Novel insights into the function of CD24: A driving force in cancer

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Abstract
CD24 is a highly glycosylated protein with a small protein core that is linked to the plasma membrane via a glycosyl-phosphatidylinositol anchor. CD24 is primarily expressed by immune cells but is often overexpressed in human tumors. In cancer, CD24 is a regulator of cell migration, invasion and proliferation. Its expression is associated with poor prognosis and it is used as a cancer stemness marker. Recently, CD24 on tumor cells was identified as a phagocytic inhibitor (“do not eat me” signal) having a suppressive role in tumor immunity via binding to Siglec-10 on macrophages. This finding is reminiscent of the demonstration that soluble CD24-Fc can dampen the immune system in autoimmune disease. In the present review, we summarize recent progress on the role of the CD24-Siglec-10 binding axis at the interface between tumor cells and the immune system, and the role of CD24 genetic polymorphisms in cancer. We describe the specific function of cytoplasmic CD24 and discuss the presence of CD24 on tumor-released extracellular vesicles. Finally, we evaluate the potential of CD24-based immunotherapy.

KEYWORDS carbohydrates, CD24, extracellular vesicles, Fc fusion proteins, phagocytosis, selectins, Siglec-

1 HOW DID THE CD24 STORY BEGIN?

Cluster of differentiation (CD)24 is a glycosyl-phosphatidyl-inositol (GPI)-anchored glycoprotein. It is a membrane protein with a small protein core but extensive N-linked and O-linked glycosylation. CD24 is expressed by many cells of the immune system1 and, relevant for this review, is frequently overexpressed in human cancers.2,3 Since its first description, the importance of CD24 has been demonstrated in different areas. In immunology, CD24 is primarily a costimulatory molecule for T lymphocyte responses and a regulator of autoimmunity.4 In cancer, CD24 regulates cell migration, invasion and proliferation.2,3,5 It is a pathology biomarker of poorer prognosis2 and, based on the pioneering work of Al-Hajj7, is a putative marker for cancer stem cells (CSCs). It is amazing that such a tiny GPI-anchored cell surface molecule has such prominent roles in biology.

What is the relationship between CD24 expression on leukocytes and its overexpression in cancer cells? Is it because tumor cells have “borrowed” a molecule that plays a role in immune cell networking.

Abbreviations: BRCA, primary breast carcinoma; CD, cluster of differentiation; CSC, cancer stem cells; DAMP, danger-associated molecular pattern; DRM, detergent-resistant membrane domains; EAE, autoimmune encephalomyelitis; EGR1, early growth response protein 1; ER, endoplasmic reticulum; EV, extracellular vesicle; GPI, glycosyl-phosphatidylinositol; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HSA, heat stable antigen; HSP, heat shock protein; LAP, Leucine-Alanine-Proline; mAb, monoclonal antibody; MM, multiple myeloma; MS, multiple sclerosis; OvCa, ovarian cancer; pCR, pathologic complete response; PDAC, pancreatic ductal adenocarcinoma; SNP, single nucleotide polymorphisms; TIC, tumor initiating cancer cell; TNBC, triple-negative breast cancers; TSA, trichostatin A; UTR, untranslated region.

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thus allowing tumor cells mimic the correct player? In the immune system, CD24 is important in the regulation of cell proliferation and clonal expansion.\textsuperscript{10-13} It was recently reported that CD24 on tumor cells can act as a brake on the immune system. Bakal et al. pointed out a novel role for CD24 at the interphase of the immune system and tumor cells. In search for new phagocytosis inhibitors, they observed that CD24 on ovarian cancer (OvCa) or triple-negative breast cancers (TNBC) acted as an antiaphagocytic surface protein, which was termed a “don’t eat me” signal.\textsuperscript{11} CD24 on tumor cells interacted with Siglec-10 on macrophages.\textsuperscript{8} The CD24-Siglec-10 axis is well known in the immune system and is exploited as a novel target for dampening of overshooting immune reactions.

Rapid progress on the functional role of CD24 has been made over the last decade. Here, we summarize the present status of the research. We outline some basic features of CD24 and then focus on the research progress with special emphasis on its role in cancer.

2 | CD24 BASIC FINDINGS

2.1 | What does the CD24 protein look like?

The murine precursor molecule and homolog for human CD24 is the mouse heat stable antigen (HSA). It was discovered and defined in the early 1980s using a panel of rat monoclonal antibodies (mAb) from different labs, including M1.69, J11D, B2A2, 79 and others. These mAbs recognized a cell surface molecule on mouse hematopoietic cells. The designation “heat stable antigen” reflected the antigen’s resistance to heat.\textsuperscript{9} In many studies during the late 1980s mAbs to HSA were used to investigate the maturation of hematopoietic cells in the mouse.\textsuperscript{10-13} Biochemical analysis showed that the antigen migrated at a molecular mass of 40 to 70 kDa in SDS-PAGE.\textsuperscript{14} In the beginning of the 1990s, HSA was cloned by two groups.\textsuperscript{15,16} Surprisingly, the mRNA encoded a small peptide containing only 30 amino acids after removal of the signal sequence and displacement of the C-terminal region by the GPI-anchor. Thus, almost all of the mass of HSA was made up by extensive N- and O-linked glycosylation at multiple sites of the short peptide.\textsuperscript{15,16}

