SH-SY5Y and the MES-phenotyped SH-EP2 cell lines. Thorough research into these distinct lineages has revealed that NBL cells possess the capability to undergo spontaneous bidirectional interconversion through epigenetic reprogramming in both *in vitro* and *in vivo* settings. Multiple studies have identified MES- and ADRN-specific genes that are able to drive adrenergic-to-mesenchymal transition (AMT). Induction of PRRX1 was found to drive SK-N-BE(2)-C cells toward a MES state [14], and NOTCH transgene induction was able to reprogram SH-SY5Y cells through a transcriptional feed-forward loop in culture and in mice[7,15]. Additionally, several studies have demonstrated inducible AMT *in vivo*. Engraftment of NBL tumor cells of a single lineage identity in mice eventually results in heterogeneous populations over time[6,16]. Nonetheless, the *in vivo* microenvironment has been shown to pressure NBL cells toward the ADRN identity in mice[11,17]. Currently, it is unclear to what extent AMT occurs in NBL patient tumors and which driving mechanisms are behind it.

MES NBL cells show a more aggressive phenotype and exhibit increased therapy resistance

Tumor cells with a mesenchymal phenotype have been associated with tumorigenic features for many years. In the well-studied process of epithelial-to-mesenchymal transition (EMT), epithelial cells lose their attachment to neighboring cells and gain migratory and invasive characteristics.[18] Tumor cell plasticity has additionally been implicated in resistance to therapy[19]. Although EMT in NBL has been redefined as AMT due to a different set of marker genes being involved in mesenchymal induction[11], similar oncogenic properties have been reported for MES cells that result from AMT. Cells with a MES-phenotype show enhanced invasion and migrational activity compared to cells with an ADRN-phenotype[20,21]. Transdifferentiation to a MES state has also been strongly associated with resistance to therapies. MES cells were more resistant *in vitro* to standard chemotherapeutic agents used to treat NBL patients, such as cisplatin, doxorubicin, and etoposide (Table 1) [6]. In addition, MES cells are enriched in biopsies of relapsed NBL tumors[6]. It has been suggested that besides resistance, this could be due to AMT plasticity upon chemotherapy treatment. Genes associated with the MES CRC have been persistently elevated in circulating NBL tumor cells of relapsed patients[22], enabling cancer progression. Anti-GD2 immunotherapy is applied during the maintenance stages of treatment to eliminate residual disease, but has, despite improved event-free survival (EFS) rates[4], been ineffective against MES-like cells. A recent study confirmed that cells that adopt the mesenchymal phenotype lose GD2 expression and become resistant to dinutuximab[23]. Additionally, inhibitors of the oncogene ALK that are currently in clinical trials have limited effectivity against mesenchymal cells due to downregulation of the ALK gene.

Recently, researchers have conducted studies to identify potential targets and agents that can effectively target mesenchymal cells and promote differentiation towards the adrenergic state, thereby decreasing tumor aggressiveness and increasing therapy sensitivity (Table 2). This includes investigating the mechanisms of AMT induction, with the aim of potentially inhibiting it. Studies have found that activating specific MES-SEs within adrenergic cells can be sufficient to induce AMT. For example, the combination of TNFα + EGF has been shown to promote AMT in the tumor microenvironment[24]. Currently, the RA derivative isotretinoin is administered in the maintenance phase of the therapy regimen to drive differentiation of MES cells towards the more favorable adrenergic state, and thought to facilitate the reversal of AMT: MAT. RA is suggested to improve NBL patient outcome[25,26], but nonetheless, its effectivity is debated on in literature.

Table 1. Studies into the effects of therapeutic agents on the MES vs ADRN cell lineage.

Agent	Target	Findings for each cell type	Tissues (in vitro / in vivo / ex vivo)	Techniques	Studies

