REVIEW

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Application of Synthetic Antigen-Encoded *Escherichia coli* Nissle 1917 Probiotic-Guided PD-1/CD28 Receptor-Integrated CAR-T Cell Therapy as Targeted Therapy for Colorectal Cancer: A Literature Review

Tegar Raharja ARIAWAN[a], IG Mahapraja DIVASTA[a], Asri BINTARI[a], Bryan Gervais de LIYIS[a], Desak Made WIHANDANI[a]

[a]Department of Biochemistry, Faculty of Medicine, Udayana University, Bali, Indonesia

ABSTRACT Colorectal cancer is a leading cause of cancer-related deaths. The traditional therapeutic strategies, such as chemotherapy and surgery, which are generally used to treat colorectal cancer are invasive and often accompanied by significant side effects. Chimeric antigen receptor-T (CAR-T) cell therapy is effective, particularly in treating hematological malignancies; however, the treatment of solid tumors such as colorectal cancer is difficult. These challenges include poor targeting, loss of function, inadequate expansion, and short-lived persistence of CAR-T cells. Some researchers have developed a novel approach to overcome these limitations by using the probiotic bacterium *Escherichia coli* Nissle 1917 (EcN) and integrating PD-1/CD28 receptors into CAR-T cells. EcN bacteria naturally target the tumor microenvironment (TME) and can be genetically engineered to release synthetic antigens at the tumor site. This improves the targeting ability of CAR-T cells, ensuring that they localize and activate precisely where needed. Additionally, PD-1/CD28 receptor integration enhances the efficacy of CAR-T-cells by converting inhibitory signals into costimulatory signals, thus increasing the activation, proliferation, and persistence of T-cells. This approach has shown promising preclinical results, indicating improved targeting, activation, and longevity of CAR-T cells in solid tumors. Researchers should next focus on optimizing bacterial engineering, enhancing CAR-T-cell design, and conducting rigorous clinical trials to validate the safety and effectiveness of this combined therapy. Their findings may revolutionize treatment for colorectal cancer.

Keywords: Synthetic antigen; *E. coli* Nissle 1917; colorectal cancer; PD-1/CD28; CAR-T cells

Cancer has a high death rate worldwide. Colorectal cancer is the second deadliest cancer in the world. In 2018, about 1.8 million new cases of colorectal cancer were expected, with the death toll reaching about 881,000.[1] The development of colorectal cancer can be triggered by genome instability, that results in genetic and epigenetic mutations, which can transform normal glandular epithelial cells into benign tumors (neoplasms) and can further transform into an invasive type of cancer (carcinoma).[2]

The available treatment options include surgery, chemotherapy, and radiotherapy; however, these techniques are invasive and have significant side effects and resistance. Therefore, immunotherapy is a promising alternative.[3,4] The complexity of cancer treatment, as well as the intricate interactions between immune cells and cancer cells, highlights the need for targeted immunotherapy, such as chimeric antigen receptor T-cell (CAR-T) therapy. Unlike the typical mechanism of action of T cells, CAR-T cells are en-

Correspondence: Desak Made WIHANDANI
Department of Biochemistry, Faculty of Medicine, Udayana University, Bali, Indonesia
E-mail: dmwihandani@unud.ac.id

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gineered to identify targets without relying on the expression of the major histocompatibility complex (MHC). CAR-T cells can express CARs that target specific antigens present on the surface of tumor cells.4 CAR-T-cell therapy was found to effectively treat blood-related cancers, including multiple myeloma, non-Hodgkin lymphoma, and acute lymphoblastic leukemia.[3,4] Although CAR-T-cell therapy can treat hematological malignancies, challenges in the context of solid tumors remain. These challenges include the expression patterns of tumor-associated antigens (TAAs) in solid tumors and the phenomenon of T-cell exhaustion.[4-6] This highlights that new methods are needed for CAR-T-cell therapy. Such methods should not depend on tumor antigens and should increase the activity of active CAR-T cells, including CAR-T-cell therapy, integrated immunostimulatory fusion protein (IFP) PD-1/CD28, and guided synthetic antigen-encoded probiotics.[4,6]

The probiotic Escherichia coli Nissle 1917 (EcN) is based on the ability of bacteria to colonize tumor cells. It is specifically localized in the nuclei of hypoxic tumor cells and is safe for human use.[7,8] These probiotic bacteria are engineered to produce and release synthetic antigens, which are subsequently responded to by CAR-T cells.[6]

The use of IFP is a new strategy to overcome T-cell exhaustion, which is triggered by the interaction between programmed death-ligand 1 (PD-L1) and the programmed cell death protein 1 (PD-1) receptor, which reduces the function and activity of T cells.[5] IFP consists of the extracellular domain PD-1 protein, as well as the transmembrane and intracellular domains of the CD28 protein.[5,9] Using this strategy, inhibitory signals can be converted into stimulatory signals, and thus it is effective in overcoming CAR-T-cell immunosuppression.[5] This literature review comprehensively examines the refinement of PD-1/CD28 receptor-integrated CAR-T-cell therapy guided by synthetic antigen-encoded EcN 1917 probiotics as a new modality for treating colorectal cancer.

