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Regulation of Vascular Endothelial Cells and Cytokines in the Tumor Microenvironment by Radiotherapy

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CONTENTS

LIST OF ABBREVIATIONS

RT	Radiotherapy
CT	Chemotherapy
TME	Tumor microenvironment
TEM	Transendothelial migration
DNA	Deoxyribonucleic acid
D-CRT	Three-Dimensional Conformal Radiation Therapy
IMRT	Intensity-modulated Radiation Therapy
TOMO	Tomotherapy
VMAT	Volumetric modulated arc therapy
TH	CD4+ helper T cells
SBRT	Stereotactic host radiation therapy
TSC	Trans Sodium Crocetinate
MHC	Histocompatibility complex
NSCLC	Non-small cell lung cancer
ECM	Extracellular matrix
TCRs	T cell receptors
MHC	Major histocompatibility complex
APCs	Antigen-presenting cells
IFN-γ	Interferon gamma
IL	Interleukin
TGF	Transforming growth factor
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
CTL	Cytotoxic T lymphocyte
ADCC	Antibody-dependent cellular cytotoxicity
TGF-β	Transforming growth factor beta
CDC	Complement dependent cytotoxicity
MDSC	Myeloid derived suppressor cell
NK	Natural killer cells
TAMs	Tumor-associated macrophages
M1	Classically activated macrophages
M2	Alternatively activated macrophages
TNFα	Tumor necrosis factor alpha

IL-1β	Interleukin-1 beta
TGF-β	Transforming growth factor beta
DCs	Dendritic cells
pDCs	Plasmacytoid dendritic cells
cDC1s	Conventional type 1 dendritic cells
cDC2s	Conventional type 2 dendritic cells
MoDCs	Monocyte-derived dendritic cells
ECs	Endothelial cells
PSGL-1	P-selectin glycoprotein ligand-1
WPBs	Weibel-Palade bodies
EGF	Epidermal growth factor
ESL-1	E-selectin ligand-1
LFA-1	Lymphocyte function-associated antigen 1
ICAM1	Intercellular adhesion molecule 1
VCAM1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen-4
VE-cadherin	Vascular endothelial cadherin
ROS	Reactive oxygen species
GM-CSF	Granulocyte-macrophage colony-stimulating factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-associated death domain
RIP1	Receptor interacting protein kinase 1
JAK	Activating Janus activated kinase
IFNR	Interferon receptor type
GM-CSFR	Granulocyte-macrophage colony-stimulating factor receptor
MAPK	Mitogen-activated protein kinase
PI3K	Phosphatidylinositol 3-kinase
2-HG	2-Hydroxyglutarate
DMEM	Dulbecco's modified Eagle's medium
IF	Immunofluorescence
PTM	Post-translational modification
WHO	World Health Organization
PD-1	Programmed cell death protein 1

PD-L1	Programmed cell death ligand 1
bFGF	Basic fibroblast growth factor
VEGF-A	Vascular endothelial growth factor
VEGF	Vascular endothelial growth factor
TGFβ	Transforming growth factor beta
FBS	Fetal bovine serum
bEnd5	Mouse-brain-derived endothelial cells
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qRT-PCR	Quantitative Real-time PCR
RNA	Ribonucleic acid
cDMEM	Complete Dulbecco's Modified Eagle Medium
MACS	Magnetic-activated cell sorting
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
CFA	Complete Freund's Adjuvant
RPMI	Roswell Park Memorial Institute
DAPI	4′,6-diamidino-2-phenylindole
HCL	Hydrogen chloride
CO_2	Carbon dioxide
BSA	Bovine serum albumin
TEC	Tumor endothelial cells
DNase	Desoxyribonuklease
EP	eppendorf
EtOH	Ethanol
ddH_2O	Sterile Double Distilled Water
OD	optical density
cDNA	Complementary Deoxyribonucleic acid
PFA	Paraformaldehyde
mRNA	Messenger RNA
MCP-1	Monocyte chemoattractant protein-1

