Epitranscriptomic Approach: To Improve the Efficacy of ICB Therapy by Co-Targeting Intracellular Checkpoint CISH

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Abstract: Cellular immunotherapy has recently emerged as a fourth pillar in cancer treatment by co-joining surgery, chemotherapy and radiotherapy. Where, the discovery of immune checkpoint blockage or inhibition (ICB/ICI), anti-PD-1/PD-L1 and anti-CTLA4-based, therapy has revolutionized the class of cancer treatment at a different level. However, some cancer patients escape this immune surveillance mechanism and become resistant to ICB-therapy. Therefore, a more advanced or an alternative treatment is required urgently. Despite the functional importance of epitranscriptomics in diverse clinico-biological practices, its role in improving the efficacy of ICB therapeutics has been limited. Consequently, our study encapsulates the evidence, as a possible strategy, to improve the efficacy of ICB-therapy by co-targeting molecular checkpoints especially N6A-modification machineries which can be reformed into RNA modifying drugs (RMD). Here, we have explained the mechanism of individual RNA-modifiers (editor/writer, eraser/remover, and effector/reader) in overcoming the issues associated with high-dose antibody toxicities and drug-resistance. Moreover, we have shed light on the importance of suppressor of cytokine signaling (SOCS/CISH) and microRNAs in improving the efficacy of ICB-therapy, with brief insight on the current monoclonal antibodies undergoing clinical trials or already approved against several solid tumor and metastatic cancers. We anticipate our investigation will encourage researchers and clinicians to further strengthen the efficacy of ICB-therapeutics by considering the importance of epitranscriptomics as a personalized medicine.

Keywords: epitranscriptomics; immune checkpoint blockage (ICB) therapy; anti-PD-1/PD-L1 drug resistance; personalized medicine; CISH; microRNAs

1. Introduction

The advent of immunotherapy given in-combination with standard chemotherapeutic drugs has greatly control the cancer-spread over decades. However, still some cancer patients develop resistance against these therapeutic approaches, alarming the discovery of further advanced medicines. The invention of programmed cell death protein-1 and its ligand-1 (PD-1/PD-L1) was breakthrough in the history of cancer treatment, but still some tumors escape these immune surveillance mechanisms and relapse to grow continuously. Therefore, a more creative and advanced treatment is required instantly to overcome the issues largely associated with high-dose antibody/drug toxicities and drug-resistances, conceivably in the form of personalized medicines. In this study, we...
have summarized epitranscriptomic mechanisms to improve the efficacy of ICB-therapy by targeting N6-A-modification machineries especially m6-A-modifiers. Moreover, we have also emphasized co-targeting immune checkpoint proteins (PD-1 and PD-L1) along with intracellular checkpoint molecules (CISH, SOCS-1 and microRNAs) in enhancing the efficacy of ICB-therapeutics by combining immunotherapy. A recent human clinical trial NCT04426669, NCT03538613 [1,2] evidenced the success of targeting CISH/SOCS-1 in NK-cells [3,4], T-cells [5,6], and DCs [7] in further strengthening the efficacy of ICB-therapy against broad range of solid tumors and metastatic gastrointestinal cancers (Tables 1–3) and (Figure 1).

### Table 1. Epigenetic modifiers and microRNAs in improving the efficacy of ICB-therapy.

| RNA Modifiers | Disease Condition | Target | Disease Mechanism | Therapeutic Strategies | Ref. |
|---------------|------------------|--------|-------------------|------------------------|-----|
| **Writers** | **Mettl3/14** | up-regulated in colorectal cancer and melanoma | IFNγ, STAT1, IRF1, Cxcl-9 and Cxcl-10 | By reducing CD8+ T-cells infiltrations in TME | CRISPR/cas9 silencing of Mettl3/14 via YTHDF2 | [8] |
| **Mettl-3** | down-regulated in M1/M2-med. lung metastasis | Spred-2 | By recruiting immunosuppressive T-reg and MDSCs | Overexpressing Mettl3 via polarizing M1/M2-macrophages | | [9] |
| **m6A** | m6A-mediated regulation of PD-L1 in HNSCC | G2M checkpoint and PI3K/AKT/mTOR signaling | Analysed via cancer genome atlas TCGA and GSE65858 cohort | By targeting m6A regulated signature genes | | [10] |
| **Erasers** | **FTO** | up-regulated in melanoma | PD-1, CXCR4 and SOX10 | Impairs anti-PD1 effect by reducing target gene expressions | Selective inhibition of FTO to enhance anti-PD1 effects | [11] |
| **ALKBH5** | up-regulated in melanoma | Mct4/Slc16a3 | By recruiting immunosuppressive T-reg and MDSCs | Anti-ALKBH5 enhances the effect of anti-PD1 therapy. | | [13] |
| **Readers** | **YTHDF1** | up-regulated in solid tumors | Lysosomal cathepsins | Degradation neo-antigen and impair dendritic cell presentation | Anti-YTHDF1 suppress cathepsins and enhance DC cross-presentation | [14] |
| **YTHDF2** | up-regulated in LGG (brain tumor) and several other immune cells | PD-1, CTLA4, TIM3 | Impair immune checkpoint signalling | Anti-YTHDF2 in combination with immunotherapies | | [15,16] |

### DNA and Histone Modifiers in ICB-Therapeutics

| Epigenetic Regulators | Disease Condition | Target | Mechanism | Therapeutic Strategies | Ref. |
|-----------------------|------------------|--------|------------|------------------------|-----|
| **DNA methylation** | down-regulates CTLA4 in HNSCC | CTLA4, CD28, CD80/86, ICOS | DNA methylation affects HNSCC | Selective DNA (DNMTs) inhibitors | [17] |
| **DNA methylation** | down-regulates PD-L1 in melanoma | Interferon signaling | cg DNA methylation regulate melanoma | | [18] |
| **DNA methylation** | up-regulates PD-1 & CTLA4 in NSCLC | PD-1 (PDCD-1) CTLA4 | Hypo-methylation increases PD-1, CTLA4 expression in NSCLC | Selective DNA (5hmC) inhibitors | [19] |
| **DNA methylation** | up-regulates PD-L1 & PD-L2 in HNSCC | PD-L1 (CD274) PD-L2 (PDCD1LG2) | Hypo-methylation increases PD-L1 & PD-L2 expression | Combining DNA inh. with Nivolumab and Pembrolizumab | [20] |
| **DNA methylation** | up-regulates PD-L1 in CRC | PD-L1 (CD274) | DNA-methylation control PD-L1 exp. | Selective DNA (TETs) inhibitors | [21] |
### Table 1. Cont.

#### DNA and Histone Modifiers in ICB-Therapeutics

| Epigenetic Regulators | Disease Condition | Target | Mechanism | Therapeutic Strategies | Ref. |
|-----------------------|-------------------|--------|-----------|------------------------|------|
| HDAC                 | up-regulates CTLA4 in B-cell associated function | CTLA4 and LAG3 | Tcf1 regulate CTLA4 expression in T<sub>H</sub>-cells | HDACi control CTLA4-mediated B-cell help | [22] |
| HDAC6                | up-regulates PD-L1 in melanoma | PD-L1 (CD274) STAT3 | HDAC6 increase PD-L1 expression by recruiting STAT3 | HDAC6-inhibitor decreases PD-L1 by de-activating STAT3 | [23] |
| Active H3K4me3       | up-regulates PD-L1 in breast cancer | EMT-induced PD-L1 expression | Active H3K4me3 modifications in Breast cancer | Selective histone inhibition enhance the efficacy of ICB-Abs | [24] |
| Active H3K4me3       | up-regulates PD-L1 in pancreatic cancer | PD-L1 (CD274) | MLL1 catalyzed H3K4me3 to bind with PD-L1 promoter and increase its expression | MLL1 inhibitor in combination with anti-PD-L1, anti-PD-1 improves efficacy | [25] |
| Repressive H3K27me3  | down-regulates PD-L1 in HCC | PD-L1, IRF1 | EZH2 negatively regulate PD-L1 exp. by recruiting repressive H3K27me3 in HCC | Selective H3K27me3 inhibitor could enhance ICB efficacy | [26] |
| HDACi (Belinostat)   | up-regulates PD-L1 & CTLA4 in HCC | Increase IFN-γ & reduce T-reg populations | Belinostat treatment increase anti-tumor immunity against HCC | Combining belinostat enhances the efficacy of ICB therapy | [27] |
| SAHA                 | Increases CTLA4 and Foxp3 expression in cardiac transplant | Foxp3, CTLA4 | SAHA increases suppressive function of T-reg to prolong allograft survival | SAHA (HDACi) could be a promising immunosuppressive agent with CNI drug | [28] |
| H3Ac                 | up-regulates PD-L1 in drug resistant cancer cell | H3Ac enhance PD-L1 exp. | drug resistant issues in cancer cells | HDACi in combination with anti-PD-L1 | [29] |

#### MicroRNAs in ICB-Therapeutics

| miRNAs        | Disease Condition | Target | Mechanism | Therapeutic Strategies | Ref. |
|---------------|-------------------|--------|-----------|------------------------|------|
| miR-15a,b, miR-16, miR-193a-3p | down-regulated in MPM | Direct target of PD-L1 | miR-15a, miR-16 and miR-193a-3p (−) vely regulates PD-L1 | Respective miRNA mimics combined ICB-therapeutics | [30] |
| miR-17-5p     | down-regulated in melanoma | Directly binds 3′-UTR PD-L1 | miR-17-5p (−) vely regulates PD-L1 | miR-17-5p mimics with anti-PD-L1 Abs | [31] |
| miR-18a (miR-140, 142, 340, 383) | up-regulated in cervical cancer | PI3K/AKT, WNK2, SOX6, p53 PTEN, MEK | miR-18a (+) vely and miR-140, 142, 340, 383 (−) vely regulates PD-L1 | Respective microRNA antagoniR & mimics with ICB-therapy | [32] |
| miR-20b-21-130b (CD4<sup>+</sup>T-cells) | up-regulated in colorectal cancer | PTEN, B7-H1 (PD-1) | miR-21 (−) vely regulates B7-H1 (PD-1) exp. | Respective miRNAs AntagoniRs in combination with ICB-therapeutics | [33] |
| miR-23a-3p    | up-regulated in (MΦ) liver cancer | PTEN, AKT pathways | miR-23a-3p (+) vely regulates PD-L1 exp. | Anti-miR-23a-3p (antagomiR therapy) with anti-PD-L1 Abs | [34,35] |
| miR-25-93-106b cluster | down-regulated in pancreatic cancer | CXCL12, PD-L1 | miR-25-93-106b<sup>−/−</sup> mice increases PD-L1 | miR-93, miR-106b mimics with BET inh. | [36] |
| miR-28        | melanoma | PD-1 | miR-28 (−) vely regulates PD-1 | miR-28 mimics | [37] |
| miR-33a       | down-regulated in Lung A. carcinoma | PD-L1, CTLA4, PD-1, CAND1 | miR-33a (−) vely regulates PD-1/PD-L1 | miR-33a mimics with combined ICB-Abs | [38] |
| miR-34a       | down-regulated in AML, lymphoma | EBF-1 and 3′-UTR PD-L1 | miR-34a (−) vely regulates PD-L1 exp. | ICB therapy combined miRNA | [39] |
### Table 1. Cont.