MATERIAL AND METHODS

This article was written after conducting a comprehensive and selective literature review. The relationships between each library were searched through online scientific database search engines such as PubMed, ScienceDirect, Google Scholar, and the Publish or Perish application by applying the Boolean "AND" and "OR" logic. The keywords used in this search included CAR-T cells, PD-1/CD28, EcN 1917, colorectal cancer, and synthetic antigens. The inclusion criteria set were references with a publication period of the last 10 years (2014-2024). This literature review also had certain exclusion criteria. We did not include studies published before 2014 to maintain data relevance.

PATHOPHYSIOLOGY OF COLORECTAL CANCER

There are three main molecular pathways related to colorectal cancer tumorigenesis, namely chromosome instability, mismatch repair, and CpG hypermethylation pathways. Imbalance between oncogenes and tumor suppressor genes leads to chromosomal instability, such as mutations that occur in adenomatous polyposis coli (APC).[10] Mutations in the APC gene lead to the translocation of beta-catenin into the cell nucleus, which then activates Wnt signaling. Translocation in the nucleus results in heterodimerization with transcription factors, which promotes intestinal epithelial cell proliferation and tumorigenesis. Moreover, Wnt signaling plays a role in activating several genes related to tumorigenesis. After APC mutation, KRAS is activated, which influences the activation of Raf-MEK-ERK, phosphoinositide 3 kinase (PI3K), and NF-kB, ultimately promoting cell proliferation. In the final stages of tumorigenesis, mutations occur in the TP53 gene, thus promoting tumor development.[11]

In contrast to the chromosome instability pathway, the mismatch repair pathway is characterized by hypermutation in somatic DNA, including mutations involved in DNA mismatch repair, such as epithelial cell adhesion molecule (EpCAM), resulting in an accumulation of repeated gene mutations that lead to microsatellite instability (MSI).[10,11] Tumor cells with the MSI phenotype are unable to recognize and repair mismatched DNA. The cell maintains and replicates the mutation. MSI mutations include mutations in the TGFB receptor-2 (TGFBR2) gene, which encodes a protein that inhibits intestinal epithelial cell

proliferation. MSI mutations also occur in other genes that encode proteins that regulate cell proliferation, apoptosis, and DNA repair.[11]

Tumors can also develop through hypermethylation of CpG islands, which are a collection of cytosine/guanine bases connected by phosphodiester bonds and are often found in the promoter regions of genes. Hypermethylation of CpG islands in the promoters of tumor suppressor genes triggers tumor development. An example of a gene that undergoes promoter hypermethylation is MLH1, which functions in DNA repair (Figure 1).[11]

CAR-T CELL THERAPY

CAR-T cell therapy is a renewable type of immunotherapy for treating non-solid and solid cancers. This treatment technique utilizes the extraction of normal T-lymphocytes from the patient's body through leukopheresis.[12] Next, a specific receptor (CAR) is integrated into T cells to increase the potential of immunotherapy. Modified T cells are multiplied using in vitro media. Then, the CAR-T cells are administered back to the patient to attack and work on the cancer location specifically based on the CAR target.[12,13]

CAR-T cell therapy is widely applied in cases of hematological malignancies such as leukemia and lymphoma. Research by Kymriah et al. showed a complete remission on 82% of the patients suffering from leukemia and lymphoma.[14] Moreover, no excessive side effects were found due to cytokine release syndrome (CRS). CAR-T cell therapy is also used to treat solid cancer cases based on specific antigen markers that match the characteristics of the type of cancer. The markers that are generally used include CD133 and carcinoembryonic antigen (CEA).[15] The CEA and CD133 proteins are predominantly found in the colon. These two proteins are candidate molecular targets in the treatment of colorectal cancer via CAR-T cells.[16] The combination of CEA with an antibody against the CD30 marker is the most effective strategy for treating colorectal cancer. In clinical trials, administering CEA CAR-T cells caused seven out of 10 patients with metastatic colorectal can-