In 1991, Kay and Humphries cloned the human CD24 antigen that turned out to be the human ortholog to mouse HSA.\textsuperscript{17} CD24, like HSA, is composed of a short 31 to 34 amino acid core protein. The mature form is decorated with extensive N- and O-glycosylation. It thus resembles cell surface mucin.\textsuperscript{2} As all GPI-anchored cell surface molecules, CD24 is confined to the outer leaflet of the cell surface via the lipid portion of the GPI-anchor. Anchor attachment occurs posttranslationally in the endoplasmic reticulum (ER). The GPI-transamidase multienzyme complex recognizes a hydrophobic signal sequence at the C-terminus (termed the GPI attachment signal) of the protein and cleaves it at the ω-site with a preference for small nonpolar amino acids in this position. The preassembled anchor is then transferred to the protein.\textsuperscript{18,19}

In polarized epithelial cells, protein homooligomerization is a key step determining the apical sorting of GPI-anchored proteins.\textsuperscript{20} The presence of both lipid anchor and protein portions confers unique trafficking features to these proteins. This allows them to partition into membrane microdomains enriched in cholesterol and sphingolipids (lipid rafts).\textsuperscript{20,21}

A number of anti-human CD24 mAbs were characterized in the V International Workshop on Human Leucocyte Differentiation Antigens.\textsuperscript{1} These include those recognizing the central Leucine-Alanine-Proline (LAP) core (SWA11, ML5, OKB2) or those requiring CD24-associated sialic acid for binding (BA-1, HB-9, VIB-E3, SN3).\textsuperscript{22} Interestingly, a recent biochemical analysis suggested that the epitope for mAb SN3 is likely present in the O-glycans or in the glycan core of the GPI-anchor, and therefore is not entirely CD24 specific.\textsuperscript{23} The CD24 core protein can be modified with carbohydrate-defined CD-antigens, such as α2-6 sialylated polyolactosamine structures.\textsuperscript{24} The highly variable glycosylation makes it essential to validate mAb specificity before drawing conclusions about CD24.

CD24 expression has been reported in many cell types in the mouse. These include hematopoietic cells (transiently in T cells, constitutively in B lymphocytes, thymocytes, erythrocytes, neutrophils, dendritic cells, macrophages and others; for a review, see Reference 4) and nonhematopoietic cells (ie, neural cells; for a review, see Reference 25). In humans, CD24 is expressed on B lymphocytes, monocytes, granulocytes and on lymphoid tumor cell lines. In contrast to the mouse, human erythrocytes do not express CD24.\textsuperscript{17} Interestingly, a genomic study revealed the conservation of CD24 across many mammalian species and its appearance prior to the reptilian-avian divergence.\textsuperscript{26}

2.2 | What do we know about CD24 associated glycans?

Given the high degree of glycosylation (CD24: 14 O- and two N-glycosylation sites, HSA: seven O- and four N-glycosylation sites), it is clear that an understanding of CD24 biology requires knowledge about associated glycans. Like other membrane proteins, the cellular glycosylation of CD24 is cell-type specific depending on the cellular repertoire of glycosyltransferases.\textsuperscript{14} Aberrant glycosylation can occur in cancer cells due to cancer-associated upregulation of certain glycosyltransferases.\textsuperscript{27-29}

Unfortunately, there is not a complete structure of CD24 associated glycans. Some studies were carried out to gain insights. N- and O-linked glycans of mouse brain CD24 were studied.\textsuperscript{30,31} For O-linked glycans, the analysis revealed a diverse mixture of mucin-type and O-mannosyl glycans carrying, in part, functionally relevant epitopes, such as 3-linked sialic acid, disialyl motifs, Le(X), sialyl-Le(X) or HNK-1 units. For N-linked glycans, a highly heterogeneous pattern mainly includes complex type glycans expressing distinct carbohydrate epitopes, like 3-linked sialic acid, Le(X) or blood group H antigens, bisecting N-acetylgalactosamine residues, and N-acetyllactosamine repeats as well as high-mannose and hybrid type species.

In another study, N-glycans released from mouse and human CD24 from lymphoblastoma, neuroblastoma and astrocytoma cell lines or from mouse brain homogenate were analyzed and compared.\textsuperscript{32} The authors described the presence of fucosylated and...
sialylated complex and hybrid N-glycans. Finally, Motari et al investigated the structure of CD24-Fc (see below) by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. The majority of the oligosaccharides were Neu5Acα2,3/6Galβ1,3GalNAc (ie, sialyl-T). Although the study provided valuable data, it is clear that CD24-Fc is released via the secretory pathway. The glycosylation pattern may differ from that of membrane-associated CD24.

In summary, the information is incomplete, most likely due to inherent technical difficulties in resolving such complex structures.

2.3 Which are the CD24 binding partners?

Several binding partners for CD24 have been identified (Table 1). The study of Kadmon et al suggested that mouse CD24 (ie, nectadrin) is a cell adhesion glycoprotein exhibiting homophilic binding. It is possible that this type of interaction is carbohydrate-mediated and reflects the ability of GPI-anchored proteins to cluster in lipid rafts. In mouse brain, CD24 can interact in a carbohydrate-dependent manner with the neural adhesion molecule L1CAM (CD171), leading to the inhibition of neurite growth and altered L1CAM signaling.