| miRNAs          | Disease Condition                  | Target                  | Mechanism                           | Therapeutic Strategies                        | Ref.                |
|-----------------|-----------------------------------|-------------------------|-------------------------------------|-----------------------------------------------|---------------------|
| miR-138-5p      | down-regulated in CRC             | Target 3′-UTR PD-L1     | miR-138-5p (−)vectors                 | miR-138-5p mimics combined ICB-Abs             | [45]                |
| miR-140         | down-regulated in NSCLC           | miR-140/ PD-L1/cyclinE pathways | miR-140 target 3′-UTR PD-L1 (−)vectors | miR-140 mimics with anti-PD-L1 therapy         | [46]                |
| miR-142-5p      | down-regulated in pancreatic cancer | miR-142-5p target 3′-UTR PD-L1 | miR-142-5p (−)vectors                | miR-142-5p mimics + anti-PD-L1 therapy         | [47]                |
| miR-145         | down-regulated in ovarian carcinoma | Cisplatin cMYc (TcF)   | miR-145 (−)vectors                  | miR-145 mimics (restoration therapy) with anti-PD-L1 Abs | [48]                |
| miR-146a        | up-regulated in melanoma          | STAT1-IFNγ axis         | miR-146a (−)vectors                 | miR-146a antagononR with anti-PD-L1 Abs        | [49]                |
| miR-148a-3p     | down-regulated in dMMR/MSI-H CRC  | miR-148a-3p binds to 3′-UTR PD-L1 | miR-148a-3p (−)vectors              | miR-145 mimics with anti-PD-L1 therapy         | [50]                |
| miR-155         | up-regulated in B-cell lymphoma    | AKT and ERK             | miR-155 (+)vectors                  | miR-155 antagononR + PD-L1 antagonists         | [51]                |
| miR-191-5p      | down-regulated in colon-adenocarcinoma | PD-L1             | miR-191-5p (−)vectors               | miR-191-5p mimics                            | [52]                |
| miR-195         | down-regulated in PC and DLBCL    | PD-L1                   | miR-191-5p (−)vectors               | miR-191 mimics                               | [53,54]             |
| miR-197         | down-regulated in NSCLC           | CJK1B/STAT3 (Bcl-2, c-Myc, CyclinD1) | miR-197 (−)vectors                 | miR-193 mimics (replacement therapy) + ICB-therapeutics | [55]                |
| miR-200b, miR-152 | down-regulated in gastric cancer (GC) | B7-H1 (PD-1)          | miR-200b and miR-152 (−)vectors     | Respective miRNA mimics combined PD-L1 antagonists | [43,56,57]         |
| miR-214         | down-regulated in B-cell lymphoma (DLBCL) | miR-214 atger 3′-UTR PD-L1 | miR-214 (−)vectors                  | miR-214 mimic in combination with anti-PD-L1 Abs | [58]                |
| miR-217         | down-regulated in laryngeal cancer | AEG-1 and PD-L1        | miR-217 (−)vectors                  | miR-217 mimics with anti-PD-L1 therapy         | [59]                |
| miR-324-5p      | downregulated in Mycobacteria-responsive hedgehog sign | PD-L1, SHH signaling | (−)vectors regulate PD-L1            | miRNA mimics                                 | [60]                |
| miR-338-5p      | downregulated in Cervical cancer   | PD-L1                   | miR-340 (−)vectors                  | miR-340 mimics                               | [61]                |
| miR-340         | down-regulated in HNSCC           | JAK2                    | Inhibits JAK2-STAT1 axis suppressing PD-L1 exp. | miR-375 mimics                               | [62]                |
| miR-424 (322)   | down-regulated in ovarian cancer   | PD-1/ PD-L1, CD80/CTLA4 | miR-424 (322) (−)vectors regulate PD-L1, CD80/CTLA4 exp. | miR-424 (322) mimics (restoration therapy) + ICB-therapeutics | [63]                |
| miR-497-5p      | down-regulated in RCC (ccRCC)      | Cell proliferation      | miR-497-5p (−)vectors regulate PD-L1 exp. | miR-497-5p mimic with anti-PD-L1 Abs           | [65]                |
| miR-513         | cholangiocytes in response to C. parvum infection | B7-H1 (PD-1)          | miR-513 (−)vectors                  | miR-513 mimics                               | [66]                |
| miR-570         | down-regulated in gastric cancer   | B7-H1 (PD-1)           | SNP (polymorphism) disrupts miR-570-B7-H1 interactions | Restoration therapy combined ICB-Abs | [43,67]             |
### Table 1. Cont.

| miRNAs     | Disease Condition | Target                | Mechanism                                    | Therapeutic Strategies                          | Ref. |
|------------|-------------------|-----------------------|----------------------------------------------|------------------------------------------------|------|
| miR-873    | down-regulated in breast cancer | PI3K/Akt, ERK1/2 pathways | miR-873 (−)vely regulates PD-L1 by binding to 3′-UTR | miR-873 mimics with PD-1/PD-L1 inhibitor [68] |      |
| miR-3127-5p| up-regulated in NSCLC | pSTAT3                | Upregulates PD-L1 by suppressing p-STAT3     | Anti-miR-3127-5p (antagomIR therapy) [69]      |      |
| miR-3609   | down-regulated in breast cancer | PD-L1                 | miR-3609 (−)vely regulates PD-L1 exp.        | miR-3609 mimics [70]                             |      |
| miR-4717   | down-regulated in HBV  | PD-1                  | miR-4717 (−)vely regulates PD-1 exp.         | miR-4717 mimics [71]                             |      |

**Figure 1.** Milestones in the development of ICB-therapeutics. Discovery of immune checkpoint markers. Year of FDA-approved ICB-antibodies. Factor affecting antibody/drug-resistance. Recent strategies to improve ICB-efficacy by combining molecular medicines. Biopharmaceutical companies developing personalized medicines co-targeting epitranscriptomics and intracellular immune checkpoint (CISH/SOCS-1) in NK-cells, TILs and DCs with relevant clinical trial were summarized.

Before coming to the main stream of this review, a very logical question arises: (i) why even after so strong therapeutic approaches still some cancer cells escape these immune surveillance mechanisms? (ii) What could be the best possible combinations to overcome the issues associated with drug-resistance and high-dose antibody toxicities [72–75] and (iii) what would be the best diagnostic biomarkers or alternative strategies to completely eliminate these cancerous cells? Such questions provoked the scientist to further understand in-depth of the molecular mechanism of immune cell regulation and ICB-drug resistance. This revealed that; immune cells contains both inhibitory (break) as well as activator (acceleratory) marker to maintain immune homeostasis, or to avoid a situation called autoimmunity and self-tolerance phenomena. This understanding led to the discovery of (i) first immune checkpoint marker PD-1 or PDCD1 (CD279) in 1992 [76] and (ii) immune cell inhibitory marker cytotoxic T-lymphocyte-associated protein-4 (CTLA-4 or CD152) in 1991 [77] or 1995 [78,79]. However the first anti-CTLA4-based therapy ‘Ipilimumab’
was approved in 2011 by (James P. Allison, Nobel laureate, physiology or medicine, 2018) Medarex and Bristol-Myers Squibb for the treatment of melanoma, and the first anti-PD-1 therapy was approved in 2014 for melanoma and in 2015 for non-small-cell lung carcinoma (NSCLC) treatment (Table 2). Later, the tumor-cell inhibitory marker PD-L1 (CD274, previously known as B7-H1) was discovered in 1999-2000 [80] and PD-L2 (CD273, previously known as B7-DC) in 2001 [81] and was considered even much better control over immune cell checkpoint-based therapeutic targets [82] (Figure 2).

Table 2. Biopharmaceutical companies developing immune checkpoint blockade (ICB)-antibodies.