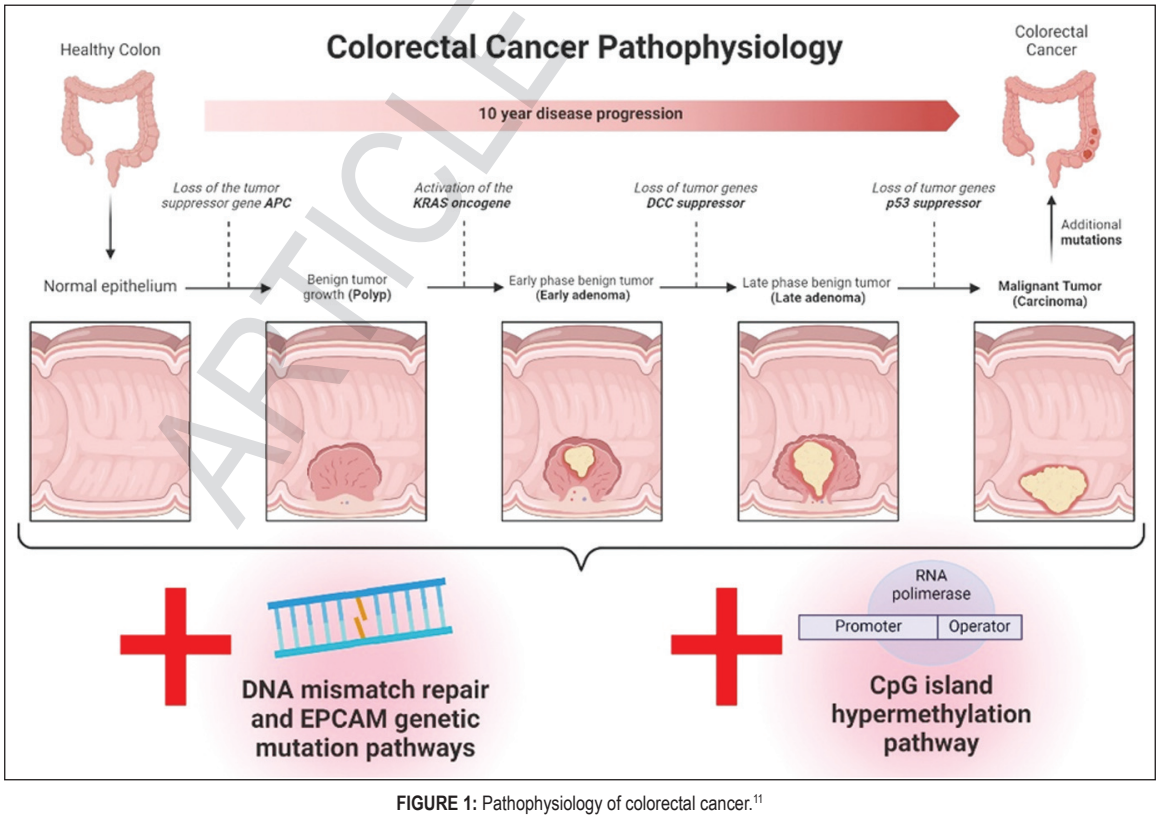

FIGURE 1: Pathophysiology of colorectal cancer.[11]

cer to achieve stable conditions within 30 weeks.[17] The ability of the CD133 component to eradicate cancer stem cells (CSCs) is also supported by the finding that 14 of 21 hepatocellular carcinoma patients achieved stable conditions for nine months on average.[18]

CAR-T cell immunotherapy is extremely suitable for treating patients with non-solid cancer. This is due to several reasons related to the weakness of CAR-T cells in solid cancer treatment. CAR-T cells cannot reach their maximum potential in the solid tumor microenvironment (TME) because T cells must have strong extracellular matrix degradation capabilities to reach the site of the tumor. Solid cancer is also known for its constituent components, which are layers of extracellular matrix with tumor-associated fibroblast, collagen, and proteoglycan components, which make it very difficult for T cells to penetrate.[19] The tumor environment also strongly influences T-cell activity, especially in the presence of cytokines, soluble proteins, and various ligand components, such as CTLA-4 and PD-L1, which can reduce the activity of T cells. These components activate anergic and apoptotic conditions in CAR-T cells, which prevents them from performing their functions.[20] Modification of CAR components specific to TAAs is also difficult because TAAs in solid cancer can cause cross-reactions and non-target specific actions with normal cells. This results in high rates of toxicity and product failure following CAR-T-cell therapy (Figure 2).[21-23]

THE POTENTIAL OF ECN 1917 AS A CARRIER OF SYNTHETIC ANTIGEN FOR CAR-T CELLS IN COLORECTAL CANCER

EcN is a serum-sensitive probiotic that does not produce enterotoxins nor cytotoxins which has been approved for use in the treatment of diarrhea and ulcerative colitis. Two types of plasmids, pMUT1 and pMUT2, from EcN are considered stable and can be used in genetic recombination.[7] This allow EcN to be genetically modified to deliver drugs to disease sites. Thus, such advantage can be utilized to increase the targeting ability of immune cells in targeted therapy for malignant diseases such as cancer.[7,24]

A study investigated the effect of EcN, under the brand name Mutaflor, administered using oral route on mice with colorectal adenomas. It revealed a more localized population of EcN in the tumor area through lipopolysaccharide (LPS) staining of EcN bacteria.[25] Moreover, experiments on colorectal cancer model mice were also conducted to test the effectiveness of integrated EcN delivery of the cytokine GM-CSF and