Alectinib	ALK	ADRN: susceptible MES: resistance, continued proliferation	*In vitro:* SH-EP2/SH-SY5Y, NBLW-MES/NBLW-ADRN cell lines	MTT assay	[12]
Cisplatin	DNA repair mechanisms	ADRN: susceptible MES: chemo-resistance *in vitro* and enrichment in post-therapy tumors induced cross-resistance to etoposide and irinotecan	*In vitro.* SH-EP2/SH-SY5Y, patient-derived cell lines *In vitro.* Kelly, SK-N-AS, CHP-212 cell lines	MTT assay, IHC of PRRX1 MTT assay, invasion assay	[6,20]
Dinutuximab	GD2	ADRN: susceptible, reduced tumor growth MES: resistance, continued proliferation	*In vitro:* Cancer Cell Line Encyclopedia (CCLE) cell lines, SH-EP2/SH-SY5Y *In vivo:* mice xenografted with heterogeneous Kelly cells	Macrophage phagocytosis assay, NK co-culture assay, FACS, RNA-sequencing analysis	[23]
Doxorubicin	DNA repair mechanisms	ADRN: susceptible MES: chemo-resistance *in vitro* and enrichment in post-therapy tumors Increased invasion and migration ability	*In vitro.* SH-EP2/SH-SY5Y, patient-derived cell lines K-N-Be(2)C and SK-N-SH WT and DoxR cell lines	MTT assay, IHC of PRRX1 Invasion and migration assay	[6,21]
Etoposide	DNA-topoisomerase-II-DNA-complex	ADRN: susceptible MES: chemo-resistance *in vitro* and enrichment in post-therapy tumors	*In vitro.* SH-EP2/SH-SY5Y cell lines + patient-derived cell lines	MTT assay, IHC of PRRX1	[6]
Lorlatinib	ALK	ADRN: susceptible MES: Resistance, continued proliferation	*In vitro:* SH-EP2 (MES)/SH-SY5Y (ADRN) cell lines + NBLW-MES/NBLW-ADRN cell lines *In vivo:* mice xenografted with inducible NOTCH3-IC expression	MTT assay, tumor volume, Immunohistochemistry (IHC) for MES + ADRN markers	[12]
TRAIL	CASP8	ADRN: resistance MES: susceptible, regression of *in vivo* established tumors	*In vitro:* SK-N-AS^{TRAIL} cells cocultured with SH-EP^{RFP} or SH-SY5Y^{GFP} cells *In vivo*: mice xenografted with inducible TRAIL expression	MTT assay, cell count assay, fluorescence assay, tumor volume	[12]

Table 2. Studies into agents that induce transdifferentiation in NBL cells.

Agent	**Target**	**Transition (AMT/MAT)**	**Additional findings**	**Tissues (in vitro / in vivo)**	**Techniques**	**Studies**
Entinostat	HDAC inhibition	AMT	Treatment induces immunogenicity of CTLs and NK-cells by	*In vitro:* SHEP21N, GIMEN, LAN5, SK-N-SH, and IMR32	RNA-sequencing, qPCR analysis, T-cell and NK-cell	[27]

			upregulation of MHC-I and many other immune genes	cell lines, patient-derived organoids	cytotoxicity assay, T cell activation assay	
NOTCH intracellular (NOTCH3-IC) transgene	NOTCH3 TF expression	AMT	NOTCH3 drives a feed-forward loop towards AMT AMT is tumorigenic *in vivo* (increased incidence of metastases and poor prognosis)	*In vitro:* SH-EP2/SH-SY5Y, patient-derived cell lines *In vivo:* mice xenografted with inducible NOTCH3-IC expression	Gene expression profiling, cell motility assay, ChIP-sequencing, Western Blot *In vivo* tumorigenicity and histological analysis	[7,12,15]
TNFα + EFG		AMT	Treatment leads to enhanced migrational ability and resistance to standard drugs cisplatin, doxorubicin, and etoposide *in vitro*	*In vitro*: SH-EP2/SH-SY5Y, patient-derived cell lines	Western Blot, cell viability assay, scratch wound cell migration assay, R2: Genomics Analysis and Visualization Platform	[24]
Tazemetostat	EZH2 (subunit of PRC2 complex) inhibition	MAT	Treated cells were more likely to be phagocytosed in presence of anti-GD2 No significant effects on immune effector cells Cotreatment with anti-GD2 significantly diminished tumor burden *in vivo*	*In vitro:* SK-N-AS, Kelly-GD2^{low}, CHLA-255-GD^{low}, and NB-SD cell lines. Ewing sarcoma cell lines, small cell lung cancer cell lines *In vivo:* mice xenografted with mesenchymal SK-N-AS cells	RNA sequencing, Gene set enrichment analysis (GSEA), Macrophage phagocytosis assay In vivo metastatic model, bioluminescence	[23]

Retinoic acid has limited success in the treatment of neuroblastoma

Retinoids such as 9-cis-retinoic acid (9-cis-RA), 13-cis-RA, and all-trans retinoic acid (ATRA) are metabolized products of vitamin A. 13-cis-RA is the preferred isomer for treating NBL due to its pharmacokinetic advantages, such as a longer half-life and consistent concentration levels[28]. Nonetheless, ATRA is the most prevalent in cells and ATRA and isomer 9-cis RA are stronger influences on cell differentiation and proliferation.