| Company                        | Antibody FDA Approval | Brand/Other Name | Combination                                                                 | Disease                                                                 | Clinical Trial | Ref.       |
|--------------------------------|-----------------------|------------------|----------------------------------------------------------------------------|------------------------------------------------------------------------|----------------|------------|
| Bristol-Meyers Squibb          | Nivolumab             | 2014             | LAG3 (BMS-986016), B7-H3 (Enoblituzumab), KIR (Lirilumab), 4-1BB (Urelumab), ICOS (TJX-2011), CD27 (Varilumab), GM.CD40L (vaccine for lung NSCLC) | Broad range of tumor types and Lymphomas                               | NCT01968109    | [83,84]    |
| Medimmune                      | MEDI0680              | 2014             | -                                                                          | -                                                                      | NCT02118337    | [85,86]    |
| Regeneron/Sanoﬁ               | REGN2810              |                  | -                                                                          | -                                                                      | Phase I/II      | [83,88]    |
| Novartis                       | PDR001                |                  | GITR (GWN323)                                                             | Melanoma, Lung, NSCLC, HNC, cervical, thyroid cancer                   | NCT02740270    | [87]       |
| Merck                          | Pembrolizumab         | 2014             | B7-H3 (Enoblituzumab), Multi-kinase inhibitor (Sunitinib)                  | -                                                                      | NCT02475213    | [83,88]    |
| Cure Tech                      | Pidilizumab           | 2014             | CT-011                                                                     | Malignant gliomas                                                      | Phase I/II      | [88]       |
| Sanofi                         | Cemiplimab            | 2018             | Libtayo®                                                                  | Cervical cancer                                                       | Phase III      | [83]       |
| Medarex/Bristol-Meyers Squibb  | Ipilimumab            | 2011             | Vervoy® (BMS-734016, MDX-010, MDX-101)                                    | Melanoma, SCLC, Bladder, prostate cancer                              | NCT00527735    | [83,89–92] |
| Pfizer/AstraZeneca             | Tremelimumab          | 2015             | Orphan drug approval, CP-675, 206                                         | Metastatic melanoma, Solid Tumor                                       | Phase III      | [93–97]    |
| Roche/Genentech                | Anti-PD-L1            | Atezolizumab (Humanized IgG1k), 2016 | Tecentriq®, MPDL3280A, RG7446, RO541267                                    | Ovarian, Urothelial, Lung Cancer, HNCLC                               | NCT02543645    | [83]       |
| Merck, EMD, Serono/Pfizer      | Avelumab              | 2017             | Bavencio® MSB0010718C                                                     | Metastatic MCC                                                        | Phase III      | [83,96]    |
| Medimmune/AstraZeneca          | Anti-PD-L1            | Durvalumab (Human IgG1k), 2017 | Imfinzi® MEDI4736                                                         | NSCLC, Solid Tumor, urothelial carcinoma                              | Reference [70] | [99–101]   |
| Bristol-Meyers Squibb          | Anti-PD-L1            | Human IgG4       | BMS-936559 (MDX1105)                                                      | HIV-1, Sepsis, NSCLC                                                  | Phase I        | [102–104] |
| Amplimmune/Glaxo Smith Klein   | Anti-PD-L2            | AMP-224          | -                                                                          | kidney cancer, melanoma                                                | Phase I        | [105]      |
Figure 2. Mechanism of immune checkpoint blockage or inhibition (ICB/ICI) therapy. The antigen presenting cells (APCs), especially dendritic cells and macrophages recognize and engulf the virus-infected or cancerous cells. The immune cells now processed and present the antigen to the naive T-cells in conjugation with MHC-I/II. The T-cell receptor (TCR) present on the immune cells recognizes this processed antigen and activates humoral as well as cell-mediated immune response. However, interestingly, immune cells, like CD8+ T-cells also express PD-1 marker which function as “immune checkpoint” before cytolytic activation. On the other hand, tumor-engulfed DCs also expresses PD-L1 and PD-L2 (ligand for PD-1) and inhibitor bypass the function of immune activation called “immune checkpoint inhibitor” and thus T-cells failed to recognize it and considered as ‘self’ rather than ‘foreign’. Therefore tumor cell escapes this immune-surveillance mechanism and proliferates rapidly. Blocking these immune checkpoint markers by means of specific antibodies endorsed the discovery of ICB-therapeutics, for example, (i) Anti-PD-1 therapy (or Immune cell targeted therapy): Nivolumab (Opdivo®), Pembrolizumab (Keytruda®), Pedilizumab (CT-011) and Cemiplimab (Libtayo®) block PD-1 receptor and bypass the ‘self-recognition’ mechanism of T-cells, and thereby allowing rapid recognition and cytolytic activation to kill tumor cells. (ii) Anti-CTLA4 therapy: Immune cell (T-cells) expresses CTLA-4 to maintain normal homeostasis by regulating the hyper activation of other immune cells and also to avoid autoimmunity, just like ‘speed breaker’. But due to its impairments under the TME it is required to be constantly activated, and so anti-CTLA4 antibodies, like Ipilimumab (Yervoy®) and Tremolimumab efficiently block the inhibitory effect of CTLA-4. Moreover, since it is highly homologous to CD28-receptor functions, thereby further activating CD8+ T effector function to enhance anti-tumor immunity. (iii) Tumor targeted therapy (or, immune checkpoint inhibitor): The anti-PD-L1 antibodies, like Atezolizumab (Tecentriq®), Avelumab (Bavencio®) and Durvalumab (Imfinzi®) blocks the inhibitory signal generated by tumor expressing PD-L1 (ligand for PD-1) to stop its self-defense mechanism, resulting in rapid tumor killing by T-cell attack. The detail mechanism of antigen presentation, ICB-therapy and strategies to overcome drug-resistance is well discribed in these articles [64,75].

1.1. Connotation of Immune Checkpoint Markers

The significance of these immune checkpoint markers (PD-1, PD-L1/L2) as a ‘remarkable discoveries’ was initially ratified after the experiments in mouse models, suggesting the requirement of these markers are equally vital in maintaining immune homeostasis by regulating a balance between ‘immune response’ and ‘immune tolerance’ via its acceleratory/co-stimulatory (CD28) as well as inhibitory (PD-1, CTLA4) receptors. For example, (i) the immune inhibitory function of PD-1 was demonstrated by characterizing autoimmune phenotype in PDI-deficient (PD-1−/−) mice, suggesting the loss of peripheral tolerance [106]. (ii) lupus like arthritis and glomerulus-nephritis in PD-1−/− C57BL/6 mice [107]. (iii) fatal myocarditis in PD-1−/− Balb/c and MRL mice [108,109]. (iv) Type-I diabetes in PD-1−/− NOD-mice [110,111]. (v) host vs graft disease in PD-1−/− mice crossed
with H-2LD-specific 2C-TCR transgenic mice [107], and (vi) hydronephrosis associated abnormalities in PD-1−/− Balb/c mice [112]. Similarly, the first report on CTLA-4 blockade (negative regulator of T-cell activation [113]) in anti-tumor immunity was demonstrated in 1996 [114] and the first clinical report of CTLA-4 against melanoma in 2003 [115,116]. These discoveries were sufficient enough to encourage scientists to investigate its human relevance and further clinical trial (CT) studies.

1.2. ICB Drug-Resistance and Toxicties

Beside patient’s age, cancer stage (I–IV) and various environmental factors; there might be several other factors for increased drug-resistance and reduced efficacy of ICB therapeutics [75]. For example, sub-optimal antibody dose, insufficient immune cell activation, intra-tumoral microenvironment, reduced memory cell formation and impaired effector cell functions after first course of treatment schedule. Sometimes, high-dose antibody toxicity also becomes a major concern for its adverse consequences (Figure 1). Therefore, a more advanced and unique therapy is required promptly to overcome this major issues.

Conclusively, our study devotes to improve the efficacy of ICB-therapy by co-targeting (i) epitranscriptomics (ii) intracellular immune checkpoints and (iii) microRNAs. More importantly, our investigation would help to design a specialized approach or custom-made strategies to improve the efficacy of ICB-therapy [7,117,118].

2. Milestones in ICB therapeutics

2.1. Discovery of ICB Therapy

The invention of ICB-therapy was started after the discovery of PD-1, PD-L1 and CTLA-4 like immune checkpoint markers. Write after that, several other immune-based markers were tested against broad range of tumor types, covered extensively in these articles [119–122]. However, this section diagrammatically simplifies the milestone in the development of ICB-therapy and recent strategies to improve the efficacy of ICB-antibodies by combining personalized medicines (Figure 1).

2.2. Mechanism of ICB/ICI-Therapeutics

The detail mechanism of ICB-therapy including current drug-resistance issues was already well described by Wei and Allison et al., 2018 [123], Jenkins et al., 2018 [73], Kalbasi et al., 2020 [75] and Barrueto et al., 2020 [124]. However, this section briefly simplifies the understanding of immune checkpoint markers and its implications in developing therapeutic antibodies. Although, our main focus is to resolve the issues associated with ICB drug-resistances by promoting personalized therapy (Figure 2).

2.3. Strategies to Overcome ICB Drug-Resistance

This section describes the strategies to overcome the issues mainly associated with drug-resistance and high-dose antibody toxicities. For example, (i) Epitranscriptomic approach: by targeting N6A modifiers: editor/writer, eraser/remover and effector/reader [125,126]. (ii) Bi-specific antibody approach: by co-targeting PD-1 and CD47 markers enlightened by ImmuneOncia therapeutics Inc. Korea [127,128] and AstraZeneca [129]. (iii) Antibody combination: by combining two antibodies targeting PD-1, PD-L1/L2 and CTLA-4 targets [123]. (iv) Precision medicines/personalized therapy: combining immunotherapy targeting intracellular immune checkpoints (CISH/SOCS-1) in specific immune cells [130,131]. (v) Molecular medicine: epigenetic modifiers targeting DNA, histone proteins and chromatin remodelers [22,132] and (vi) microRNAs [41,133,134] (Tables 1 and 3, Figure 1). The detail of ICB-therapy and strategies to overcome ICB-drug resistance is well described in this review [75], however covering all is out of scope of this review.
Table 3. Biotech companies/Universities entering into personalized medicine targeting intracellular immune checkpoints in combination with ICB-therapeutics.

| Biopharmaceutical Company/University | Target | Combined Therapeutic Approach | Clinical Trial | Indication | Ref. |
|-------------------------------------|--------|--------------------------------|----------------|------------|-----|
| ONK therapeutics (Ireland) 2015 www.onktreatments.com | CISH−/− NK-cells | CISH−/− NK-cells in combination with ICB-antibodies | ONK102, ONK103, ONK104 | M. Myeloma, NSCLC, AML | [135] |
| Fate Therapeutics San Diego, USA | iPSC-derived NK Cells (FT500) | Nivolumab (anti-PD-1), Pembrolizumab (anti-PD-1), Atezolizumab (anti-PD-L1), Interleukin-2 (IL-2) | NCT03841110, NCT04106167 | Advanced solid tumors and lymphoma | [136–140] |
| InnovaPharma S. A | NK cell (NKG2A) | Durvalumab (Phase-I/II), Nivolumab (Phase-I), Ipilimumab (Phase-I), Nivolumab + 5-Aza (Ph-I) | NCT02671435, NCT01929270, NCT01750580, NCT02599649 | Metastatic Cancer | [141,142] |
| Alter Biosciences corporation | IL-15 super agonist mediated NK-cells | Nivolumab (anti-PD-1) | NCT02523469 | NSCLC | [142] |
| ImmunityBio, Inc. | High-affinity Natural Killer (haNK) Cell | Avelumab (Bavencio®) (anti-PD-L1) | NCT03387936, NCT03387932 | Triple Negative Breast Cancer | - |
| SignalRX Pharmaceuticals, Inc. | SFI126 (dual inhibitor of PI3K and BRD4) | Nivolumab (anti-PD-1) | NCT03059147 | Advanced HCC | [83] |
| Efector Therapeutics | Tomiluzotinib (eFT-508) | Pembrolizumab (anti-PD-1) | NCT03616834, NCT03616836, NCT03616837, NCT03616838 | Solid tumors and NSCLC | [83] |
| NantKwest Inc., and Chan Soon-Shiong Institute for Medicine, USA | CD16-targeted NK-cell (haNK™) with N-803 (IL-15 superagonist) | Avelumab (Bavencio®) (anti-PD-L1) | NCT03853317 | Merkel cell carcinoma | [139,143] |
| National Cancer Institute, Naples | NK-cells (Tregs and NKS) | Nivolumab (anti-PD-1) | NCT03891485 | Renal cell carcinoma | [144] |
| Gachon University & Severance hospital, Republic of Korea | Allogeneic NK-Cells (SMT-NK) | Pembrolizumab (anti-PD-1), Keytruda | NCT03937895, NCT03937896 | Biliary tract cancer | [139] |
| Fox Chase Cancer Center, USA | NK-cells and T-cells | Pembrolizumab (anti-PD-1) | NCT02535247 | Lymphoma | [144–146] |
| Jilin University Hospital, China | NK-cells | PD-1 Ab | NCT03958097 | Non-small cell lung cancer | [139] |
| MD Anderson Cancer Center, USA | DF1001 (a new molecule targeting NK-cell activations) | Drug: DF1001 Pembrolizumab (anti-PD-1) | NCT04137111 | Advanced Solid Tumors | [139,144] |