CD24 was identified as a ligand of P-selectin in mouse myeloid and endothelial cells. In human cancer cells, the CD24-P-Selectin interaction supported the rolling of breast carcinoma cells on endothelial cells and adhesion to platelets and facilitated adhesion of ovarian cancer cells to mesothelium. One study also reported the rolling of CD24 positive MCF-7 cells on immobilized E-Selectin. To bind to P-selectin, CD24 required appropriate modifications with carbohydrates, such as sialyl-Lex. Tumor cells forced to overexpress CD24 and FucT7 (synthesizes sialyl-Lex) bound to endothelial cells and platelets in vitro and in vivo, as mediated by P-selectin. The CD24-P-Selectin pathway may enhance the metastatic spread of tumor cells.

In addition to P-selectin, CD24 can interact with Siglecs, a class of sialic acid binding receptors on immune cells. Siglec-5 and Siglec-10 are important immune-inhibitory receptors on monocytes, granulocytes and lymphocytes. Initial experiments in mice identified CD24 in a trimolecular complex together with Siglec-10 and the danger-associated molecular pattern (DAMP) molecule HMGB1. The CD24 and Siglec-10 axis selectively apparently repressed tissue damage-induced immune responses. The sialic-acid-based pattern recognition may discriminate infections from tissue injuries.

Importantly, the diverse and heterogeneous glycosylation of CD24 might bind a diverse array of DAMPs. Mass spectrometry of CD24-associated proteins revealed several prominent DAMPs including heat shock protein (HSP)70, HSP90 and Nucleolin. Interestingly, human CD52, a small GPI-anchored molecule that is structurally similar to CD24, can also bind via its glycans to the proinflammatory B box of HMGB1 to engage the Siglec-10 receptor and suppress human T cell function. Specifically, glycoforms were also required for the immune suppressive activity of soluble CD52. In humans, purified CD24 derived from tumor cell lines can bind to Siglec-5 whereas CD24 isolated from human placenta clearly bound to Siglec-10. Finally, as discussed below, CD24 on OvCa and TNBC cells bound to Siglec-10 on human monocytes. These findings suggest that Siglecs can exquisitely discriminate between different glycoforms of CD24.

2.4 How does CD24 act as a signaling molecule?

Antibody-mediated crosslinking experiments showed that CD24 itself has signaling capacity. An increase in free cytoplasmic Ca²⁺ was observed in B lymphocytes and monocytes, and augmented hydrogen production was induced by CD24 crosslinking in granulocytes.

As discussed above, CD24 is localized in detergent-resistant membrane domains (DRMs; also termed lipid rafts). These membrane

| Ligand | Type of assay | Reference |
|--------|---------------|-----------|
| mCD24 | mCD24 | Protein-protein interaction |
| | | Carbohydrate mediated? |
| | | Homoaeggregation for vesicular transport? |
| mCD24 | L1CAM | Functional cooperation |
| | | Protein-protein interaction |
| huCD24 | P-selectin | Adhesion to platelets, rolling on endothelial cells |
| mCD24 | P-selectin | Adhesion to endothelial cells, protein-protein interaction |
| huCD24 | E-Selectin | Rolling on immobilized E-Selectin protein-protein interaction |
| mCD24 | Siglec-10 | Functional cooperation |
| huCD24 | Siglec-5 | Protein-protein interaction |
| huCD24 | Siglec-10 | Protein-protein interaction |
| huCD24 | Siglec-10 | Inhibition of phagocytosis |
The expression of CD24 in cancer cells leads to Src-mediated activation of signal transducer and activator of transcription 3 and focal adhesion kinase.\textsuperscript{56-65} CD24-induced Src-activation could act as starting point for many other signaling events. For example, altered activations of extracellular signal-activated kinase-2, Akt and other signal signaling molecules were reported in response to changes in CD24 expression.\textsuperscript{66-68} In gastric cancer cells, CD24 can affect epithelial growth factor receptor signaling by promoting internalization and degradation of this receptor.\textsuperscript{69} In HER2-positive breast cancer cells, CD24 can support both the expression of HER2 and the subsequent activation of phosphoinositide 3-kinase-Akt signaling.\textsuperscript{70} A more elaborate review on CD24-mediated signaling events was published recently.\textsuperscript{66}

### 3.2 What is known about CD24 genes and genetic polymorphisms?

In humans, the CD24 gene is located on chromosome 6q21. Three intronless pseudogenes are located on chromosome 15q21-q22 and Yq11 was discovered by in situ hybridization.\textsuperscript{85} Two homologs exist. One is located on chromosome 1p36 and one has been tentatively mapped to chromosome 20q11.\textsuperscript{85}
In the mouse, CD24 was mapped to chromosome 10.\textsuperscript{86} In addition, two other genes appeared to be intronless retrotransposons. Despite numerous sequence changes, an open reading frame was maintained. However, no expression of these pseudogenes was detected.\textsuperscript{16,86} Ayre et al recently demonstrated that the diversity of the CD24 genomic structure between and within species, with varying numbers of exons, introns and the presence of untranslated regions (UTRs). Of note, the authors found no obvious criteria distinguishing CD24 genes from those annotated as CD24-like.\textsuperscript{26}