RA is an important metabolite during embryonal differentiation

RA is a ligand for nuclear retinoic acid receptors (RARs) that regulate the transcription of many developmental genes by binding to RA response elements (RAREs) on the DNA[29]. Most *in vivo* studies on working mechanisms of RA in signaling and development are done in genetically manipulated mice and zebrafish[29]. RA is reported to have an important role in embryonic development through its regulatory effects on target genes such as *HOX* genes[29]. Loss of RA in mice results in altered differentiation of

neural progenitor cells[30]. Additionally, RA directs body axis extension through caudal repression of Fgf8 transcription[31]. Lastly, an important function for RA signaling in early development is closure of the neural tube[29]. Considering that NBL originates from neural crest-derived cells and is seen as a tumor with disrupted differentiation[32], clinical use of retinoids are a logical approach for halting NBL disease progression by stimulating neuronal cell differentiation and apoptosis.

Clinical research into effectiveness of isotretinoin in NB

Efficacy of isotretinoin and other retinoids in treatment of NBL has been extensively researched. Bayeva et al. recently documented the available data on RA derivatives in NBL in a thorough systematic review[33]. Initial research *in vitro* and in murine models showed promising results for 13-cis-RA in NB. Unfortunately, limitations of *in vitro* models like a lack of interaction with the extracellular matrix (ECM) or other cell types, impede applicability of their results in *in vivo* NB. Furthermore, differences in drug efficacy and toxicity between mice and humans pose a challenge in extrapolating results from murine models to the clinic. In this review, we will focus on the available data regarding isotretinoin in NBL *in vivo*, as it is the only RA derivative currently included in the therapy regimen for high-risk NB.

Isotretinoin as single-use treatment

The availability of clinical research on isotretinoin efficacy in NBL is limited. The first clinical studies were focused on optimizing dosing of 13-cis-RA in order to achieve acceptable plasma levels, but did not yield any clinical results[34,35]. The first large cohort study into isotretinoin researched the treatment of 539 initial patients with myeloablative therapy combined with autologous bone marrow transplantation, as well as maintenance therapy with isotretinoin to clear residual tumor cells. Of a total of 319 patients, 130 were assigned to receive isotretinoin treatment after cytotoxic therapy, and 128 received no further treatment. Patients treated with isotretinoin showed a significant increase in 3-year EFS rate (46 ± 6% vs. 29 ± 5%), but no significant increase in OS[25]. A follow-up study for the same patient cohort reported a significant increase in the 5-year survival rate, but again no significant OS[26]. In another study, 175 high-risk stage 4 patients participated in a trial testing the administration of a low dose of isotretinoin as continuation therapy. Here, 3-year EFS rates as well as OS were not significantly higher in the group receiving isotretinoin[36]. Despite limited efficacy demonstrated in these studies, isotretinoin has been recommended for high-risk NBL patients post consolidation therapy in the clinic.

Isotretinoin as combinational treatment

Similarly to single-use, there has been very little research into whether RA works synergistically with immunotherapy. In 2010, Yu et al. combined 13-cis-RA therapy with immunotherapy consisting of anti-GD2 antibody, Interleukin-2 (IL2) and GM-CSF and compared it to treatment with solely isotretinoin in a phase III trial with 226 patients. The treatment relied on tackling residual tumor cells both through RA-induced differentiation and increasing tumor immunogenicity, for instance through antibody-dependent cell-mediated toxicity (ADCC). Addition of immunotherapy increased both 2-year EFS rates and OS rates significantly (66 ± 5% vs. 46 ± 5% and 86 ± 4% vs. 75 ± 5%, respectively)[4]. The study led to FDA-approval of the drug dinutuximab for treatment of NB. A follow-up study confirmed the improved outcomes using immunotherapy, although EFS and OS rates were decreased due to late relapses[5]. Combinational therapy of isotretinoin and immunotherapy has been considered promising for the outlook of NBL patients. Nevertheless, the function of isotretinoin in maintenance therapy has not been concretely investigated. All clinical data together suggests that isotretinoin has a somewhat positive effect in neuroblastoma. However, its positioning together with immunotherapy leading to any additive effect in this regimen has not been established.