**T-Cells: Tumor-Infiltrating Lymphocytes (TILs)**

| Biopharmaceutical Company/University | Target | Combined Therapeutic Approach | Clinical Trial | Indication | Ref. |
|-------------------------------------|--------|--------------------------------|----------------|------------|-----|
| Intima Bioscience, Inc. with University of Minnesota | CISH-deleted Tumor-Infiltrating Lymphocytes (TILs) | CISH checkpoint-deleted TILs combined with ICB-antibodies, Fludarabine, Aldesleukin and ICB-therapeutics | NCT0426669 | Solid tumors & gastrointestinal cancers | [1,147] |
| | CISH−/− T-cells (TILs) | NCT03538613 | Gastro-intestinal cancers | [2,5] |
| Hangzhou Cancer Hospital in collaboration with Anhui Kedgene Biotechnology Co., Ltd | PD-1 Knockout T-Cells | CRISPR/Cas9-deleted PD-1 in T-Cells with hydrocortisone | NCT03081715 | Advanced Esophageal Squamous Cell Carcinoma | [2,144] |
| Sichuan University in collaboration with Chengdu MedGenCell | PD-1 Knockout T-Cells | CRISPR/Cas9-deleted PD-1 in T-Cells with Cyclophosphamide | NCT02793856 | Metastatic Non-small Cell Lung Cancer | [2,144,148] |
| Peking University and (Cell Biotech) | PD-1 Knockout Engineered T Cells | PD-1-KO, T-cells with IL-2 and Cyclophosphamide | NCT02863913, NCT02867345, NCT02867332 (Phase-I) | Bladder, Prostate and Renal Cell Carcinoma | [2,5] |
| University of Pennsylvania, with Tmunity Therapeutics | NY-ESO-1 redirected autologous T cells | TCR-deleted and PD-1-deleted T cells | NCT03399448 | Myeloma, melanoma and several cancers | [2,5,149] |
| Nanjing University Medical School | PD-1 Knockout EBV-CTLs | PD-1-KO, EBV-CTL with IL-2, Fludarabine and Cyclophosphamide | NCT03044743 | EBV associated Malignancies | [2,5] |
### Table 3. Cont.

| Biopharmaceutical Company/University | Target | Combined Therapeutic Approach | Clinical Trial | Indication | Ref. |
|-------------------------------------|--------|-------------------------------|----------------|------------|-----|
| **Dendritic Cells (DCs)**           |        |                               |                |            |     |
| H. Lee Moffitt Cancer Center, BMS and MultiVir, Inc. | DC-based p53 Vaccine | Ipilimumab (anti-CTLA4) Nivolumab (anti-PD-1) | NCT03406715 (Phase-II) | Small Cell Lung Cancer | [137] |
| Allife Medical Sc. and Technology Co., Ltd. | DC-NK YNYY-01 (DC-NK Cells) | Pembrolizumab (anti-PD-1) Keytruda | NCT03815084 (Phase-I) | Solid tumors |     |
| Bristol-Myers Squibb and Duke Cancer Inst. | DC Vaccines | Nivolumab (anti-PD-1) | NCT02529072 (Phase-I) | Recurrent Brain Tumors |     |
| Northwest Biotherapeutics, BMS and JCCC | Autologous DCs pulsed with tumor lysate | Nivolumab (anti-PD-1) | NCT03014804 (Phase-II) | Recurrent Glioblastoma |     |
| University of Pennsylvania | Autologous DC pulsed peptide | Pembrolizumab (anti-PD-1) | NCT03092453 (Phase-I) | Advanced Melanoma |     |
| Mayo Clinic in collaboration with National Cancer Inst. | Autologous DC pulsed tumor Ags | Pembrolizumab (anti-PD-1) | NCT03053331 (Phase-I/II) | Aggressive Non-Hodgkin Lymphoma |     |
| Oslo University Hospital in collaboration with NCS and MSDC | Autologous DC | Pembrolizumab (anti-PD-1) Rituximab, GM-CSF and anti-TNF-alpha therapy | NCT0267155 (Phase-II) | Follicular Lymphoma |     |
| Capital Medical Univ. in collaboration with Duke Univ. | Autologous DC-CIK cell | Pembrolizumab Anti-PD-1 + DC-CIK (Ph-I) Anti-PD-1 alone (Ph-II) | NCT03190811 NCT03360830 | Advanced Solid Tumors and NSCLC | [144,150] |
| Sun Yat-sen University | DC-CIK cell (Cytokine-induced Killer Cell) | Anti-PD-1 antibody | NCT02886897 (Phase-I) Completed, 2019 | Refractory Solid Tumors |     |
| Beth Israel Deaconess Medical Center | Dendritic Cell Fusion Vaccine | Pidilizumab (anti-PD-1) | NCT01067287 (Phase-I) | Multiple Myeloma |     |
| Cancer Insight in collaboration with Elios Therapeutics, LLC | Autologous DC (TLPLDC Vaccine) | Checkpoint Inhibitor | NCT02675741 (Phase-I/II) | Metastatic Melanoma |     |
| Grupo Espanol Multidisciplinario del Cancer Digestivo | Autologous DC Vaccine (AVEVAC) | Avelumab (Bavencio®) (anti-PD-L1) | NCT03152565 (Phase-I/II) Completed, 2020 | Metastatic Colorectal Carcinoma |     |
| Dana-Farber Cancer Institute in collaboration with Celgene | DC/AML Fusion Vaccine | Durvalumab (Imfinzi®) (anti-PD-L1) | NCT03059485 (Phase-II) | Acute Myelogenous Leukemia |     |
| Radboud University in collaboration with Dutch Cancer Society | MIHA-loaded PD-L1/L2 silenced DC Vaccination | PD-L1/L2-silenced DC (siRNA silenced) | NCT02528682 (Phase-I/II) Completed, 2021 | Hematological Malignancies |     |
| Johns Hopkins University, USA | TLR3 agonist enhance DC activation | Anti-PD-1 in combination with DCs | - | Glioblastoma | [16] |

### 3. Epitranscriptomics in ICB-Therapeutics

Epirtranscriptomics has contributed greatly to the clinico-biological practices due to its diverse role in regulating at post-transcriptional and translational level. Epirtranscriptomics generally referred to chemical modifications in the RNA molecule without changing the nucleotide sequence. So far more than 160 chemical modifications have been identified [151] playing a crucial role in regulating various biological processes, for example, in acute myeloid leukemia treatment [125], lung adenocarcinoma [152] gastric cancer [153] and broad range tumor types [151,154,155]. The major epitranscriptomic machineries (writer/editor, eraser/remover and readers/effectors [156] not only regulate RNAs by specific regulatory mechanism [157,158] but also decide the fate of the cells and its associated immune disorders in cellular context-dependent manner. In this section, we have described the clinical application of epitranscriptomics in overcoming the issues associated with ICB drug-resistance by combining personalized approach.
3.1. Editors (Writers):

3.1.1. Mettl-3/14 in Anti-PD-1 Resistance (Colorectal Cancer)

Wang, et al., 2020 [8] demonstrated the role of Mettl-3/14 (m6A-writer enzyme) in improving the efficacy of anti-PD-1 therapy. They found that even after standard anti-PD-1 treatment, still some patients with colorectal cancer and melanoma develop resistance, because of insufficient immune response generated by the tumors with low mutation burden issues (mismatch-repair-proficient or microsatellite instability-low ‘pMMR-MSI-L’) constituting ~85% of the patients [159]. They found that, these patients have significantly increased level of Mettl-3/14, which has impaired the function of certain crucial genes under the tumor microenvironment (TME). Interestingly, CRISPR/cas9-mediated deletion of Mettl-3/14 in colorectal cancer cell line (CT26) and murine melanoma cell line (B16) has not only increased cytotoxic CD8\(^+\)T-cell (CTL) infiltrations in the TME but also provided durable adoptive immune response. Mechanistically, they justified that, the loss of Mettl-3/14 augmented mRNA-stability of IFN\(\gamma\), STAT-1 and IRF-1 by promoting IFN\(\gamma\)-STAT1-IRF1-signalling through YTHDF2 reader proteins [157,158], leading to prolong secretion of these cytokines in the TME, resulting in strong immune response. These investigations suggest the key role of Mettl-3/14 in inhibiting the efficacy of anti-PD-1 therapy by decreasing IFN\(\gamma\), Cxcl-9 and Cxcl10-mediated immune response. Conclusively, this study endorsed the immunotherapeutic potential of m6A-writer in improving the efficacy of anti-PD-1 antibody by silencing Mettl-3/14 in the TME [8]. Moreover, overexpressing FTO (m6A-demethylase) or by targeting intracellular YTHDF2 (m6A-reader protein) in decreasing Mettl-3/14 methylation could be considered as an alternative strategy to improve anti-PD-1 therapeutics (Figure 3).