Studies of autoimmune diseases have identified two polymorphisms within the CD24 gene as modifiers of disease risk and progression.\textsuperscript{87-91} The first is a C > T single nucleotide polymorphism (SNP; rs52812045, also termed P170) at position 170 from the CD24 translation start site. It is located in the putative GPI-anchor cleavage site (–1 position) and results in the substitution of an alanine (A) by the more bulky amino acid valine (V). The P170T/T (CD24 V/V) genotype is associated with an increased risk and more rapid progression of multiple sclerosis (MS).\textsuperscript{92}

The second polymorphism is a dinucleotide deletion within the 3′UTR (rs3838646, also termed P1527). This polymorphism results in reduced CD24 mRNA stability and is protective in both MS and systemic lupus erythematosus.\textsuperscript{93} Additional SNPs have been found in the CD24 promoter region. Jia et al. reported shorter survival times of gastric cancer patients with tumors harboring the P-534 SNP in the promoter region of CD24.\textsuperscript{94} A hypermorphic SP-1-binding CD24 variant was associated with MS risk and progression.\textsuperscript{95} A potential role for SP-1-mediated transcriptional regulation in MS pathogenesis was proposed.\textsuperscript{95}

Several studies have investigated the role of CD24 SNPs in cancer incidence and progression. A significant association of CD24 genetic variants with onset and progression was noted in prostate cancer.\textsuperscript{96} In esophageal cancer, the incidence of metastasis in regional lymph nodes was markedly higher in patients carrying the V/V variant compared to those not carrying it.\textsuperscript{97} The influence of CD24 polymorphisms on breast cancer prognosis and risk was investigated in a cohort of 2514 patients and 4858 controls.\textsuperscript{98} The CD24 V/V genotype affected the outcome, but not risk, of primary breast cancer.\textsuperscript{98}

The clinical relevance of CD24 polymorphisms and their potential to predict a pathologic complete response (pCR) to neoadjuvant chemotherapy was studied in primary breast cancer.\textsuperscript{99} The CD24 V/V genotype was the only significant predictor of pCR following doxorubicin treatment. Interestingly, a significant correlation was evident between CD24 V/V and intratumoral lymphocyte aggregates.\textsuperscript{99} However, these findings were not confirmed in a second study in breast cancer where pCR to anthracycline- and taxane-based neoadjuvant chemotherapy was investigated.\textsuperscript{100} A metaanalysis of the role of CD24 in cancer showed that two SNPs (rs52812045 and rs3838646) did not modify the risk of cancer.\textsuperscript{101}

In summary, CD24 SNPs have not been proven to be strong and independent predictors of cancer incidence, progression and responsiveness to therapy. These findings differ from the observations in autoimmune disease and suggest that the CD24 polymorphisms affect immune cell functions more profoundly than tumor cell characteristics. It remains unclear how CD24 polymorphic forms affect immune functions. P170 polymorphisms gives rise to an A-V amino acid exchange in the GPI-anchor cleavage size. It was proposed that such exchange could cause changes in the efficacy of cleavage and processing, resulting in altered cell surface density. Indeed, patients carrying the CD24V/V SNP expressed higher levels of CD24 on peripheral blood T cells than did CD24A/A patients. In addition, transfection with CD24A and CD24V cDNA demonstrated that the CD24V allele was expressed at higher efficiency than the CD24A alleles.\textsuperscript{87} However, since these experiments were only performed in CHO cells, a more careful analyses in different cell types are needed.

Finally, a prostate cancer study described that tumors with P170 (T) or P-534(C) alleles had a 2-fold increased protein expression of CD24 compared to tumors with P170(C) or P-534(A) alleles. Likewise, tumors with a combination of P170(T/T) and P-534(C/C) genotypes were associated with a high mRNA level of CD24.\textsuperscript{96} Thus, it appears that small changes in expression levels of CD24 can result in measurable physiological effects.

### 4 | How is CD24 Expression Regulated?

In mice, CD24 displays tissue-specific as well as developmental regulation. During the maturation of several hematopoietic lineages, HSA expression is generally high in immature precursor cells and low or absent in terminally differentiated cells. The sequence and methylation analysis of the murine CD24 promoter revealed characteristics of both "housekeeping" and tissue-specific promoters, including multiple putative SP-1 and AP-2 consensus binding sites.\textsuperscript{86}

As mentioned above, SP-1 binding sites were also reported in the human CD24 promoter.\textsuperscript{95} In human urothelial carcinoma, CD24 expression is under the control of androgen receptor. Androgen treatment led to increased CD24 promoter activity. Androgen receptor and androgen-response elements upstream of the CD24 start codon were considered responsible.\textsuperscript{102} In contrast, in primary breast cancer, CD24 was downregulated and involved estrogen receptor-α and two estrogen responsive elements in the CD24 promoter, one of which was able to bind estrogen receptor-α.\textsuperscript{103}

In another study on breast cancer cell lines MCF-7, MCF-10 and MD-MB-231, the overexpression of Twist suppressed the expression of CD24.\textsuperscript{104} Twist promotes the generation of a breast CSC phenotype characterized by the high expression of CD44, little or no expression of CD24, and increased aldehyde dehydrogenase 1 activity.\textsuperscript{104} In contrast, in breast cancer cells the truncated glioma-associated oncogene homolog 1, known as the terminal effector of the Hedgehog pathway, can upregulate CD24 gene expression, thereby contributing to enhanced migration and invasiveness.\textsuperscript{105}