Possible explanations for clinical results for isotretinoin in NB

There are many considerations to take into account when speculating about why isotretinoin shows limited effectiveness in the current treatment regimen. RA has many effector functions in regulating the immune response in various tissues, yet its effects could also be proinflammatory in some cases. Signaling and functions of RA are broad and complex, and the mechanisms used can depend greatly on the local microrenvironment[37]. Therefore, the role of RA in NBL cannot be easily researched in *in vitro* models. Some *in vitro* research supports the notion of RA contributing to immunotherapy effectiveness. Susceptible NBL cells pretreated with ATRA show an increased ADCC in response to anti-GD2[38]. Additionally, RA has been implicated in neutrophil differentiation and maturation through complex formation with RAR, and was shown to induce anti-tumor cytotoxicity of neutrophils[39,40]. One study suggested that RA may induce MHC class I promoter activity in NT2 embryonal carcinoma cells[41]. Because MHC class I downregulation is a well-known escape mechanism of many cancers[42], restoring this pathway could be promising for eliciting an immune response in the treatment of NB. On the other hand, there are studies that suggest a restricting role for RA in immune activity. The use of RA is considered for treating several autoimmune diseases due to its anti-inflammatory functions such as regulatory T cell (T_{reg}) induction and promotion of tolerance. Rather than immune activation, RA has appeared an important player in inducing immune homeostasis[37]. This is supported by the implication that RA inhibits production of inflammatory cytokines, thereby modulating macrophage activity[43]. Combination of the ATRA derivative with IFNα2 has been tested in a phase II trial but showed no beneficial effects from the treatment[44], implying a lack of synergy between RA derivatives and immunogenic components. Additionally, RA indirectly recruits polycomb repressive complex 2 (PRC2) through repression of the Fgf8 RARE[29], which has been identified as a repressor of immune genes and described as a barrier to anti-tumor immunity[45]. TFs HIC1 and SMAD3 are also activated by RA and, as mentioned before, part of the retino-sympathetic CRC. These proteins modulate the TGFβ signaling pathway, which was shown to create an immune-resistant, pro-tumorigenic environment in NBL tumors[46].

RA differentiation is a process distinct from mesenchymal-to-adrenergic differentiation

In addition to immune-related functions, the limited effect of retinoic acid may also be due to its differentiation mechanism. Despite the decade-long application of isotretinoin in the clinic, it has not yet been demonstrated that retinoids effectively induce differentiation of MES cells towards an ADRN phenotype. In the past, induction of NC cell differentiation by retinoids has mostly been measured *in vitro* by monitoring phenotypical characteristics, such as neurite formation and outgrowth, the hallmarks of neuronal differentiation[33]. As a consequence, changes in transcriptome landscape and SEs induced by RA were barely described, until recently. Zimmerman et al treated ADRN MYCN-amplified NBL cells with ATRA and reported that ATRA reprograms the adrenergic CRC into a CRC distinct from ADRN and MES cells, in which some ADRN CRC-associated TFs are involved while others are downregulated. The induction of this 'retino-sympathetic' CRC coincided with arrested proliferation, MYCN downregulation, and induced differentiation in to mature neurons[47]. In another study, ADRN cell lines were found more susceptible to ATRA when the ADRN CRC TF ASCL1 was knocked down, suggesting that the ADRN CRC inhibits further maturation of NBL cells[48]. Gomez et al. studied the effects of ATRA treatment on the SE-associated CRCs of MYCN-amplified ADRN cells (SK-N-BE(2)C), MYCN-non-amplified ADRN cells (SH-SY5Y), and MYCN-non-amplified MES cells (SH-EP). They confirmed earlier findings on rewiring of the ADRN CRC by ATRA and reported responsiveness of both MYCN-amplified and MYCN-non-amplified ADRN cells to ATRA, with downregulation of ADRN markers HAND2, ASCL1, and IRF1 upon treatment. Additionally, they identified consensus CRC components of ADRN cells responsive to ATRA treatment: HIC1 and SMAD3, RARA, and RARB. Interestingly, SH-SY5Y cells lost their differentiated morphological features and resumed proliferation after ATRA withdrawal[49]. Collectively, these studies show that ADRN are capable of responsiveness to retinoid treatment. On the other hand, MES cells have been demonstrated unresponsive to RA-induced differentiation. Treatment of SH-EP with ATRA did

not result in phenotypic neuronal features or upregulation of differentiation-associated SEs, even though the RA signaling pathway seemed constitutively active and proliferation was slowed down[49]. Loss of MES marker SNAI2 sensitized NBL cells to RA, while also reducing self-renewal and metastatic spread *in vivo*[50]. It has been hypothesized that MES resistance to RA may be associated with a lack of overlap between the retino-sympathetic CRC and the MES CRC[32]. These findings together show that RA-induced differentiation is an entirely different process than MAT, thereby emphasizing the intricate nature of treating a heterogeneous disease such as NB.