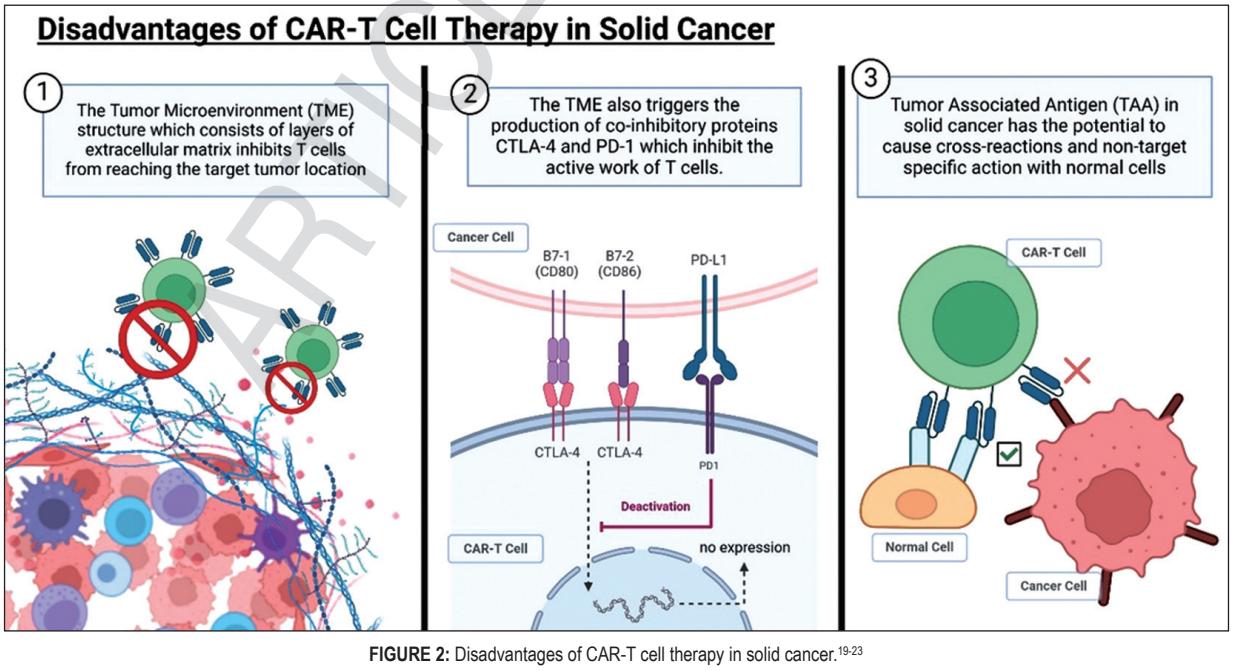

FIGURE 2: Disadvantages of CAR-T cell therapy in solid cancer.[19-23]
CAR-T: Chimeric antigen receptor T.

the nanoantibodies PD-L1 and CTLA-4. Histological examination revealed a 47% decrease in the size and number of tumor cells. This finding highlighted the potential of EcN in the colonization and degradation of colorectal cancer.[26,27] This ability of EcN can be used to deliver synthetic CAR-T antigens in targeted therapy for colorectal cancer.[6]

Genetic modifications applied to EcN for guiding CAR-T-cell involve various types of synthetic antigen components. One experimental result revealed that synthetic antigen consisting of heparin binding domain originating from placental growth factor-2 (PlGF-2) and connected with superfolder green fluorescent protein (sfGFP) via a glycine-serine linker has a great potential. The reason lies in the fact that the specific diffusion of the synthetic antigen in the TME with its unique fibronectin and collagen population will limit the CAR-T-cells to only act inside the TME, thus increasing the safety.[28,29] In addition, using the aforementioned synthetic antigen revealed a more effective activation period extension of the CAR-T-cells.[30,31] As a response to these components, CAR modification involving several appropriate antibodies is also needed for T cells. Specific antibodies against sfGFP in CARs can be used and linked with CD28 and CD3 domains by IgG4 linker.[31]

The mechanism of EcN and CAR-T cells in colonization and immunological activation cannot be separated from the mechanism of controlled bacterial replication and multiplication. When EcN with specific antigens is administered and colonizes the tumor site, EcN grows to a certain bacterial population density threshold (quorum threshold). In detecting the quorum threshold, several sensing genes, such as luxI and ϕX174E, play a role. Next, some of the bacteria in the population undergo lysis and release specific antigens, which are targeted by CAR-T cells. The population that is not lysed continues to grow until it reaches the quorum threshold and repeats the cycle. This mechanism is known as the Synchronized Lysis Circuit (SLIC).[7,32] Studies on the colonization ability of EcN can be found in Table 1.

To determine the targeting ability of CAR-T cells, Vincent et al. constructed dye-linked sfGFP-PlGF-targeting CAR-T cells. Then, the CAR-T cells were incu-

TABLE 1: Studies of EcN colonization ability in tumor tissue.