Figure 3. Therapeutic model targeting ‘Mettl-3/14’ in colorectal cancer. (A) Biological mechanism: Mettl-3/14 is up-regulated in colorectal cancer and melanoma, and inhibits the expression of IFN\(\gamma\)-STAT1-IRF1 signaling via YTHDF2-mediated (decreased mRNA decay) mechanism and thereby decreases the efficacy of anti-PD-1 effect by lowering CD8\(^+\)T-cell infiltrations in the TME, and thus facilitated disease progression. (B) Therapeutic model: Anti-Mettl-3/14 therapy: CRISPR/cas9-silencing of Mettl-3/14 increases the expression of its target IFN\(\gamma\)-STAT1-IRF1 genes/signaling by reducing the recruitment of YTHDF2-mediated decay mechanism, and thus enhances the efficacy of anti-PD-1 antibody by increasing infiltrations of CD8\(^+\)T-cell in the TME. Moreover, FTO overexpression might decrease Mettl-3/14 level via balancing mechanisms, and ‘anti-YTHDF2 therapy’ by directly augmenting target gene expressions, via its mRNA stability mechanisms, might have therapeutic benefits.
3.1.2. Mettl-3 in Anti-PD-1 Resistance (Lung Metastasis)

Yin et al., 2021 [9] demonstrated the molecular mechanism of anti-PD-1 resistance by Mettl3-mediated macrophage polarization, and enlightened the significance of decreased Mettl3-level in lung metastasis. Yin and colleagues identified that the in vitro co-culture of bone marrow derived macrophages (BMDM) with B16 (skin melanoma) or LLC (lewis lung carcinoma) cell lines decreases the expression of Mettl-3. Moreover, the in vivo implantation of B16 and LLC cell lines into syngeneic mice also decreases Mettl-3 expression in tumor associated macrophages (TAM: CD11b\(^+\)F4/80\(^+\)) and tumor associated macrophages (TAM: CD11b\(^+\)F4/80\(^+\)) expressing Mettl-3. In addition, abnormal macrophage polarization characterized by increased M1-pro-inflammatory/anti-tumor (CD11b\(^+\)F4/80\(^+\)NOS2\(^{\text{high}}\)IL-12\(^{\text{high}}\)) and decreased M2-anti-inflammatory/pro-tumor (CD11b\(^+\)F4/80\(^+\)ARG1\(^{\text{high}}\)IL-10\(^{\text{high}}\)) were also noted in Mettl3\(^{\text{cKO}}\) mice, along with impaired response to effector T-cell functions. More importantly, the flow cytometry analysis of tumor bearing mice (TBM) revealed increased infiltration of immunosuppressive cells like, regulatory T-cells (T-reg: CD4\(^+\)CD25\(^+\)Foxp3\(^+\)) and myeloid derived suppressor cells (MDSCs: CD11b\(^+\)Gr1\(^+\)) in the TME evidenced by increased expression of CCL22-migratory marker in Mettl3\(^{\text{cKO}}\) mice. Reciprocally, selective depletion of T-reg (anti-CD25) and macrophages (clodronate liposomes) significantly decreased both tumor growth and lung metastasis. This result clearly suggests that anti-PD-1 resistance has occurred due to (i) increased abundance of immunosuppressive populations, (ii) impaired CD8\(^+\) T-cell effector function and (iii) hyper-polarization of M1/M2-macrophage in the TME of Mettl3\(^{\text{cKO}}\) mice mimicking diseased model. Mechanistically, m6A-methylated RNA-immunoprecipitation followed by high throughput sequencing (MeRIP-Seq) of the RNA isolated from BMDMs from Mettl3\(^{\text{WT}}\) and Mettl3\(^{\text{cKO}}\) mice revealed ‘spred2’ as a potential downregulated target of Mettl-3 overlapping MAPK/ERK pathways. This suggests that spred2 is an upstream target of NF\(\kappa\)B and STAT3 pathway in polarizing M1/M2-macrophages, as well as negative regulator of ERK/MAPK-signaling [160]. The above findings were further validated by reverting the M1/M2-polarizations by selective inhibition of STAT3 (S3I-201) and NF\(\kappa\)B (BAY-11-7082) pathways, justified by chromatin immunoprecipitation (ChIP) for increased STAT3 binding to Arg1 promoter (M2-polarization marker) in Mettl3\(^{\text{cKO}}\). Next, with regard to epigenetic regulation, the overexpression of Mettl-3 increases the translation of ‘spred2’ by YTHDF1-mediated mechanism, confirmed by increased binding of YTHDF1 to spred2 via RNA-IP. Conversely, knockdown of YTHDF1 (siRNA) diminishes spred2 level. This result further supports ‘spred2’ as a target of Mettl-3 and is regulated by YTHDF1-mediated mechanism [126] rather than by targeting mRNA-stability or promoter-dependent translation mechanisms [161], and thereby activated ERK-mediated (being spred2 as a negative regulator of ERK signaling) other downstream signaling pathways in polarizing M1/M2-macrophages. Additionally, polysome profiling for translation-active (>80S) regulatory site and m6A-conserved motif ‘GGAC’ analysis further authenticate spred2 regulation by Mettl3-methylation mechanisms, validated by decreased spred2 expression in mutant (G\(\rightarrow\)C)C motif as compared to the wild-type (GGAC) motif. Lastly, the link between Mettl3-driven spred2 and ERK1/2-NF\(\kappa\)B-STAT3 signaling confirms the polarization of M1/M2-macrophage by aggravating TNF\(\alpha\) and IL-6 (M1: pro-inflammatory) and IL-10, Arg1 (M2: anti-inflammatory) cytokines, validated by diminished expression of the same by selective signaling inhibitors. Taken together, these results suggest the crucial role of Mettl-3 in impairing anti-PD-1 efficacy by (i) polarizing M1/M2-macrophage via activating spred2-mediated ERK1/2-NF\(\kappa\)B-STAT3 signaling cascade through cytokine milieu and (ii) by recruiting immunosuppressive cell populations in the TME. Conclusively, Mettl-3 is key player in reducing the efficacy of anti-PD-1 therapy, and therefore targeting (overexpressing) Mettl-3 could be a promising approach to
control cancer metastasis by enhancing the efficacy of anti-PD-1 antibodies (Figure 4) [9]. This hypothesis was further supported by Yi, et al., 2020 in regulating PD-L1 mediated HNSCC control by implicating m6A-modifiers, and thus potentiating its therapeutic value by targeting G2M checkpoint, mTORC1 and PI3K/AKT/mTOR signaling analyzed via cancer genome atlas TCGA (n = 499) and GSE65858 (n = 270) cohorts [10].

**Figure 4.** Therapeutic model targeting ‘Mettl-3’ in lung metastasis. (A) Biological mechanism: Mettl-3 is significantly down-regulated in tumor associated macrophage (TAM) and thereby alters M1/M2-macrophage-polarization and thus increases the infiltration of immunosuppressive populations (T-reg and MDSCs) in the tumor microenvironment, resulting in increased tumor growth and lung metastasis. (B) Therapeutic model: overexpression therapy: overexpression of Mettl-3 recruited YTHDF1-reader protein which increases the expression of its target ‘spred2’ gene, resulting in decreased infiltration of immunosuppressive cells by reducing ERK1/2 signaling, that finely reduces lung metastasis by improving the efficacy of anti-PD-1 therapeutics.

3.2. Erasers (Removers):

3.2.1. FTO in Anti-PD1 Resistance (Melanoma):

Yang et al., 2019 [11] demonstrated the role of m6A-eraser protein ‘FTO’ in melanoma progression, a type of skin cancer, and enlightened the intrinsic mechanism to improve the efficacy of anti-PD-1 therapy by targeting FTO. Yang and colleagues found that FTO is significantly up-regulated in human melanoma patients (metastatic skin samples n = 65) including human (Mel624) and mouse (B16F10) cell lines, and facilitated rapid tumorigenesis, caused by metabolic starvation stress in mice requiring autophagy and NFκB pathway [162]. However, selective depletion of ‘FTO’ not only increases sensitivity to anti-PD-1 therapy but also increases m6A methylation-inhibition of critical pro-tumorigenic (tumor-promoting) genes. Mechanistically, they proved that FTO-deficiency increases m6A-methylation at 5′UTR and 3′UTR of target genes; PD-1 (PDCD1), CXCR4 and SOX10, and thereby causing rapid mRNA-degradation by recruiting YTHDF2-reader proteins [155,157,158], confirmed by YTHDF2-knockdown in ‘increasing’ and YTHDF2-overexpression in ‘decreasing’ melanoma growth. Moreover, FTO-deficiency enhances the sensitivity of anti-PD-1 treatment by IFNy-mediated cytokine response. These results clearly suggest that FTO plays a crucial role in melanoma tumorigenesis by regulating mTOR signalling through limiting the nutrient supply to the tumours [162]. Therefore, co-targeting FTO in combination with ICB-antibodies would be a promising approach to control melanoma progression [11]. This hypothesis was also supported by Singh et al., 2016 in controlling triple-negative inflammatory breast cancer cells using FTO (MO-I-500) inhibitor [125,163,164].
ically, targeted overexpression of Mettl-3 might also control melanoma progression by decreasing FTO via balancing mechanism, and also by directly inhibiting the expression of pro-tumorigenic genes via recruiting YTHDF2 reader proteins (Figure 5).

**Figure 5.** Therapeutic model targeting ‘FTO’ in melanoma. (A) Biological mechanism: FTO is highly up-regulated in melanoma (due to starvation stress through NFκB-pathways and autophagy) leading to increased mRNA transcript of the critical pro-tumorigenic genes (PD-1, CXCR4 and SOX10) by decreasing m6A-methylation mark, resulting in increased melanoma progression. (B) Therapeutic model: (i) Anti-FTO therapy: selective inhibition of FTO (FTO inhibitor [125]) or intracellular silencing of ‘FTO’ controls melanoma progression by selectively increasing the methylation-inhibition of its pro-tumorigenic genes, including PD-1 immune checkpoint markers by increasing the efficacy of anti-PD-1 antibody [163]. (ii) YTHDF2 therapy: YTHDF2 overexpression would control melanoma progression by accelerating the mRNA-decay of critical pro-tumorigenic genes. Moreover, targeted overexpression of Mettl-3 might control melanoma progression by destabilizing critical tumor-promoting genes by recruiting YTHDF2-reader proteins. Furthermore, targeting NFκB/mTOR signaling might also control melanoma progression by limiting nutrient supply to the tumors.

3.2.2. FTO in Anti-PD-1 Resistance (Colon Cancer)

Tsuruta et al., 2020 [12] demonstrated the role of FTO in colon cancer progression and enlightened the molecular mechanism to control cancer carcinogenesis by targeting FTO. They found that FTO is aberrantly expressed in colon cancer cell line (HCT-116). Moreover, immune checkpoint molecule ‘PD-L1’ expression was also highly up-regulated. Therefore, targeting FTO by selective depletion (siRNA) not only reduced FTO-level but also significantly decreased PD-L1 expression in IFNγ signaling-independent manner at both mRNA and protein levels. This result clearly suggests that FTO facilitates colon cancer progression by promoting the expression of PD-L1 markers. Mechanistically, they proved (via RNA immunoprecipitation) that FTO binds to m6A-marked PD-L1 mRNA and elevates its expression probably by decreasing mRNA-decay mechanism. Taken together, this study reveals the critical role of FTO in facilitating colon carcinoma by increasing PD-L1 expression, and therefore targeting FTO by means of either selective FTO inhibitor [125] or CRISPR/Cas9-based methods could hold the potential to control colon cancer in combination with anti-PD-L1 therapeutics (Table 1) [12].
3.2.3. ALKBH5 in Anti-PD-1 Resistance (Melanoma)