The characteristics of RA as an inducer of differentiation and proliferation arrest have deemed its derivative isotretinoin a promising candidate for the treatment of NB. However, evidence on its clinical success is minimal. The current strategy to use RA in combination with anti-GD2 therapy has improved short-term survival in high-risk NBL patients, but not yet in the long-term. RA function has been shown to promote, as well as inhibit, immunogenicity in cell and murine models, leading to the question of what role RA specifically has in the NBL TME of patients, in terms of immunity. Moreover, we describe here how RA-induced differentiation does not apply to MES cells and thus does not contribute to MAT. Its reasons for application in maintenance therapy therefore immediately seem a lot less convincing, as RA does not increase target availability for dinutuximab. In conclusion, RA does not appear to be the solution to skewing to the ADRN state for improved sensitivity to conventional therapy.
Meanwhile, the identification of other agents is also pursued for the induction of MES-to-ADRN transition (MAT) in NBL. EZH2 inhibitors (tazemetostat) could pose as suitable candidates as they have been reported to reprogram cells in the adrenergic direction, towards differentiation[23]. Another promising strategy for eradicating MES cells is not to change their phenotypic identity, but to instead look for therapy options to which MES cells are sensitive. Immune-mediated therapy might be well suited for this.

Mesenchymal targets for immunotherapy are needed

Anti-GD2 has improved NBL outcome, but may not be optimal for immunotherapy

Immunotherapy has been a major advancement in the treatment of numerous cancers, and may offer a means of targeting MES cells in NBL. High-risk NBL has been viewed as a classically “cold” tumor, evading the immune system through MHC class I downregulation and establishing an immunosuppressive tumor microenvironment (TME)[51]. Yet recent research has explored novel strategies for immunotherapy in NB, including chimeric antigen receptor (CAR) T cell therapy, checkpoint inhibition, and antibody-mediated therapy, with dinutuximab, an anti-GD2 antibody, already yielding promising results. It has been administered for years in combination with isotretinoin, GM-CSF, and IL-2, the latter two with the aim of increasing cytotoxicity of anti-GD2[4,52]. However, in 2021, Szanto et al. discovered that IL-2 could function as a two-edged sword, stimulating both immune-activating and immunosuppressive processes [53]. Now, GM-CSF and IL-2 have been cut out of the regimen and only dinutuximab and isotretinoin are administered as maintenance therapy to eliminate residual disease. GD2 is a ganglioside commonly expressed on neural and mesenchymal stem cells, and is believed to play a role in neural differentiation [54]. However, there is also evidence suggesting that GD2 has tumorigenic properties, including immune evasion and promotion of metastasis in osteosarcoma[55,56]. Anti-GD2 antibodies increase immune engagement by recruiting immune effector cells (NK-cells macrophages, monocytes, neutrophils) bearing Fc receptors[57]. In addition, the complement cascade was shown to be activated by anti-GD2 antibodies, leading to complement-mediated cytotoxicity (CMC)[58]. This, together with GD2’s limited expression in most postnatal tissues and stable expression on NBL tumor cells even after therapy has made GD2 an attractive candidate for NBL therapy. Despite the addition of maintenance therapy, long-term EFS of high-risk NBL patients still remains 40-50%, demonstrating that anti-GD2

immunotherapy is suboptimal for a large group of patients. A recent study has revealed that even though NBL tumors ubiquitously express GD2, a subset of NBL cells lacks GD2 expression and correlates with the MES identity. It further demonstrated that transition to the MES state is paired with loss of GD2-expression and subsequent resistance to dinutuximab and continued proliferation[23,59]. Considering the association of MES cells with chemoresistance and the limited ability of RA to differentiate them, this would make MES cells in NBL a significant challenge in terms of therapeutic elimination. Yet, there are also studies that challenge the association between MES lineage and GD2 expression. Van den Bijgaart et al, treated NBL murine models with HDAC inhibitor vorinostat and showed an increase in GD2 expression[60,61], even though HDAC inhibitor treatments were also associated with AMT[27]. Nevertheless, based on the available data, it is still preferable to choose targets that are equally or more expressed on MES cells for optimal immunotherapy efficacy, as recent findings have added another layer of complexity to the story that offers new opportunities for targeting MES cells.