In human bladder cancer, CD24 is reportedly an effector of hypoxia-inducible factor 1 driven tumor growth and metastasis mediated by a functional hypoxia response element in the CD24 promoter.\textsuperscript{106} In colorectal cancer, CD24 expression is apparently under the control of the β-catenin/TCF-dependent transcription machinery.
via the activation of cyclooxygenase-2.\textsuperscript{107} In contrast, CD24 expression in melanoma cell lines is low but can be induced by BRAF inhibitors, such as Vemurafenib.\textsuperscript{108} The upregulation of CD24 is reportedly mediated by SOX2 that can bind to the CD24 promoter.\textsuperscript{106} The forced overexpression of either SOX2 or CD24 can significantly increase the resistance to BRAF inhibitors, while SOX2 or CD24 knockdown rendered cells more treatment sensitive.\textsuperscript{108}

A recent study involving HCC examined in more detail transcripts from the CD24 gene located on chromosome 6q21.\textsuperscript{109} Two transcripts, termed CD24A and CD24B, were identified. They differed in length and only shared approximately 47% amino acid identity.\textsuperscript{109} The variant CD24A, but not CD24B, which was barely detected by qPCR and western blotting, was significantly upregulated in HCC tissue.\textsuperscript{109} When overexpressed in recipient cells, both CD24A and CD24B contributed to cell proliferation, migration and invasion. However, CD24A was more effective than CD24B. CD24A was demonstrated to be a direct target gene of early growth response protein 1, a nuclear transcription factor that binds to the GC enrichment region of DNA sequences and functions as a transcriptional regulator.\textsuperscript{109}

A role of epigenetic regulation was investigated in MCF-7 and MDA-MB-231 cells expressing different levels of CD24.\textsuperscript{71} Treatment of cells with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) significantly increased CD24 transcript levels.\textsuperscript{71} In contrast, treatment with the DNA methyltransferase inhibitor 5-aza-dC had little effect. Methylation analyses revealed that the promoter region of CD24 was unmethylated in both cell lines regardless of mRNA expression levels.\textsuperscript{71} It was concluded that CD24 is epigenetically regulated in association with histone modification in breast cancer cells.\textsuperscript{71}

CD24 is overexpressed in multiple cancer types. A recent study by Zhang et al indicated that amplification of the CD24 gene is an important mechanism.\textsuperscript{110} The authors investigated the copy number status and expression level of CD24 in primary breast carcinoma (BRCA), OvCa, lung cancer and prostate cancer. CD24 gene amplification was observed in carcinoma of breast, ovarian and lung, but not in the prostate. Importantly, the copy number amplification was strongly correlated with CD24 mRNA overexpression and gene amplification seemed to be the most influential genetic alteration for the prognosis of BRCA.\textsuperscript{110}

### 4.1 Is CD24 a good marker for EVs?

Cells in the body (but also tissue cultured cells) release vesicles into the surroundings. These are termed EVs.\textsuperscript{111-115} This term refers to all vesicles released by various cell types including erythrocytes, platelets, leukocytes and cancer cells.\textsuperscript{116,117} The process of EV secretion is particularly active in proliferating cells, such as cancer cells.\textsuperscript{116,117} Small vesicles (50-150 nm) are released from the cell surface (shed microvesicles) or from the endosomal system (exosomes). Fusion of late endosomes with the plasma membrane results in the secretion of exosomes into the extracellular space.\textsuperscript{118,119} In contrast, apoptotic vesicles (bodies) are larger (1000-5000 nm) and can be separated from smaller vesicles based on size and density.\textsuperscript{118,119} Depending on the cell of origin, EVs can transport different biologically active molecules, such as proteins, mRNA, microRNA and lipids, and appear to be important mediators of intercellular communication.\textsuperscript{120,121}

Due to its GPI-anchor and raft localization, CD24 is recruited into EVs and can be an EV marker protein. CD24+ EVs have been described in urine (derived from the urinary tract)\textsuperscript{122} and amniotic fluid (derived from the fetus).\textsuperscript{122} In cancer, CD24+ EVs have been detected in the serum of OvCa cancer patients\textsuperscript{123} and patients with breast cancer.\textsuperscript{124} There are many examples of tumor tissue and isolated serum EVs positive for CD24. Immunohistochemistry has demonstrated that many melanoma tissues are positive for CD24\textsuperscript{108} and CD24+ EVs were detected in melanoma patient sera (X. Hu and P. Altevogt, unpublished results). However, the origin of these EVs from either the melanoma or immune cells was not determined. In another melanoma study, EVs were discovered in the serum\textsuperscript{114} and lymphatic drainage\textsuperscript{125} of patients.

There is optimism that the analysis of EVs could contribute to the "liquid biopsy" method. Due to the overexpression of CD24 in cancer, it is increasingly used as a marker to identify tumor-derived EVs in plasma or other body fluids for diagnostic purposes.\textsuperscript{123,126-128}

#### 4.2 Is soluble CD24-Fc a novel immunotherapeutic?

In line with its expression as a hematopoietic cell surface antigen, the function of CD24 has been intensively studied in the immune system. The important findings have been already summarized in a previous review\textsuperscript{6} and will not be mentioned again.