Li et al., 2020 [13] explained the role of another m6A-eraser protein ‘ALKBH5’ in progression of melanoma-associated metastatic cancer, and enlightened the molecular mechanism to overcome anti-PD-1 resistance by targeting ALKBH5. Based on their previous studies [8] for the role of Mettl-3/14 in melanoma progression, the authors hypothesized that ALKBH5 might also have significant role in regulating the efficacy of anti-PD-1 therapeutics. To this end, Li and colleagues used B16 (mouse melanoma) and CT26 (colorectal carcinoma)-induced TBM model, and selectively depleted ALKBH5 and/or FTO (CRISPR/Cas9-mediated silencing) in B16 and CT26 cell lines respectively, and injected subcutaneously into wild-type C57BL/6 and BALB/c mice to create tumor, followed by 1-day prior vaccination with irradiated B16 cells secreting GM-CSF ‘GVAX’ to induce sufficient antitumor T-cell response, and finally anti-PD-1 antibody treatment was given to check its efficacy. Interestingly, ALKBH5−/− TBM showed prolonged survival and slower tumor growth as compared to the non-transfected (NTC) control mice, suggesting the direct involvement of ‘ALKBH5’ in interfering with the efficacy of anti-PD-1 antibody. To further elucidate the role of ALKBH5 in modulating GVAX/anti-PD-1 treatment, they analysed tumor infiltrating lymphocytes (TILs) by FACS and found that among total CD45+CD4+CD8+ gated populations, ALKBH5−/− mice have elevated granzyme-B (GZMB)+CD8+ CD45+CD4+CD8+ T-cell, NK-cell (CD56+) and dendritic cell (DCs: CD45+Ly6C+MHC-II+CD24hiF4/80lo) numbers, but more importantly, T-reg (CD4+Foxp3+) and polymorphonuclear myeloid derived suppressor cell (PMN-MDSCs: CD45+CD11b+Ly6G+Ly6CloF4/80−MHC-II−) populations were drastically reduced as compared to the control mice. This was further validated by immunohistochemistry staining (IHC) of the MDSC-mLy6G, however, no differences in other immune cell populations (MDSC and macrophage) were noted. This suggests that ALKBH5 has the potential to recruit immunosuppressive (T-reg and PMN-MDSCs) populations in the TME during ICB therapy. Again, to stamp the selective function of immunosuppressive cells in inhibiting anti-PD-1 effect, they specifically depleted T-regs (anti-CD25) and PMN-MDSCs cells in the NTC control mice, resulting in delayed tumor progression as compared to the ALKBH5−/− model (due to already fewer T-reg numbers), confirming the immunosuppressive function of T-regs (induced by MDSCs) in impairing the efficacy of anti-PD-1 antibody by inhibiting CD8+ T-cells effector functions through decreasing DC-differentiation (CD45+Ly6C+MHC-II+CD24hiF4/80lo) markers [165]. These observations clearly suggest that ALKBH5 recruits immunosuppressive populations in the TME and thereby interfering with the efficacy of anti-PD-1 therapy. Next, to identify the molecular targets, they sequenced RNA isolated from ALKBH5/FTO−/− B16 tumors and compared it with the NTC-control TBM on day-12 after GVAX/anti-PD1 treatment. Interestingly, the gene ontology (GO) analysis of the differentially expressed genes (DEG) revealed ALKBH5 is associated with metabolic genes especially ‘Mct4/Scl16a3’ involved in lactate metabolism, whereas, FTO is associated with IFNγ and chemokine signalling pathways. This was validated by increased IFNγ intermediates (qRT-PCR expression) upon in-vitro stimulation of IFNγ to the FTO−/− B16 cells. Moreover, the comparison of mouse DEGs with human melanoma patients (n = 21 anti-PD1 therapy responder) and (n = 17 non-responder) reveals eight common genes associated with ALKBH5-deficiency and eleven common genes with FTO-deficiency, indicating ‘conserved’ and potential targets of ALKBH5 and FTO in mouse as well as human receiving anti-PD1 therapy. This suggests that ALKBH5 modulates anti-PD-1 resistance by recruiting immunosuppressive T-reg cells and by modulating metabolic genes whereas FTO works by targeting IFNγ and by modulating inflammatory chemokine-mediated signalling pathways in the TME. Next, epigenetic analysis via LC-MS/MS reveals higher m6A-abundance in ALKBH5-deficient as compared to FTO-deficient B16 tumours, which meaningfully suppresses the expression of m6A-mediated ‘Mct4/Scl16a3’ in ALKBH5 alone and ‘Mex3d’ in ALKBH5 and FTO both. Moreover, MeRIP-seq reveals enriched SRSF motif (a subunit of SAG core involved in RNA splicing [166]) in ALKBH5-deficient tumors as compared to FTO, suggesting different mechanisms of action of these two de-methylases in modulating anti-PD1 efficacies. Collectively,
these results suggest that ALKBH5 and FTO target metabolic genes and increase the expression of Mct4/Slc16a3 and Mex3d (supplementing lactate to the tumour) by inhibiting m6A methylation-mediated RNA-splicing mechanisms, supported by Zaho et al., 2014 [167], (Figure 6, therapeutic model). Furthermore, to dig out the m6A-modulated genes via RNA-splicing mechanism, they identified m6A-enriched transcripts around 5’-3’ splice sites by m6-CLIP and found the involvement of three immunotherapeutic resistance genes Eif4a2, Arid4b and USP15 affecting the response of anti-PD-1 therapeutics by regulating transcription, translation and T-reg activation via TGF-β signalling in the TME. (Figure 6)

Taken together, ALKBH5 is playing a crucial role in promoting tumour metastasis, and therefore intracellular silencing of ALKBH5 in the TME would hold the potential to control tumor metastasis via increasing the efficacy to anti-PD-1 therapeutics [13].

Figure 6. Therapeutic model targeting intracellular checkpoint ‘ALKBH5’ in melanoma. (A) Biological mechanism: The ALKBH5 abnormally expressed in melanoma and colorectal cell carcinomas, and impairs the efficacy of anti-PD-1 therapy by (i) recruiting immunosuppressive; regulatory T-cell (T-reg) and polymorphonuclear myeloid derived suppressor cells (PMN-MDSC) abundances in the TME. (ii) by impairing DC-differentiation resulting in decreased CD8+ T-cell effector functions. (iii) by increasing extracellular lactate availability to the tumors by up-regulating the expression of Mct4/Slc16a3 genes due to decreased m6A-methylation mark associated mechanism. (B) Therapeutic model: (i) Anti-ALKBH5 therapy: selective inhibition of ALKBH5 [168] by increasing m6A methylation-mediated inhibition of crucial genes essential to increase the efficacy of CD8+ T-effector cells. (ii) T-reg/PMN-MDSCs depletion therapy: could also show the therapeutic propensity by rescuing the immunosuppressive environment. (iii) Increasing DC-differentiation: could be also a promising approach to enhance DC-mediated CD8+ T-cell effector function. (iv) Targeting metabolic genes: could be an alternative approach to control melanoma tumorigenesis by limiting extracellular lactate accumulation in the TME. Collectively, all these approach seems promising in overcoming the issues associated with ICB drug-resistance.

3.3. Effectors (Readers):
3.3.1. YTHDF1 in Anti-PD1 Resistance (Solid Tumors)

Han et al., 2019 [14] demonstrated the synergistic role of dendritic cells expressing ‘m6A-writer’ and ‘YTHDF1-readers’ proteins in anti-tumor immunity. They found that despite the presence of numerous neo-antigens, some patients still failed to generate sufficient anti-tumor response. To this end, in discovering the intrinsic molecular mechanism, they generated dendritic cell-specific conditional knockout mice depleted with YTHDF1 (YTHDF1cKO) gene. Surprisingly, the loss of YTHDF1 enhances antigen-recognition and cross-presentation ability of DCs in-vivo, resulting in elevated CD8+ T-cell infiltration in the TME as compared to the control wild type (YTHDF1WT) mice. Moreover YTHDF1cKO mice showed enhanced response to anti-PD1 therapy [159]. Mechanistically, they proved that the
wild type mice, in the presence of m\textsuperscript{6}A mRNA-methylation machineries recruited YTHDF1 reader proteins at the lysosomal-cathepsins mRNA axis, resulting in increased mRNA-stability, and thereby increased the abundance of cathepsin proteins in the phagosomal compartments of the DCs, causing severe degradation of the neo-antigens and thus limiting the antigen availability to the DCs for antigen-recognition and further cross-presentation to CD8\textsuperscript{+} T-cells in the cytosol. This result suggests that YTHDF1 is playing a crucial role in suppressing anti-tumor immunity [14], and therefore intracellular silencing of YTHDF1 in DCs designates its potential to enhance anti-tumor immunity. Collectively, this discovery reveals two important mechanisms to enhance anti-tumor immunity by co-targeting (i) anti-YTHDF1 therapy: where, YTHDF1-deficiency protect ‘antigen-degradation’ and allows efficient recognition and presentation by DCs, in-turn, further increases the abundance of DC-mediated effector CD8\textsuperscript{+} T-cells by cross-presentation mechanism, supported by Ding et al., 2021 [169] and (ii) by enhancing anti-PD-1/PD-L1 efficacy: which further potentiates the efficacy of anti-PD-1 immunotherapy by enhancing the effector function of CD8\textsuperscript{+} T-cells in the TME [159] Figure 7.

Figure 7. Therapeutic model targeting intracellular checkpoint ‘YTHDF1’ in enhancing DC-mediated anti-tumor immunity. (A) Biological mechanism: The ‘YTHDF1’ reader protein recognizes m6A-marked cathepsin transcript and increases it’s mRNA and protein level, which translocate into the phagosome and degrades neo-antigens, and thus limiting its recognition and cross-presentation by the DCs, and thereby the impaired DCs decreases CD8\textsuperscript{+} T-cell effector function, leading to decreased efficacy of anti-PD-1 therapy, resulting in increased tumor growth. (B) Therapeutic model: Anti-YTHDF1 therapy: inhibits cathepsin level and thus unable to degrade neo-antigens, resulting in effective antigen recognition and cross-presentation by DCs and thereby enhanced CD8\textsuperscript{+} T-cell effector function which improves the efficacy of anti-PD-1 therapy.

3.3.2. YTHDF2 in Anti-PD1 Resistance (Brain Tumors)

Lin et al., 2020 [15] demonstrated the role of YTHDF2 in progression of lower-grade glioma (LGG) also called ‘pilocytic astrocytoma’, a type of early stage brain tumor. They showed that YTHDF2 is abnormally expressed in various types of cancers and reduces overall longevity and survival. The higher expression of YTHDF2 has been positively correlated with immune cell (B-cells, T-cells, DCs, MΦ and neutrophils) expressing PD-1, TIM-3 and CTLA-4 markers. Therefore, targeting YTHDF2 in DCs would hold the potential to enhance anti-tumor immunity in combination with ICB-therapeutics [14,15]. A similar pre-clinical trial was proposed by jubilant-therapeutics targeting PD-1 inhibitor (with brain penetrant PRMT5) in controlling LGG, potentiating the scope to utilize in
combinations with immune cells targeting intracellular checkpoints as targeted therapy. Moreover, Garzon-Muvdi et al., 2018, have supported the prominence of DC activation in enhancing the efficacy of anti-PD-1 immunotherapy against glioblastoma [16]. Taken together, this study reveals the importance of targeting YTHDF2 in combination with DC-immunotherapy [7] to enhance the efficacy of ICB therapy against early stage brain tumors [15], (Table 3).