EcN variation	Subject	Method (Route, Dosage, Period)	Results
EcN is normal	8 female NMRI Nude mice with head and neck squamous cell carcinoma model	Intravenous, 1×107 CFU, 3 days	The ratio of bacterial colonization in tumor tissue and normal tissue is almost 1000:1.[47]
EoN luxCDABE cassette encoder (EcN-lux)	ApcMin/+ mice as a precursor model for colorectal cancer	Oral, 1010-1011 CFU/mL, 7 weeks	Bioluminescent bacteria are more abundantly observed in the distal colon where the adenoma burden is greatest.[26]
EcN is normal	BALB/c mice bear 4T1 tumors	Intravenous, 2×104 CFU, 3 days	2 out of 4 mice had tumors colonized with bacteria.[48]
EcN with pMUT-gfp Knr modification	5 BALB/c mice with 4T1 breast cancer cell line xenograft with/without pretreatment with antibiotics	Intranasal, 1×107 CFU, once every 3 days for 18 days	The results of a study of tumor samples showed that there were ~50 times more EcN colonies in the group pretreated with antibiotics, with the highest being 7.37×103±3.39×102 CFU per 1 g of tumor tissue.[49]
Normal EcN and EcN conjugated CA-Dox-Hyd-SH/AuNRs	BALB/c nude mice with MCF-7 breast cancer cell line xenograft	Intravenous, 2×108 CFU, Injection only on the first day	In the group injected with EcN, there was a high accumulation of EcN in tumor tissue on days 2 to 4 after EcN injection. High accumulation of EcN also occurs in the kidneys and liver.[50]
EcN with modified pMut1 plasmid	CB6F1 mice with CT26 colorectal cancer cell line xenograft	Intravenous, 2×108 CFU, injection only on the first day	In the group given modified EcN, there was a significant increase in IL-2 cytokines in tumor tissue compared to the normal EcN group (p≤0.001).[51]

EcN: *E. coli* Nissle 1917.

bated with the MDA-MB-468 cell line that was treated with pure sfGFP-PlGF synthetic antigen (100 ng/mL) and observed. The results were then compared to those of the control sample.[33]

The findings showed that cluster formation was more prominent in the group treated with pure synthetic antigen than in the diGFP group. The large number of clusters indicated the number of sfGFP-PlGF antigen receptors that formed synapses on target cells after the pure synthetic antigen was administered. Synapse formation was lower in the diGFP group than in the pure synthetic antigen group because the diGFP group did not have the PlGF domain, which functions to attach synthetic antigens to tumor cells.[33]

Cytotoxicity assessment of sfGFP-PlGF-targeting CAR-T cells was performed using the luciferase lysis assay. Cytotoxicity assessment was conducted on several cancer cell lines with an effector-to-target (E:T) ratio of 3:1 and incubated with a synthetic antigen (100 ng/mL) for 20 h. The results showed that the level of cytotoxicity (in RLU) in all cell lines treated with the pure synthetic antigen was highly significant compared to that in the control cells treated with PBS ($p<0.0001$). These findings indicated that synthetic antigens act as universal antigens for sfGFP-PlGF-targeting CAR-T cells regardless of the tumor type and origin.[33]

Assessment of the antitumor activity of sfGFP-PlGF-targeting CAR-T cells in vivo was performed using NSG mice with a Nalm6 cell line xenograft model. Groups of mice then received intratumor injections of EcN (1×105 CFU). Intratumor administration of sfGFP-PlGF-targeting CAR-T cells (2.5×106 CFU) was performed two days after EcN was injected, while the control group was administered PBS.[33] The results on day 28 after treatment revealed a decrease in tumor growth in the sfGFP-PlGF-encoded EcN group which tumor volume was <600 mm^3. This result was significantly different from that of the normal EcN group which tumor volume was >600 mm^3 ($p<0.01$). The results in the sfGFP-PlGF group were also highly significantly different from those in the diGFP-encoded EcN group and the control group which tumor volume was >1,200 mm^3 ($p<0.0001$). This significant difference between the diGFP and sfGFP-PlGF groups matched the results of the cytotoxicity test, which further validated the role of the PlGF domain in attaching synthetic antigens to tumor cells. Additionally, sfGFP and diGFP levels in serum were estimated by ELISA on day 14 after EcN injection, and the results revealed a significant difference between the serum levels of sfGFP (<0.02 ng/mL) and diGFP (>0.06 ng/mL) ($p<0.01$). These findings indicated that sfGFP-PlGF can reduce the systemic release of the synthetic antigens. The IFN-γ and TNF-α levels also increased significantly ($p<0.01$) in the sfGFP-PlGF group compared to their levels in the control group. These findings further supported the increase in antitumor activity *in vivo*.[33]

The adaptive response of the endogenous immune system was assessed using three groups of immunocompetent mouse models of MC38 colorectal cancer cell line xenografts at two different sites. Then, one of the tumors from each group was intratumorally-injected with PBS, normal EcN, and sfGFP-PlGF-encoded EcN (2×106 CFU). Then, all groups were intratumorally injected with 1.5×106 CFU of sfGFP-PlGF-targeting mouse CAR-T cells on day 2 and 5 after EcN was administered.[33] The results on day 23 after the administration of EcN sfGFP-PlGF at one tumor site alone revealed not only the inhibition of tumor growth at the injection site but also a reduction in tumor growth at the other and more distal site (>200 mm^3; $p<0.01$) compared to those of the control group and the normal EcN group (~600 mm^3). The results of the immunophenotyping of tumor samples at the site injected with EcN sfGFP-PlGF revealed a significant increase in CD69 expression on CD8+ cells and conventional T cells (CD4+Foxp3-) compared to that in the control group. Significant differences were also recorded in the increased frequencies of Ki67+ ($p<0.05$) and CD44+ ($p<0.01$) conventional T cells. Based on these results, Vincent et al. confirmed that administering sfGFP-PlGF-encoding EcN and sfGFP-PlGF-targeting CAR-T cells can propagate endogenous immune cells, enabling them to induce a systemic antitumor response.[33]