It was initially shown that mouse CD24 on antigen presenting cells can act as a CD28-independent costimulatory molecule for both CD4 and CD8-T cell responses.\textsuperscript{129-132} Furthermore, CD24 on T cells was found to be essential for T cell homeostatic proliferation.\textsuperscript{133} Considering the various possible binding partners (see Table 1), the identity of the binding partners of CD24 on nonhematopoietic cells remains an open question. The homotypic interaction of CD24 was reportedly not responsible for costimulation mediated by CD24 expressing antigen presenting cells.\textsuperscript{134} Of note, in this setting CD24 acted as a positive stimulus.

Based on the essential role of CD24 in autoimmune disease, animal models for autoimmune encephalomyelitis (EAE) were established using immunization with the myelin oligodendrocyte glycoprotein peptide.\textsuperscript{135} This experimental model is frequently used to study MS in animals and is mediated by autoreactive T cells. An important finding was that CD24 knockout mice, in contrast to wild-type mice, did not develop EAE.\textsuperscript{135} Underscoring the importance of CD24, CD24 knockout mice and transgenic mice with CD24 expressed only on T cells were used to demonstrate the necessity of CD24 on both T- cells and non-T cells in order for pathogenic T cells to execute their effector function.\textsuperscript{135}

Next, the same authors blocked CD24-mediated cell-cell interactions in vivo. Since mAb to CD24 in mice are toxic due to the presence of CD24 on erythrocytes, a CD24-Fc fusion protein composed of the extracellular part of CD24 and the human IgG-Fc portion was
generated. The systemic application of CD24-Fc to EAE mice significantly ameliorated the disease progression. Similar results were recently reported in another mouse model of central nervous system degeneration. CD24-Fc administration protected mice against cuprizone-induced oligodendrocyte loss. In both experimental settings, the CD24-Fc stimulated the signaling of Siglec G (i.e., Siglec-10 in humans) and suppressed the inflammatory response in vivo via SHP-1 activation. Thus, the CD24-Fc fusion protein provided a negative signal to the immune system.

Human CD24-Fc was robustly generated (http://grantome.com/grant/NIH/R44-NS041692-04A2) and tested in rhesus monkeys suffering from chronic immune activation and inflammation of human immunodeficiency virus type-1/simian immunodeficiency virus infection. CD24-Fc was well tolerated and the results suggested that it conferred protection to simian immunodeficiency virus-infected animals regarding progression to AIDS. CD24-Fc may prove to be a novel tool to modulate the immune response in disease conditions where chronic immune activation and systemic inflammation are the underlying causes. Several clinical trials CD24-Fc in immune diseases that include graft-vs-host, MS and others are underway (http://www.oncoimmune.com).

Interestingly, in a recent COVID-19 ClinicalTrial.gov summary (NCT0431704), CD24-Fc was listed as an inflammatory cytokine inhibitor. CD24Fc is being tested as nonantiviral biological modifier for COVID-19 therapy. A phase III trial will involve 230 patients randomized into blinded placebo and CD24-Fc arms, with time to clinical improvement from severe to mild symptom as the primary endpoint.

Importantly, CD24-Fc is also being explored for therapy of various types of human cancers (NCT04060407). In metastatic melanoma, CD24-Fc is being tested for safety and efficacy in combination with the checkpoint inhibitory mAbs ipilimumab and nivolumab, to reduce the toxicity of immunotherapy. In addition, a phase Ib/II study plans to investigate the use of CD24-Fc in combination with ipilimumab or nivolumab for renal cell carcinoma and colon cancer.

In cancer it is important to consider the mode of action of CD24-Fc. The binding to Siglec-10 on immune effector cells as an agonist could render cells more immunosuppressive. In contrast, as an antagonistic compound, CD24-Fc might be able to inhibit the interaction of cell-bound CD24 on tumor cells with Siglec-10 on immune cells (see below).

4.3 What function does CD24 have at the tumor-immune cell interface?

Suppression of the immune system may be desirable in a situation of overactivation and systemic inflammation. The ability to evade immune destruction is a defining hallmark of cancer. Tumor cells have developed multiple strategies to suppress the immune system and secure survival.

Phagocytosis is an important mechanism that allows the innate immune system to clear the body of dangerous or toxic substances. Phagocytic cells include neutrophils, monocytes, macrophages, dendritic cells, osteoclasts and eosinophils. In the cancer setting, monocytes and macrophages participate in radio- and chemotherapy by removing apoptotic and necrotic tumor cells and debris with or without inflammation. Moreover, tumor antigen-induced cellular immunity is dependent on phagocytosis by antigen presenting cells.

A recent paper has shed light on the adverse function of the CD24-Siglec-10 axis in human cancer. Barkal et al demonstrated that in OvCa or TNBC CD24 can act as an antiphagocytic surface protein (i.e., "don't eat me" signal). CD24 on tumor cells reportedly interact with Siglec-10 on tumor-associated macrophages. The authors described that genetic ablation or therapeutic blockade of CD24 resulted in macrophage-dependent reduction of tumor growth in vivo and increased survival.