Mesenchymal cells may be more susceptible to immunotherapy than ADRN cells

Increasing evidence conveys that immunotherapy targeting MES cells may be more effective than targeting ADRN cells. Sengupta et al. have demonstrated that a subset of mesenchymal cells exhibits more immunogenic features compared to adrenergic cells. Using RNA-sequencing on a cohort of 394 patient NBL tumors, they identified a cluster of NBL cells with enriched immunogenic scores, and found that this cluster's epigenetic state corresponded with the MES signature. Moreover, MES cell clusters had higher predicted TIL numbers in the TME, confirmed by the presence of more CD8+ T-cells in primary tumors with superior MES enrichment compared to those with more adrenergic enrichment. Converting ADRN SH-SY5Y cells to the MES state using PRRX1 overexpression was sufficient to increase the expression of immune regulatory genes involved in MHC class I presentation (including the antigen-presentation pathway), NK-cell ligand detection, and type I interferon signaling. A subsequent study by Cornel et al. confirmed that transition to the MES identity correlates with increased expression of MHC class I presentation genes through inhibition of histone deacetylases (HDAC) in NBL cells[27]. Sengupta et al. additionally showed that MES cells were able to recruit T-cells through stimulation of TLR3 and NK-cells due to expression of NK-cell ligand NKG2D, which is downregulated in ADRN cells by PRC2[45]. Another recent study by Wolpaw et al. demonstrated a similar TLR3-mediated inflammatory response and a greater basal inflammatory state specifically linked to MES cells[62]. The increase in T cell activity was also reproduced by Cornel et al., who increased T cell-mediated cytotoxicity against NBL with HDAC inhibitors which was linked to induction of AMT. Notably, the increased immunogenic phenotype of MES encompasses higher immune activation scores as well as higher immune evasion scores, including activation of immune checkpoints, indicating a general boost in immune engagement[45]. Interestingly, Engler et al. have demonstrated a similar connection between mesenchymal-like cells and activation of immune response genes in glioblastoma a decade earlier. Here, this immunogenic subgroup was associated with poorer prognosis[63]. These data together form a strong indication that the MES subgroup in NBL tumors has a greater immune activity than ADRN-type cells and has a higher potential to initiate anti-tumor immune responses. This suggests that MES cells are more susceptible to immunological interventions like immune checkpoint therapies. Unfortunately, with the current regimen targeting mostly ADRN cells in chemotherapy as well as maintenance therapy, we do not make good use of this MES-cell characteristic. It is now crucial that we identify novel markers specific for MES cells that can be targeted by immunotherapy. That way, we can fully take advantage of the Achilles heel of MES cells that is elevated immunogenicity and improve patient survival for neuroblastoma.

Potential MES-specific immunotherapeutic targets

Selecting targets for immune strategies like CAR T cell therapy and antibody-mediated therapy is a challenging practice. A suitable target antigen should be sufficiently and homogeneously expressed on

NBL tumor cells and its expression should be restricted to only tumor cells, to prevent off-tumor toxicity to healthy tissues. The most clinically studied targets for NBL are GD2 and anaplastic lymphoma kinase (ALK), both of which have recently been discovered not to be expressed on the surface of MES cells[12,23]. Yarmakovich et al. also proposed an interesting strategy, targeting non-immunogenic intracellular oncoproteins with CAR T cells, but used PHOX2B as a target for this practice, which is another ADRN-specific target[64]. This emphasizes the demand for new potential immunotherapy targets that can treat this tumorigenic subgroup. Even so, the method may have promising implications as it greatly broadens the pool of possible immune targets, both MES and ADRN. One could identify suitable MES-specific antigens for this strategy and create CARs against them. PRRX1 is a logical protein to investigate here as it is a hallmark for MES identity, but the data available on its broad tissue expression suggests that off-target toxicity for this protein will be too high[65,66]. Fortunately, the study by Westerhout et al., which described MES resistance to ALK inhibitors (ALKi) for the first time, also introduced a MES-specific protein in the apoptotic pathway, caspase-8 (CASP8). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) was shown to activate this pathway in NBL cell lines and in mice, leading to complete regression of MES-cell tumors[12]. This makes TRAIL a promising candidate for immunotherapy. However, much research on TRAIL and this pathway stills need to be done. After all, the pharmacokinetic profile of TRAIL was not yet favorable in this study. Other targets for NBL have been described, but lack data on ADRN- or MES-specific expression. B7-H3 is an immune checkpoint protein highly expressed in NBL and has been proposed as a promising candidate for immune checkpoint therapy[67,68]. B7-H3 is involved in oncogenic signaling and high expression of B7-H3 has been associated with chemotherapy resistance. Additionally, B7-H3 was reported to promote EMT in glioma and hepatoma cells[69]. These features logically give rise to the hypothesis that B7-H3 is expressed on the surface of MES cells, although this is something that still needs to be investigated. For now, TRAIL and B7-H3 are the immunotherapy targets with the greatest potential to eradicate MES cells in immunotherapy.