PROGRAMMED CELL DEATH-1 (PD-1) INHIBITORY AGENTS ON T LYMPHOCYTE CELL IMMUNE ACTIVITY

Under normal conditions, CD8+ cytotoxic T cells and CD4+ helper T cells have antigen receptor components that are associated with costimulatory and coinhibitory molecules. Activating these components results in the activation or tolerance of T cells.[34] The most common coinhibitory secondary signaling component found on T cells is PD-1, also known as CD279, and cytotoxic T lymphocyte antigen 4 (CTLA-4). When T cells are activated, PD-1 is expressed on the cell surface and interacts with the PD-L1 ligand (CD274) found on target cells or tumors. The interaction of PD-1 with PD-L1 leads to the activation of the active inhibitory pathway of T cells, thereby triggering apoptotic conditions and reducing the cell survival rate.[12,35,36]

Regarding adverse inhibitory conditions, the use of CAR-T cells, which are essentially T lymphocytes, is highly challenging in cancer treatment. The TME has acidic, hypoxic conditions and high levels of oxidative stress substances, which trigger the production of inhibitory immune molecules (PD-1 and CTLA4) and inhibitory immune cells (Tregs). This reduces the invasion and infiltration ability of immune cells. Additionally, the presence of excess PD-1 and CTLA4 inhibits the activation of T cells. As a result, tumor cells are easily released without degradation by immune cells.[37]

THE ROLE OF PD-1/CD28 IN THE IMMUNOSTIMULATORY MECHANISM OF CAR-T CELLS IN COLORECTAL CANCER

Various techniques have been tested to overcome the inhibitory effects of PD-1 immune checkpoint inhibitors, starting with the use of anti-CD19 and systemic antibodies (nivolumab or pembrolizumab). However, their implementation resulted in various side effects related to excessive immune activity. Therefore, a component that is more specific and safe is needed to inhibit PD-1/PD-L1 activity.[38,39]

A new approach involving the use of an IFP in the form of the CD28 domain can inhibit the PD-1/PD-L1 inhibitory pathway. CD28 is located in the intracellular domain of extracellular PD-1, which functions as a secondary signaling pathway stimulator. This suppresses the activity of inhibitory proteins in T cells and activates costimulatory pathways when PD-1/PD-L1 interacts.[40] The implementation of PD-1/CD28 on TRuC-T cells that target tumor cells also increases the production of the cytokines IFN-γ and IL-2 in the TME.[41] On the other hand, the use of PD-L1 on CAR-T cells increases the sensitivity of the contact area with PD-L1 to the tumor cell surface such that the T-cell activation response increases further.[5]

An increase in T-cell activity due to PD-1/CD28-type IFPs is supported by several studies on leukemia, lymphoma, and even solid cancer.[5,42-44] An in vivo leukemia study involving the administration of PD-1/CD28 CAR-T cells revealed an increase in leukemic clearance and survival of NSG mice. The results revealed that compared to the mice administered CAR-T-cell therapy alone, mice with central and peripheral T-cell modulation (IFP modulation) had a longer life expectancy of 90 days.[5] A trial of PD-1/CD28 CAR-T cells in 17 PD-L1-positive B-cell lymphoma patients reported a good response. In total, 10 patients experienced an objective response to treatment, and seven others experienced complete remission from their lymphoma. Additionally, no signs of neurological toxicity or CRS were found in the patient.[9] Application in solid cancer was also performed by introducing IFP PD-1/CD28 TRuC-T cells into NSG mice with inoculation of pancreatic cancer target cell lines (SUIT-MSLN and mesothelioma (MSTO-MSLN). The results revealed smaller tumors (20 mm^3) with TRuC-T PD-1/CD28 treatment than with TRuC-T treatment alone. Moreover, in MSTO-MSLN mice, no significant difference was found in changes in tumor size between TRuC-T PD-1/CD28 and TRuC-T alone.[41] These findings indicated that the use of CAR-T cells with PD-1/CD28 can increase tumor cell eradication ability, treatment response, and patient survival rates. Studies on PD-1/CD28 integration are listed in Table 2.

MECHANISM OF GENETIC MODIFICATION OF ECN 1917 AND CONSTRUCTION OF PD-1/CD28-INTEGRATED CAR-T CELLS

Genetic modification of EcN begins by combining an AS component consisting of an sfGFP homodimer

TABLE 2: Studies of PD-1/CD28 on CAR-T cells.