The discovery of immune checkpoints as targets for successful therapeutic interventions has transformed oncological practice in the last decade. In addition to Programmed cell death protein 1 and Cytoxic T-lymphocyte-associated protein 4, "don't eat me" signals were discovered as valuable targets. Such surface molecules include CD47, programmed cell death ligand and β2-microglobulin associated with class I major histocompatibility complex molecules. mAbs to these molecules that antagonize the interaction of the "don't eat me" signals with their macrophage-expressed receptor (termed phagocytic checkpoints) have demonstrated therapeutic potential in several cancers. Barkal et al suggested that CD24 might be a novel innate immune checkpoint and a novel target for cancer immunotherapy. Indeed, in preclinical animal models, mAbs to CD24 have shown remarkable therapeutic efficacy (see below).

4.4 Is CD24 a good marker for CSCs?

CSCs represent a subpopulation of cancer cells responsible for tumor initiation, maintenance and recurrence. Initially, Fogel et al reported that primary breast cancer in humans are positive for CD24. Subsequently, it was shown that the ability of primary breast cancer cells to form tumors in NOD/SCID mice was far greater in the CD44+/CD24- fraction compared to the CD44+/CD24+ fraction. Owing to this enhanced tumorigenicity, the CD44+/CD24- fraction was termed "breast CSC." Since the original paper was published, many studies have used CD24 to define CSC or "tumor initiating cancer cells" (TICs).

The cellular markers for CSC and epithelial-mesenchymal transition are often overlapping. Epithelial-mesenchymal transition describes the cellular transition from adherent epithelial cells into mesenchymal-like cells with the ability to migrate and invade adjacent tissues. The reverse process is termed mesenchymal-epithelial transition. In breast cancer it was reported, that CSC transit between the epithelial and mesenchymal state playing an important role during metastasis. Mesenchymal-like breast CSCs are CD44+ CD24−, are primarily quiescent and localized at the tumor invasive front, while CD44+/CD24+ cells tend to associate with more differentiated epithelial features.

In contrast to breast cancer, in pancreatic cancer the putative CSC population was CD24+. The CD24+ phenotype of CSCs has also been demonstrated in other tumors including cervical
cancer, cholangiocarcinoma, colorectal cancer, and gastric cancer. A recent study described that TICs in multiple myeloma (MM) were positive for CD24. CD24+ MM cells exhibited increased clonogenicity, drug resistance and tumorigenicity. As few as 10 CD24+ MM cells were required to develop plasmacytomas in mice. CD24+ MM cells were enriched after chemotherapy in complete remission MM patients with minimal residual disease. The cells displayed increased drug resistance. Genes of induced pluripotent or embryonic stem cells, such as NANOG, OCT4, KLF4 and SOX-2, were significantly upregulated in CD24+ MM cells.

Several papers reported the involvement of CD24 in the regulation of the Notch signaling pathway. This pathway is important for self-renewal, differentiation, proliferation, survival and migration of CSCs. Using cancer cell lines or cancer tissues, many studies have used cell populations that are positive or negative for CD24 or other putative CSC markers, such as CD44, epithelial cell adhesion molecule or CD133. For example, only a small percentage of cells (<0.5%) were reportedly CD24+ in human and mouse melanoma cell lines. The same study demonstrated that the CD24+ subpopulation had self-renewal properties in vitro and in vivo using soft agar assays and xenograft tumor models. In addition, CD24 expression was accompanied by activation of the Notch1 signaling pathway.

In MCF10DCIS basal-like breast cancer cells, the CD44+/CD24−/low subpopulation showed elevated Notch1 signaling and increased cell proliferation compared to the CD44+/CD24+ subpopulation. In HCC patients, the CD133 + CD24+ subpopulation displayed stem cell characteristics of high SOX2 and NANOG expression and short survival time. Activation of the Notch1 signaling pathway was promoted by induced nitric oxide synthase-mediated activation of the metalloproteinase ADAM17. Similar results were reported in a study on renal cell carcinoma, in which the CD133 + CD24+ subpopulation was isolated from cell lines.

Finally, mouse models for human cancers are often based on particular genetic alterations that recapitulate the human disease. In a prostate cancer model harboring conditional KrasG12D mutations