4. Immune Cells: Targeting Intracellular Checkpoint ‘CISH’ in Combination with ICB-Therapeutics and Recent Clinical Trials

Cytokine-inducible SH2-domain containing protein (CISH or CIS) is one of the eighth members of SOCS family of proteins, recently gaining high attention due to its widespread regulatory role in cytokine signalling [170,171] and its involvement in more than 349-diseased (https://platform.opentargets.org/target/ENSG00000114737/associations; accessed on: 25 July 2021) [172–174] phenotypes. The therapeutic significance of ‘CISH’ can be evidenced by a recent clinical trial (NCT04426669, NCT03538613 by Intima Bioscience, UK; and ONKT102, ONKT103 and ONKT104 by ONK therapeutics, Ireland) targeting NK-cells, TILs and DCs for the treatment of broad range of metastatic cancers [1,2]. The so-called personalized medicine targeting ‘CISH’ in immune cells has shown promising effect in improving the efficacy of ICB-therapeutics [3,5]. Therefore, this section highlights another layer of strengthening ICB-therapy by targeting intracellular immune checkpoint ‘CISH’ in different immune cells. A few important links/references are also provided in (Box-2) supporting ‘CISH/SOCS’ to be used as potential markers in developing personalized medicine [7,175–178] (Table 3, Box-1).

4.1. NK-Cells Targeting CISH in ICB Therapeutics

Delconte et al., 2016 [3] demonstrated the therapeutic benefit of anti-PD-1 and anti-CTLA-4 antibodies in combination with intracellular checkpoint targeting CISH (CISH-deletion) in NK-cells for the treatment of lung metastasis and melanomas in murine model. They showed that intravenous (i.v.) administration of melanoma cell line (B16/F10) and prostate cancer cell line (RM-1) into CISH-deficient (CISH\(^{-/-}\)) NK-cells have significantly reduced melanoma growth and metastatic nodule formation as compared to the wild-type (CISH\(^{+/+}\)) mice, indicating the critical role of CISH in NK-cell cytotoxicity. The specificity of NK-cell function was confirmed by selective depletion of NK-cells (anti-asiolo GM1) in rendering susceptibility to B16F10 metastasis in CISH\(^{-/-}\) mice. Moreover, the adoptive transfer of CISH\(^{-/-}\) NK-cells into NK-cell\(^{-/-}\) recipient mice (Mcl1\(^{f/f}\)Ncr1-i\(^{Cre}\)) showed fewer B16F10 metastases as compared to the mice receiving CISH\(^{+/+}\) NK cells. These results clearly suggest that (i) CISH is playing a crucial role in NK-cell activation. (ii) CISH is a negative regulator of NK-cell cytotoxicity and (iii) CISH\(^{-/-}\) NK cells are intrinsically more active. Moreover, combining anti-PD-1 and anti-CTLA-4 antibody treatment with CISH\(^{-/-}\) NK-cells drastically reduced lung metastasis as compared to the IgG control and CISH\(^{+/+}\) NK-cells alone in the adoptive transfer model, highlighting the potential therapeutic benefit that could be achieved when anti-PD-1 and anti-CTLA-4 therapy was combined with loss of CISH function. A similar result targeting intracellular checkpoint ‘CISH’ in NK-cells’ in combination with ICB-antibodies in increasing anti-tumor immunity was described by Putz et al., 2017 [4], Bernard et al., 2021 [179], Felices et al., 2018 [180] and Andre et al., 2018 [181]. Furthermore, a recent phase-I clinical trial (ONKT102, ONKT103 and ONKT104) targeting CISH-deletion in NK-cells was proposed by ONK therapeutics, Ireland, until 2021–2022 against hematological malignancies (multiple myeloma and acute myeloid leukemia) and solid tumors (ovarian, NSCLC and breast cancers) [127,135,142] (Figure 8A,D). In addition to ‘CISH’, other immune checkpoint markers in NK-cells were nicely described by Chiossone et al., 2018 [121].

4.2. T-cells Targeting CISH in ICB Therapeutics

Palmer et al., 2020 [5] demonstrated the improved efficacy ICB-antibodies when combined with CISH-depleted (CISH\(^{-/-}\)) TILs. Palmer and colleagues showed that the adoptive
transfer of neoantigen specific TILs, derived from antigen expressing tumors, was failed in constantly eliciting durable tumor regression. Moreover, an altered expression of CD39, Tox and PD-1 marker was observed, suggesting the impaired function of effector CD8+ T-cells in the TME. Interestingly, depletion of CISH (CRISPR/Cas9) in TILs significantly improved neoantigen recognition, TCR avidity, T-cell activation/expansion and tumor cytolysis, resulting in rapid-control over tumorigenesis. However an increased expression of PD-1 marker was also observed. Thus, co-targeting CISH−/− TILs in combination with anti-PD1 antibody has proficiently controlled the tumor progression. This result clearly suggests the negative regulatory role of CISH in impairing T-cell effector functions, supported previously by Palmer et al., 2015 [6] and Periasamy et al., 2011 [147]. Therefore, co-targeting CISH−/− TILs in combination with ICB-therapy would hold the potential to control tumor progression by improving the efficacy of ICB-antibodies as well as CD8+ T-cell effector function. A relevant human Phase-I/II clinical trial (NCT04426669, NCT03538613) targeting CISH−/− TILs in combination with ICB-therapy was proposed by Intima Bioscience, UK, until 2021–2022 against wide range of tumor types and gastrointestinal cancer [1,2,115] (Figure 8B,E, Table 3, Box-1).

4.3. Dendritic Cells Targeting SOCS-1/CISH in ICB-Therapeutics

Wang et al., 2018 [7] demonstrated the therapeutic benefit of targeting intracellular checkpoint SOCS-1, one of the members of CISH family, in DCs (SOCS-1−/− DCs [182]) in controlling relapsed acute leukemia (RAL). They showed that the adoptive transfer of genetically modified DCs plus CIK cells is safe & effective in prolonging the survival of RAL patients (n = 48), by increasing DC activation, DC-maturation and TAA-induced CTL response. A relevant human phase-I/II clinical trial (NCT01956630) was conducted by the academy of military medical sciences, China and recommends it safe in-use [7]. A similar result was observed by Shen et al., 2004 in increasing anti-tumor immunity by silencing SOCS-1 in DCs [183]. More relevantly, Miah et al., 2012, demonstrated the importance of CISH-expressing DCs in increasing anti-tumor immunity by enhancing CTL activity in CISH−/− CD11c mouse model [184], however it would be interesting to further investigate the role by combining ICB-antibodies. These findings suggest that targeting intracellular checkpoints ‘CISH/SOCS-1 in DCs’ would hold the potential to treat several cancers even-in-combination with ICB-therapeutics [7,185] (Figure 8C,F and Tables 1 and 3).

In addition, several researches support the improved efficacy of DC-immunotherapy when combined with ICB-antibodies. For example; Zhang et al., 2019 [186] demonstrated the role of PD-1 blockade in increasing anti-tumor activity of specific DCs called DC-stimulated cytokine-induced killer cells (DC-CIK) generated in presence of anti-CD3-antibody, IFNγ, poly-hydroxyalkanoates and IL-2; characterized by co-expression of CD56 and CD3 or CD3 and CD8 markers. The authors have shown that the adoptive transfer of pre-treated DC-CIK with PD-1 inhibitor (Pembrolizumab) block PD-1/PD-L1 axis and therefore increased its cytotoxic activity as compared to the null-DCs. Moreover, an increased infiltration of effector CD8+T-cells was noted in a nude mouse xenograft model with hepatocellular carcinoma (HCC), resulting in reduced tumor growth. This study suggests the improved efficacy of pre-treated ‘PD-1 inhibitor DC-CIK’ in controlling HCC recurrence [186]. Similarly, Lim et al., 2016 supported the above hypothesis and further emphasized the anti-tumor activity of PD-1−/− DCs in controlling HCC [187]. They showed that PD-1 expression on DCs reduces T-cell proliferation and suppresses CD8+T-cells effector function, resulting in decreased anti-tumor immunity. The adoptive transfer of PD-1−/− DCs increases CD8+T-cells infiltrations along with IFNγ, IL-2, perforin and GZMB secretions in the TME and thereby causing rapid tumor control. This result suggests the improved efficacy of PD-1−/− DCs in controlling HCC [187]. Next, Garzon-Muvdi et al., 2018 showed improved efficacy of anti-PD-1 when given in combination with DC-immunotherapy in controlling glioblastoma [16]. They showed that DC-activation through TLR3 agonist increases anti-tumor immunity in vitro. Moreover, TLR3 agonist
poly (I:C)-injected mice showed increased DC-activation, antigen presentation and T-cell proliferation and thus enhancing the efficacy of ICB-therapy against glioblastoma [16].

Furthermore, Peng et al., 2020 [188] demonstrated the role of PD-L1 (ligand for PD-1) in the impairment of DCs. They showed that in response to antigenic exposure and IFN-II, type-I DCs (cDC1) increases the expression of PD-L1 and suppress CTL activity. Interestingly, the blocking of PD-L1 significantly improves DC-mediated T-cell infiltration and killing abilities in vitro. This result clearly suggests that PD-L1 expression is playing a crucial role in cDC1-impairment, and therefore targeting PD-L1 would hold the potential to enhance therapeutic benefits [188]. Similarly, Go et al., 2021 underlined the role of PD-L1-expressing DCs in reducing helicobacter-induced gastritis [189]. Go and colleagues showed that the treatment of anti-PD-L1 or PD-L1−/− in bone marrow transplantation enhances gastritis. Upon a closer look, the loss of Fli3 (Flt3−/−) or Zbtb46-diphtheria toxin receptor (DTR) mice showed decreased DC-abundances causing severe mucosal metaplasia, and suggesting the protective role of PD-L1 expressing DCs in controlling gastritis [189]. Furthermore, miRNA-200b and miRNA-152 have been found to be downregulated in HP-induced gastric cancer tissues, suggesting a negative correlation of miRNA-200b and miRNA-152 in B7-H1 (PD-1) expression. Therefore, targeting miRNA (miRNA-restoration or miRNA-mimics) would have therapeutic benefit against gastric cancer [57] (Table 1). Collectively, these investigations suggest the potential therapeutic benefit of targeting intracellular immune checkpoint ‘CISH/SOCS-1’ in enhancing the efficacy of ICB-therapy if combined with DC-immunotherapy (Table 3, Figure 8C,F).