CAR-T cell type	Subject	Dose	Period	Results
Mesothelin-specific CAR-T cells	NSG mice with SUIT-2 pancreatic cancer cell line xenograft	1×107 CFU	Injection only on the first day	In in vitro studies, there was a significant increase in IFN-γ and IL-2 by CAR-T cells with PD-1/CD28 compared to CAR-T cells without PD-1/CD28. (IFN-γ, IL-2= p<0.001).[41] In *in vivo* studies, CAR-T cells with PD-1/CD28 were able to significantly inhibit tumor growth compared to regular CAR-T (p=0.05).[41]
CD-19, mesothelin, and PSCA specific CAR-T cells	Mice with EMMESO, PC3-PSCA-PD-L1, and PC3-PSCA cancer cell line xenografts	2×107 CFU	Injection only on the first day	There was a significant inhibition of tumor size by CAR-T cells with PD-1/CD28 compared with regular CAR-T cells (SS1BBz/PD1CD28= p<0.05; PSCABBz/PD1CD28= p<0.05).[42]
CD-19 specific CAR-T cells	17 patients with PD-L1+ B-cell lymphoma	0.5×106-4×106 CFU/kg	Injection only on the first day	The results of the study on 17 patients showed that 10 out of 17 had an objective response and 7 out of 10 had a complete response within 3 months. Some of the adverse events most frequently experienced by patients were granulocytopenia (100%), pyrexia (100%), anemia (76.47%), thrombocytopenia (70.59%), hypotension (41.17%), CRS Grade 1 (47.06%), and CRS Grade 2 (41.18%). No patient experienced symptoms of neurotoxicity.[9]

CAR-T: Chimeric antigen receptor T.

that binds to the CAR receptor, linker, and PlGF-2. These components are then incorporated into a plasmid and inserted into EcN. The EcN strain is also equipped with a synchronized lysis chain (SLIC) system.[6,29] After EcN is modified, the bacteria are cultured and then administered.[6]

The generation of PD-1/CD28 CAR-T cells begins with sampling normal T cells from patients through leukapheresis.[12] Next, a CAR component, consisting of specific sfGFP nanobodies, immunoglobulin G4 (IgG4), a CD28 transmembrane domain, and a CD3ζ intracellular domain, is created. Then, the gene for the CAR receptor is cloned and processed into a lentiviral vector that carries the CAR receptor gene. This lentiviral vector is then transduced into the normal T cells of the patient to obtain CAR-T cells. The cells are then cultured for expansion before they can be used.[6]

The PD-1/CD28-integrated CAR-T cells can be constructed using additional lentiviral vectors. PD-1/CD28 receptor generation, according to Liu et al., involves combining pieces of the extracellular domain of PD-1 and pieces of the transmembrane and intracellular domains of CD28 in the mouse stem cell virus (MSCV) promoter, which is then cloned and inserted into a lentivirus transgenic transcription vector.[9] Lesch et al. showed that the simultaneous transduction of two lentiviral vectors into normal T cells did not interfere with CAR expression or PD-1/CD28 expression. This study also showed that 58.3% of T cells successfully expressed CAR and PD-1/CD28 after being transduced simultaneously with two different lentiviral vectors (Figure 3).[41]

ADMINISTRATION OF A SYNTHETIC ANTIGEN-ENCODED ECN 1917 PROBIOTIC

Intravenous injection of EcN provides an opportunity for EcN to be released systemically and colonize tumors. The main challenge that needs to be overcome in the intravenous route involves determining how to transport EcN to the tumor site before it is eliminated by the reticuloendothelial system. Cao et al. coated EcN with erythrocyte membranes for intravenous administration. They found that retention of EcN increased in the blood and reported a nearly fivefold greater yield on imaging compared to the yield of EcN without erythrocyte membranes. In addition, analysis of the immune inflammatory response by checking serum levels of IL-6, IL-10, and TNF-α, found higher levels in EcN without erythrocyte membrane coating. From this result, the study conclude erythrocyte coating