| Name          | Type of therapy                                      | Remarks                                      | Year | Outcome                                      | Reference |
|---------------|------------------------------------------------------|----------------------------------------------|------|----------------------------------------------|-----------|
| SWA11         | mAb IgG2a mAb mouse anti-human CD24                  | CD24 targeting of human cancer cells in SCID mice | 2013 | Retardation of tumor growth, altered cytokine milieu | Salnikov et al159 |
| ALB9          | mAb IgG1 mAb mouse anti-human CD24 (this isotype is not optimal for ADCC) | Treatment of human bladder cancer cells Lu-L2 in nu/nu mice | 2011 | Reduction of tumor growth and metastasis | Overdevest et al160 |
| SWA11         | IgG2a mAb mouse anti-human CD24                      | Treatment of myeloma cells in immunodeficient mice | 2019 | Inhibition of multiple myeloma cell growth and prevention of tumor progression | Gao et al153 |
| SWA11.dgA     | ADC between mAb SWA11 and deglycosylated ricin A-chain | Treatment of small cell lung-cancer xenografts in a sponge matrix | 1993 | Selective elimination of clonogenic SW2 cells from small tumor-cell deposits in sponge matrices | Zangemeister-Wittke172 |
| SWA11.dgA     | ADC between mAb SWA11 and deglycosylated ricin A-chain | Treatment of human BL-38 Burkitt’s lymphoma in SCID mice | 1996 | Extended survival time | Schnell et al161 |
| SWA11-ZZ-PE38 | ADC between mAb SWA11 and Pseudomonas exotoxin        | ADC not toxic in mice                         | 2011 | Reduction of growth of human HT29 CRC cells | Shapira et al162 |
| HN-01         | Anti-CD24-ADC, antibody-nitric oxide conjugate        | HN-01 in hepatic carcinoma-bearing nude mice | 2019 | Induction of apoptosis and suppression of tumor growth | Sun et al163 |
| cG7-MICA      | Bi-specific antibody (BsAb) composed of the NKG2D ligand MHC class I-related chain A (MICA) and a mAb to CD24 | In vivo, cG7-MICA on NK cells NK and antitumor efficacy | 2019 | Recruitment of NK cells, augmented response to sorafenib | Han et al164 |
| Anti-CD24-CAR | NK cells expressing CAR derived from single-chain variable fragment of mAb SWA11 | Targeting of CD24+ ovarian carcinoma cells in vitro | 2019 | Effector cells exhibited efficient killing | Klapdor et al165 |
| Anti-CD24-CAR | T cells expressing CAR derived from single-chain variable fragment of mAb SWA11 | Intratumoral injection in SCID mice bearing different PAC xenografts | 2012 | CD24-specific effector cells prolonged survival of mice | Maliar et al166 |
and prostate-specific abrogation of p53 function, the mutant Kras induced upregulation of CD24 in cancer cells and enhanced cancer stemness and bone metastasis. CD24 was identified as a key driver of tumorigenesis and metastasis in vivo. The data demonstrated that specific factors involved in cancer stemness are critical for metastatic conversion of prostate cancer. In another study using the same oncogenic KrasG12D model for pancreatic ductal adenocarcinoma (PDAC) in mice, CD24 expression was upregulated in both murine and human PDAC and during acute pancreatitis. CD24 was expressed exclusively in differentiated PDAC with an epithelial phenotype, whereas CD24 absence was associated with undifferentiated tumors.

**FIGURE 1** The functional role of CD24 in oncoimmunology. A, CD24 promotes tumor progression by activating signaling molecules involved in proliferation and survival of cancer cells. CD24-mediated raft clustering leads to activation of Src kinases that are of central importance for downstream signaling. Activated Src can phosphorylate STAT3, FAK but can also trigger other signaling pathways. B, By interaction with Siglec-10 on the surface of phagocytic cells, CD24 protects cancer cells from phagocytosis. C, CD24 is expressed on tumor derived EVs and could protect EVs from phagocytosis. D, Antibodies against CD24 could be used to counteract the function of CD24 on the surface of tumor cells or EVs and restore phagocytosis. CD24-Fc used as immune-therapeutic could trigger immune suppression in Siglec-10 expressing immune cells (T-, B- and phagocytic cells) and thereby protect from autoimmunity. In the cancer setting this could be detrimental. However, CD24-Fc might also act as soluble inhibitor for CD24-Siglec-10 interaction.
In summary, these collective data indicate that although CD24 is widely used as a marker, we are only starting to understand its relevance and function in CSCs.

4.5 Can CD24 be a target for cancer immunotherapy?

Due to the abundant expression in human cancer, and its putative role as a CSC marker, CD24 might be a good target for cancer therapy. However, its expression on immune cells might cause deleterious side effects. Despite this, several attempts have sought to analyze the efficacy of anti-CD24 based tumor therapy in preclinical models. These models are summarized in Table 2.

Different formats were used for therapy. These included unconjugated mAbs, where the effector mechanism mainly relies on antibody-dependent cell-mediated cytotoxicity of SWA11,153-159 ALB9,160 antibody-drug conjugates,161-163 bispecific antibodies,164 chimeric antigen receptor T cells166 or chimeric antigen receptor-natural killer cells.167 In all these experiments more or less efficient killing of tumor target cells was observed in vitro or in vivo in human tumors grafted into immunodeficient mice. It remains to be seen if any of these approaches can also efficiently eliminate TICs in vivo. Blocking of CD24 to prevent phagocytic inhibition via Siglec-10 might be feasible with naked CD24 mAbs.

5 CONCLUDING REMARKS

In the present review, we have summarized recent advances of CD24 in human tumors. A graphical summary is presented in Figure 1. We have tried to be as careful as possible not to overlook essential contributions. If we have, we apologize.

As in any progress report, a number of important questions and problems remain. Although the original characterization of CD24−/− mice did not reveal a dramatic phenotype,167 it is surprising to see how many cellular defects were observed both in immunological133,135,168,169 and nonimmunological studies.107,170,171 It is unclear to what extent these observations are due to the lack of CD24-mediated raft signaling or to a failure of interaction with cognate ligands.

The processing of CD24 in cancer has not been carefully studied. In particular, the function and fate of the GPI-anchor displaced tail and the presence of cytoplasmic CD24 is of interest. If indeed cytoplasmic CD24 interferes with p53 stability, it is important to know which part of CD24 is involved. Due to its intensive and variable glycosylation, CD24 may have additional ligands, as suggested.26 It will be interesting to study whether other Siglecs or Galectins that are endowed with carbohydrate binding ability are able to interact with CD24 glycoforms.

Finally, the use CD24 as a target for cancer therapy has just begun. Time will tell if this approach is feasible.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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