Figure 8. Immunotherapeutic model co-targeting intracellular checkpoint (CISH/SOCS-1) in combination with ICB-therapeutics. (A) NK-cell biological mechanism: CISH expression in natural killer cell impairs its cytolytic function by reducing NK-cell proliferation, fitness and survival inside the TME [190] and thus insufficient in protecting lung metastasis [3].
B) Tumor infiltrating lymphocytes (TILs) biological mechanism: CISH expression in TILs also impairs T-cells function by reducing TCR avidity, cytokine poly-functionality and CD8+ T-cells effector functions and thereby facilitating tumorigenesis [6]. (C) Dendritic cell biological mechanism: SOCS1-deficiency and CISH-expression in DCs has been proven in regulating DC-mediated anti-tumor immunity against broad range of solid tumors by increasing DC-activation and CTL-activity [7,184]. (D) Therapeutic model targeting CISH in NK-cells: (i) anti-CISH therapy: CISH deletion (CRISPR/Cas9) in NK-cells improves overall NK-cell survival, proliferation, fitness and effector functions, and thereby potentiating its cytotoxic activity. (ii) Personalized therapy: co-targeting CISH−/− NK-cells with ICB-antibodies significantly improves ICB-efficacy in controlling lung metastasis [190]. A relevant human clinical trial (ONKT102, ONKT103 and ONKT104) targeting CISH-deficient NK-cells has been proposed by ONK therapeutics, Ireland, against hematological malignancies and solid tumors [135] (Box-1). (E) Therapeutic model targeting CISH in TILs: (i) anti-CISH therapy: CISH deletion (CRISPR/Cas9) in TILs improves T-cell effector functions by increasing TCR avidity and cytokine poly-functionality, and thereby potentiating its effector function. (ii) Personalized therapy: co-targeting CISH−/− TILs with ICB-antibodies significantly improves ICB-efficacy in increasing anti-tumor immunity. A relevant human clinical trial (NCT04426669, NCT03538613) targeting CISH−/− TILs has been proposed by Intima Bioscience, Inc. UK, against solid tumors and metastatic gastrointestinal cancers [1,2] (Box-1). (F) Proposed therapeutic model targeting SOCS-1/CISH in DCs: (i) SOCS-1/CISH regulated therapy: Regulation of SOCS-1/CISH DC-mediated anti-tumor immunity has been already shown in increasing CTL-activity against broad range of solid tumors [7,182–184], however, (ii) Personalized therapy: Co-targeting SOCS-1/CISH in DCs in combination with ICB-antibodies would further potentiate the efficacy of ICB-therapy, and therefore would be efficient in solving the issues associated with high-dose antibody toxicity and drug-resistance. A relevant human clinical trial (NCT01956630) targeting SOCS1−/− in DCs has been conducted by academy of military medical sciences, China against leukemia [7] (Box-1). Moreover, a future research targeting epitranscriptomic machineries (m6A-modifiers) and microRNAs might further potentiate DCs by regulating CISH diversity [191], (Table 1).

5. MicroRNAs and Epigenetic Modifiers (DNA and Histone Proteins) in ICB-Therapy

5.1. MicroRNAs in ICB-Therapeutics

In addition to co-targeting epitranscriptomics, intracellular immune checkpoints (m6A-modifiers, CISH/SOCS-1) and microRNA can be another potential targets to overcome the issues associated with ICB-drug resistances and high-dose antibody toxicities. The significance of ‘microRNAs’ also cannot be ignored because of their versatile roles in regulating numerous genes associated with immune checkpoint inhibitors. Kumar and colleagues have extensively described the therapeutic potential of microRNAs in treating DC-mediated Th1/Th2-associated immune disorders [41,192]. However, this section highlights some of the recent advancements in the utilization of ‘microRNAs’ in improving the efficacy of ICB-therapeutics. For example, miR-21, miR-34, miR-146a, miR-155 including many others microRNAs (Table 3) [193,194] have shown astonishing results by targeting PD-1, PD-L1 and CTLA4 immune markers summarized well in these references [132,133].

5.2. Epigenetic Modifiers (DNA and Histone Proteins) in ICB-Therapeutics

Like microRNAs, other epigenetic modifiers such as DNA and histone modifiers also hold great potential to increase the efficacy of ICB-therapeutics. Therefore, this section summarizes in brief about the systemic utilization of DNA-modifiers alone or in combination with other immunotherapeutic procedures. The DNA modification machineries, also known as writers/editors: DNMTs; removers/erasers: TET-proteins; readers/effectors: histone proteins HATs (acetylases) & HDACs (de-acetylases) [195] and chromatin remodelers: SWI/SNF chromatin remodelling complexes [196,197] have significant role in modulating the genes associated with immune checkpoint markers. Collectively, section-5 nurtures the potential of DNA-epigenetic modifiers and microRNAs in developing efficient molecular medicines in resolving the issues associated with ICB-drug resistance and toxicities [198–201] (Table 3).
6. Biopharmaceutical Companies Developing Personalized Medicines: Targeting Intracellular Checkpoint ‘CISH’ in Combination with ICB-Therapeutics and Recent Clinical Trials

In this section, we have described some of the biopharmaceutical/cell-therapy companies entering into developing personalized medicines by targeting immune cells expressing ‘intracellular checkpoint CISH’ in combination with ICB-antibodies to overcome the issues associated with ICB drug-resistance and high-dose antibody toxicities in several caners.

6.1. ONK Therapeutics Limited

ONK therapeutics is an Ireland-based cell therapy company, founded in 2015, conducting phase-I clinical trial against multiple myeloma, NSCLC and AML by targeting intracellular checkpoint ‘CISH’ in NK-cells. It was disclosed that the deletion of CISH improves the cytotoxic activity of NK-cells and therefore can be used efficiently to enhance the efficacy in combination with ICB-therapeutics. The respective phase-I clinical trials (ONKT102, ONKT103 and ONKT104) are estimated to complete until 2021–2022 (https://www.onktherapeutics.com/pipeline; accessed on: 25 July 2021). A relevant patent (US10034925B2 and EP3434762A1) was also filed for securing global license to use CISH knockout NK-cells from Australia’s WEHI” on 28 May 2021 (www.onktherapeutics.com; accessed on: 25 July 2021) [135].

6.2. Intima Bioscience, Inc.

Intima Bioscience is a UK-based Biotechnology Company, founded in 2021, conducting phase-I/II clinical trials (NCT04426669) against metastatic gastrointestinal (GIT) cancer patients by administering CISH-inactivated TILs by CRISPR/Cas9 system [1]. It is estimated to complete the trial by 31 October 2022 in collaboration with Masonic Cancer Center, University of Minnesota, USA [5].

In addition to the above companies some other biopharmaceutical companies are also involved in encouraging personalized medicines by targeting immune cells are: AstraZeneca, Acepodia, Affimed, Avid Biotics, Bristol-Myers Squibb, Celgene, Cellular Therapeutics, Celularity, Crispr Therapeutics, Dragonfly Therapeutics, Effector Therapeutics, Fate Therapeutics Inc., Fortress Biotech Inc., Genentech, Glycostem Therapeutics, Green Cross Lab Cell Korea, Gamida Cell, GT Biopharma, ImmuneOncia therapeutics, Korea [127,128], Innate Pharma, ImmunityBio, Inc. (NCT03387085), Intima Bioscience, Inc. (NCT04426669 and NCT03538613), Juno Therapeutics Inc., Kyowa Hakko Kirin, Kiadis Pharma, Mentrik Biotech, Multimmune GmbH, NantKwest Inc., Nektar Therapeutics, Nkarta Therapeutics, NOXXON Pharma, Northwest Biotherapeutics, ONK therapeutics, Roche Glycart, Rubius Therapeutics, Sanofi, Senti Biosciences, SignalRX Pharmaceuticals Inc., Sorrento Therapeutics Inc., XNK Therapeutics in collaboration with Sanofi’s and NextGenNK competence center coordinated by Karolinska institute conducting (EudraCT No: 2010-0223330-83 phase-I/II and NCT04558853) clinical trial, and Ziopharm Oncology Inc. [83,139,142,143,175], (Tables 1 and 3).

Box 1. Immune cells targeting intracellular checkpoint ‘CISH/SOCS-1’ in improving ICB-efficacy.

- **NK-cells targeting intracellular checkpoint CISH**: ONK therapeutics, Ireland, estimated to conduct Phase-I clinical trial (ONKT102, ONKT103 and ONKT104) by 2021–2022 for the treatment of haematological malignancies (multiple myeloma and AML) and solid tumors (ovarian, NSCLC and breast cancers) [135].
- **TILs targeting intracellular checkpoint CISH**: Intima Bioscience, UK, estimated to conduct Phase-I/II clinical trial (NCT04426669, NCT03538613) by 2021–2022 for the treatment of wide range of tumor types and gastrointestinal cancer [1,2,5].
- **DCs targeting intracellular checkpoint SOCS-1**: Military medical sciences, China, conducted Phase-I clinical trial (NCT01956630) in 2018 for the treatment of RAL [7,182].
Box 2. Important links targeting intracellular checkpoint ‘CISH’ in developing personalized medicine (accessed on: 28 July 2021).

https://clinicaltrials.gov/ct2/show/NCT04426669
https://www.onktherapeutics.com/pipeline/
https://youtu.be/-ejrnuT_yo4
https://acir.org/weekly-digests/2020/october/a-new-internal-t-cell-checkpoint-cish
https://crisprmedicinenews.com/news/crispr-cas9-knockout-of-a-novel-cancer-checkpoint-unleashes-t-cell-reactivity-against-solid-tumours/
https://www.onktherapeutics.com/onk-therapeutics-secures-exclusive-global-license-to-patent-for-cish-knockout-in-nk-cells-for-the-treatment-of-cancer-from-australias-wehi/
https://www.evaluate.com/node/13152/pdf
https://www.nkartatx.com/news/06-22-20/
AIA  Ag-induced arthritis
ALKBH5  alpha-ketoglutarate-dependent hydroxylase
CAND1  Cullin-associated NEDD8-dissociated protein 1
EZH2  Enhancer of zeste 2 polycomb repressive complex-2 subunit
EBF-1  Early B-cell factor-1
FTO  Fat Mass and Obesity Associated Protein
MPM  Malignant pleural mesothelioma
Sprend2  Sprouty related EVH1 domain containing protein-2

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