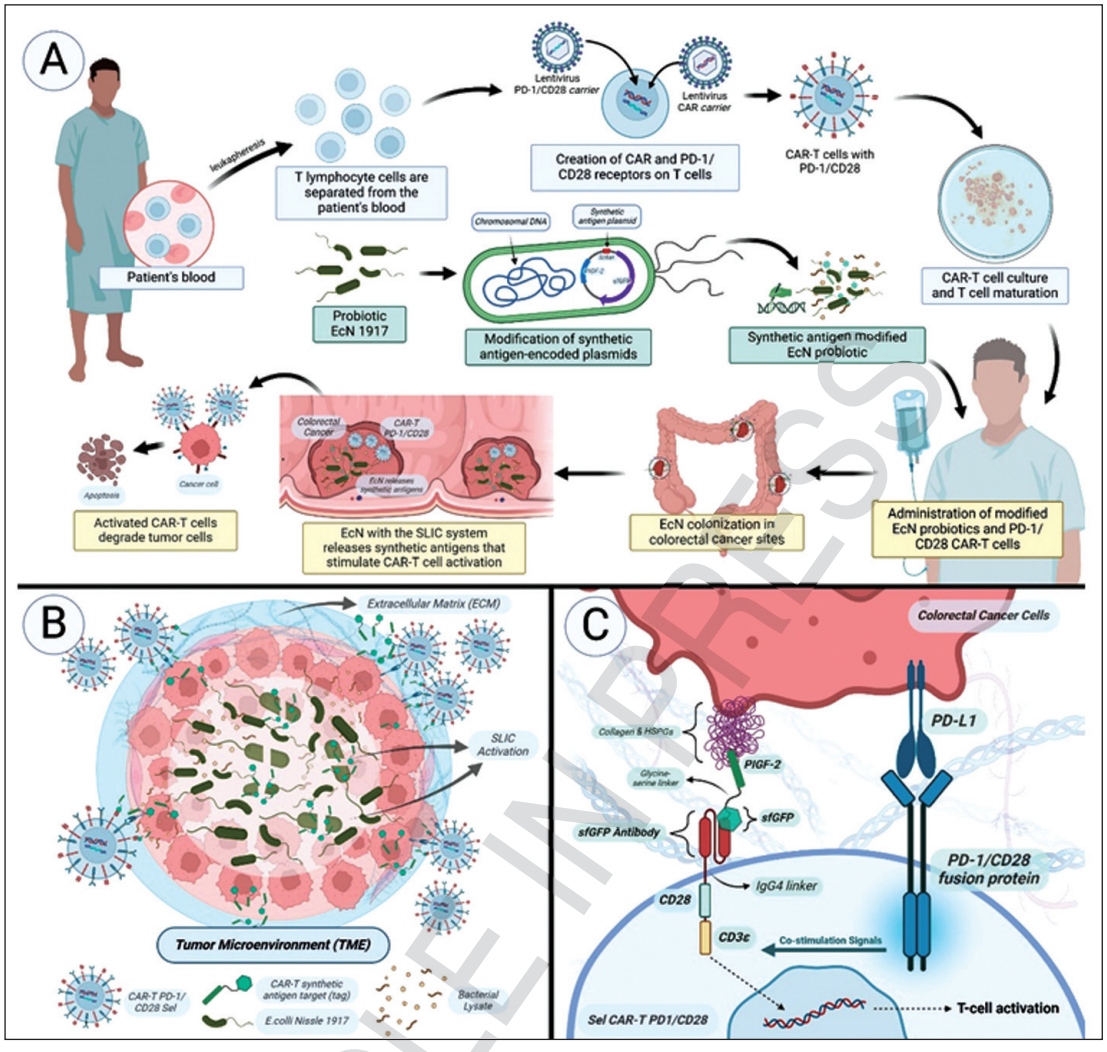

FIGURE 3: Overall molecular activation of PD-1/CD28 CAR-T cells.[6,8,9,33,40-42]
A) Mechanism underlying the construction and administration of PD-1/CD28-integrated CAR-T cells in colorectal cancer; B) Activation of PD-1/CD28 CAR-T cells against synthetic antigens produced by EcN via the SLIC; C) Structure of the synthetic antigen, CAR-T receptor, and PD-1/CD28

membrane has an effect to preserving EcN bioavailability from natural human inflammatory immune response.[45]

Colorectal cancer in the gastrointestinal tract provides an opportunity for EcN to be administered orally. Gurbatri et al. administered EcN orally to orthotopic colorectal cancer (MSS and MSI) mouse models and found that EcN accumulated in colorectal cancer tissue (~108 CFU/g). The level of EcN accumulated in colorectal cancer tissue was significantly higher than that accumulated in the normal colon (~106 CFU/g) and other organs, such as the liver and spleen (0 CFU/g) (normal colon, liver, and spleen: $p<0.0001$).[26] The main challenge in the oral administration of EcN involves ensuring that EcN reaches tumor sites that are influenced by various factors, such as gastric acid. One strategy to solve this problem is to use a double-layer polysaccharide hydrogel. The imaging results revealed an increase in the retention of encapsulated probiotics even after 48 h of treatment compared to that of nonencapsulated probiotics, which lasted only 4 h. This occurred due to the nature of the double-layer hydrogel, which completely disintegrated upon reaching the colon.[46] Based on these facts, double-layer hydrogel encapsulation is optimal for the oral administration of EcN.

CONCLUSION

The combination of CAR-T-cell immunotherapy with PD-1/CD28 has promising potential as a new therapeutic option for treating colorectal cancer. Immunotherapy based on sfGFP-PlGF-targeting CAR-T cells has a lytic cytotoxic effect specifically on cancer cells and can effectively suppress the formation and growth of tumor cells. The effectiveness of CAR-T cells can be inhibited by PD-1 molecules in the TME of colorectal cancer. The use of PD-1/CD28 can increase the activity of CAR-T cells. Through more specific and personalized integrated sfGFP-PlGF PD-1/CD28 CAR-T-cell therapy, a new, more effective method for treating colorectal cancer can be obtained.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Tegar Raharja Ariawan;* ***Design:*** *Tegar Raharja Ariawan;* ***Control/Supervision:*** *Desak Made Wihandani;* ***Data Collection and/or Processing:*** *Ig Mahapraja Divasta;* ***Analysis and/or Interpretation:*** *Asri Bintari;* ***Literature Review:*** *Ig Mahapraja Divasta;* ***Writing the Article:*** *Asri Bintari;* ***Critical Review:*** *Bryan Gervais De Liyis;* ***References and Fundings:*** *Bryan Gervais De Liyis;* ***Materials:*** *Desak Made Wihandani.*

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