Conclusion / Discussion

In this review, we explored the plasticity of neuroblastoma cells and implications for responsiveness to therapy, particularly isotretinoin. High-risk NBL has, despite continuous advancements in chemotherapy and immunotherapy, a poor chance of survival. Understanding the mechanisms that make NBL cells with a MES phenotype resistant to most therapies is crucial for optimization of the therapy regimen for these patients. Our findings emphasize the resistance of the MES lineage to RA-induced differentiation, but also its higher immunogenic properties compared to the ADRN lineage. These insights offer new perspectives for targeting MES cells with immune therapies and highlight the necessity for MES-specific targets. TRAIL and B7-H3 are currently the most promising candidates for targeting of MES-cells, but the development of therapies against these antigens is still in its infancy. Yet, the fact that conventional therapies and immunotherapy can be designed in such a way that they target different cell subsets, greatly increases the promise of immunotherapy in NBL. Other reviewed targets for NBL are CD171, GPC2 and CD56, but their expression has yet to be established on a cell lineage basis. Target expression in both MES and ADRN cell identities is a crucial factor in predicting therapy effectiveness and possible resistance. Therefore, we appeal for a clear differentiation in outcomes for MES and ADRN cells when reporting results in neuroblastoma research.

Research into effectiveness of isotretinoin in NBL has been disappointing and notably limited, with only two large clinical trials conducted of which none led to a significant increase in OS rates. Nevertheless, the positive effect of isotretinoin on patient's 3-year EFS led to incorporation of isotretinoin post-consolidation. We call into question if maintenance therapy is the optimal use of RA and speculate whether its application might be more effective elsewhere in the therapy regimen. Anti-GD2-mediated

therapy is thought to benefit from RA-treatment, seeing as ADRN cells, unlike MES cells, express the GD2 target and are more differentiated compared to MES cells. The discovery that the MES lineage is associated with a more immunogenic phenotype, suggests that immunotherapy could better be focused on targeting MES cells, because they have more potential on eliciting an anti-tumor response. Consequently, anti-GD2 may not be the optimal choice as a target for immunotherapy. We also describe that MAT, which anti-GD2 treatment could benefit from, cannot be induced by RA. There is limited evidence to support that isotretinoin potentiates the effect of dinutuximab in NB, and the aforementioned findings only suggest otherwise. In the case of administering a new therapy that is able to also target MES cells, there could be arguments for and against supplementation of isotretinoin. While studies indicate that RA has an immunosuppressive effect that may hinder the anti-tumor inflammatory response in the current regimen, it is possible that checkpoint therapy could reverse this effect. Additionally, a retrospective analysis of studies exploring the immunostimulatory effects of RA on heterogeneous NBL populations, may offer new insights that are relevant for MES-targeted immunotherapy. Conflicting results here can possibly be attributed to the cell lineage (MES or ADRN) on which RA was used. For instance, a 2002 study reported that resistance to ATRA-induced differentiation correlated with ATRA-induced NF-κB signaling, which was not detected in responsive cell lines. The resistant cell line SK-N-BE 9N was described as lacking neural extension and ganglion-like structures and may have been MES cells. A more recent study discovered a synergetic effect of RA with polyinosinic-polycytidylic acid (poly (I:C)) in mice, leading to increased innate immune activity and type I interferon (IFN)-dependent apoptosis by activation of CASP8[70]. Although cell lineage identities were not examined, CASP8 was recently identified as a component of the MES-specific apoptotic pathway[12]. These findings together lead to the tentative hypothesis that RA, although not contributing to MAT, may enhance MES cell immunogenicity. In our current treatment proposal, we therefore continue administering RA along with immunotherapy, but plan to shift immunotherapeutic targeting towards the MES side (Figure 2).

It might also be useful to consider RA as a potentiating agent in the chemotherapy phase of high-risk NBL treatment. Adding ATRA to chemotherapeutic agents has achieved an immense increase in 5-year complete remission rates in acute promyelocytic leukemia, a classification of acute myeloid leukemia[71]. Furthermore, *in vitro* studies have shown that ATRA potentiates the chemotherapeutic effect of cisplatin and doxorubicin in liver cancer cells and breast cancer cells, respectively[71–73]. Further research should see if a similar benefits can be yielded in NBL tumors.

The field of research on NBL has substantial limitations which must be considered when interpreting the findings of this review. Most of the studies discussed here were conducted in cell lines, biopsies from patients, or mice. We cannot conclude that these models are an accurate representation of what occurs in actual patient tumors, as the extracellular matrix and other cell types present in NBL tumors can heavily impact the outcome of therapeutical interventions and might also influence MES/ADRN composition. Technological advancements in 3D models such as organoids have made it possible to create more accurate systems that represent the entire TME, providing new opportunities for researching the immune landscape of NBL and finding targets for NBL treatment[74,75]. Still, NBL is characterized by its heterogeneity and patients therefore display high epigenetic and phenotypic variability. Combined with the limited number of patients available for trials, this can make it difficult to interpret results and complicates prediction of therapeutical outcome.

In conclusion, additional investigation is necessary to elucidate the differential mechanisms by which RA affects MES cells and ADRN cells specifically. That way, we will understand how we can determine the optimal application of isotretinoin in the treatment regimen for high-risk NB.

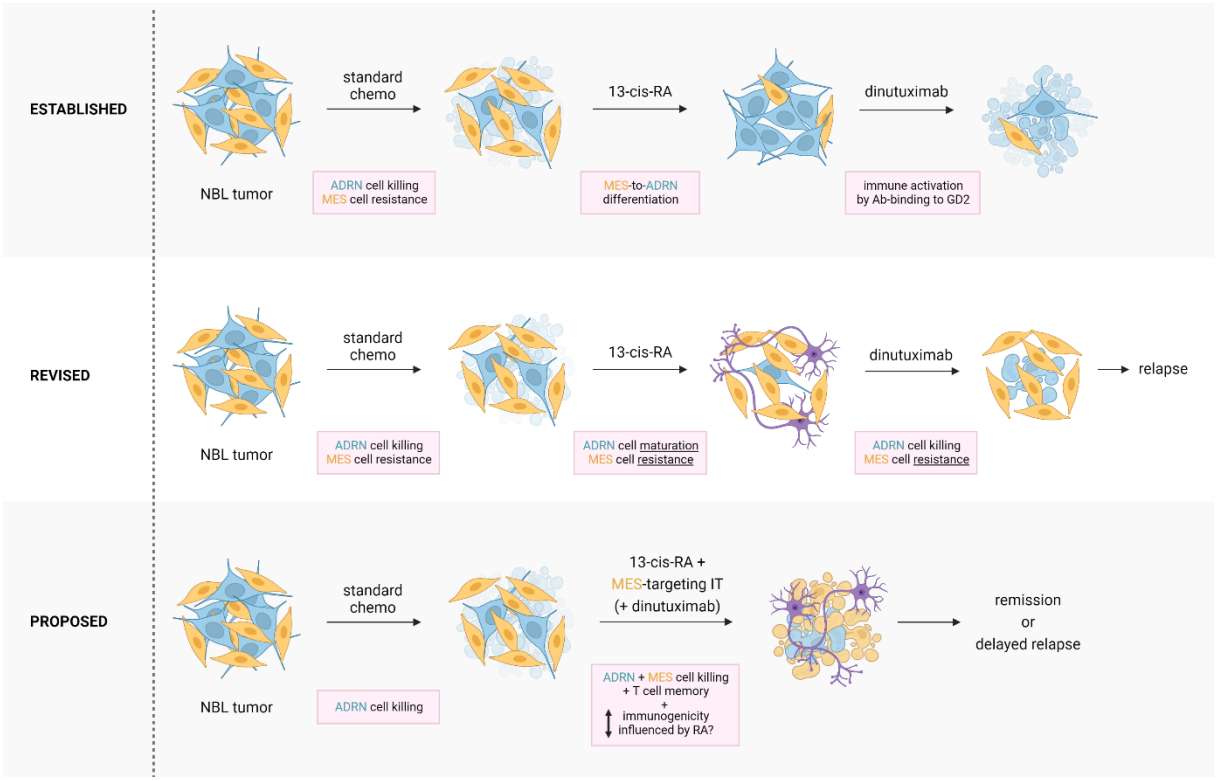

Figure 2. Proposed treatment regimen for NBL, based on recent findings in therapeutic responsiveness of ADRN and MES cells. New immunotherapeutic targets must be found that strongly target MES cells and enhance intrinsic anti-tumor activity. Created with Biorender.com.

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