1. Zusammenfassung

Krebs ist nach wie vor eine große Bedrohung für die menschliche Gesundheit. In den letzten Jahrzehnten hat die Forschung zur Behandlung von Tumoren große Fortschritte gemacht. Traditionelle Krebsbehandlungen wie Chirurgie, Strahlentherapie (RT) und Chemotherapie (CT) werden ständig verbessert. Darüber hinaus wird mit der Entwicklung der wissenschaftlichen Forschung und der Vertiefung des Verständnisses auf molekularer Ebene die Forschung zur Krebsbehandlung auf molekularer Ebene zu einer der wichtigsten Richtungen, beispielsweise ist die Immuntherapie zu einem Meilenstein in der Krebsbehandlung geworden. Chirurgie, RT und CT zielen darauf ab, bösartige Zellen direkt abzutöten, während die Immuntherapie hauptsächlich den Zweck erfüllt, Krebszellen zu zerstören, indem sie die Immunflucht des Tumors eliminiert und die Immunantwort des Körpers aktiviert. Obwohl im Laufe der Jahre große Durchbrüche und Fortschritte in der Krebsbehandlung erzielt wurden, bleibt Krebs eine der größten Bedrohungen für die Gesundheit der Menschen. Seit entdeckt wurde, dass Strahlen die Desoxyribonukleinsäure (DNA) von Zellen schädigen können und verwendet werden können, um Krebszellen abzutöten und das Wachstum von Tumoren zu kontrollieren, hat die RT-Technologie mit der Entwicklung von Wissenschaft und Technologie ständig Innovationen hervorgebracht. Von der konventionellen Strahlentherapie zur intensitätsmodulierten Strahlentherapie (IMRT) und von der externen Strahlentherapie zur Brachytherapie und Partikelimplantation werden Genauigkeit, Sicherheit und Wirksamkeit der Strahlentherapie ständig verbessert. Aufgrund der Verbreitung von Krebszellen wird Krebs jedoch oft resistent gegen bestimmte Behandlungen und Krebszellen wachsen weiter. Seit die Immuntherapie in der klinischen Behandlung angewendet wird, wurde auf dem Gebiet der Tumorbehandlung ein Meilenstein erreicht. Verwirrend ist jedoch, dass es immer noch viele Patienten gibt, die von der Immuntherapie nicht profitieren können. Es ist erwähnenswert, dass klinische Studien gezeigt haben, dass die therapeutische Wirkung einer kombinierten RT und Immuntherapie besser ist als die einer Immuntherapie allein, und sogar einige Patienten, die nicht auf die Immuntherapie ansprechen, können von der Immuntherapie profitieren, nachdem sie eine kombinierte RT und Immuntherapie erhalten haben. Im Vergleich zu einer Einzelbehandlung werden mit einer Kombinationstherapie oft bessere Ergebnisse erzielt, und RT, CT und Operation werden oft bei Kombinationsbehandlungen eingesetzt. Der Unterschied besteht jedoch darin, dass die Kombination von RT, CT und Operation vor allem auf der Überlagerungswirkung der verschiedenen Behandlungsmethoden auf die Abtötung maligner Zellen beruht, während die Förderung einer kombinierten Therapie mit RT und Immuntherapie das Immunsystem beeinflussen kann, was eine lohnende Erforschung dieses Bereichs darstellt. Damit eine Anti-Tumor-Immunantwort aktiviert werden kann, muss das Immunsystem Tumorantigene erkennen und Immunzellen müssen durch das Gefäßendothel zu den Krebszellen wandern,

um eine Immunwirkung auszuüben. Immuntherapien wie Checkpoint-Inhibitoren können verhindern, dass der Krebs dem Immunsystem entkommt und die Reaktion des Immunsystems auf maligne Zellen reaktivieren. Manche Patienten sprechen nicht auf eine Immuntherapie allein an, profitieren aber von der Kombination aus Immuntherapie und RT. Wir vermuten, dass ein Grund für dieses Phänomen darin liegen könnte, dass RT das Tumormikromilieu (TME) verändern und die transendotheliale Migration von Immunzellen fördern kann. TME ist einer der wichtigsten Bereiche der Tumorbehandlungsforschung und umfasst die Aggregation und Migration von Immunzellen, die Funktionsstörung und Aktivierung von vaskulären Endothelzellen und verschiedene Zytokine wie TNF-α, IFN-γ, IL-6 usw. Daher ist die Aktivierung von vaskulären Endothelzellen (ECs) für die Krebsimmuntherapie sehr wichtig, und die Adhäsionsmoleküle wie P-Selectin, E-Selectin, ICAM1 und VCAM1 auf der Oberfläche des vaskulären Endothels spielen eine sehr wichtige Rolle im Prozess der transendothelialen Migration (TEM) von Immunzellen. In dieser Arbeit untersuchte ich den Einfluss von RT auf TME, insbesondere auf die Regulierung von Adhäsionsmolekülen, die auf den vaskulären ECs exprimiert werden, durch In-vivo- und In-vitro-Experimente. Die Ergebnisse zeigten, dass RT die Expressionsniveaus von E-Selectin, P-Selectin, ICAM1 und VCAM1 auf ECs hochregulierte und auch die Niveaus einiger entzündlicher Zytokine GM-CSF, IL-1α und IL-1β hochregulierte. Die Hochregulierung von vaskulären endothelialen Adhäsionsrezeptoren und entzündlichen Zytokinen könnten Ziele und neue Punkte für die Untersuchung einer Kombinationstherapie mit RT und Immuntherapie sein.

2. Abstract

Cancer is still an essential threat to human health. In recent decades, research on the treatment of tumors has made great progress. Surgery, radiotherapy (RT) and chemotherapy (CT) are traditional cancer treatments. In addition, with the development of scientific research and the deepening of the understanding of the molecular level, research on cancer treatment at the molecular level is becoming one of the most important directions, and immunotherapy has become a milestone in cancer treatment. Surgery, RT and CT are aimed at directly killing malignant cells, while immunotherapy mainly achieves the purpose of destroying cancer cells by eliminating the tumor's immune escape and activating the host's immune response. Although great breakthroughs and progress have been made in cancer treatments over the years, cancer remains one of the biggest threats to people's health. Since rays were discovered to damage the deoxyribonucleic acid (DNA) of cells and were used to kill cancer cells and control the growth of tumors, RT technology has been constantly innovating with the development of science and technology. From conventional radiotherapy to intensity-modulated radiotherapy (IMRT), and from external radiotherapy to brachytherapy and

particle implantation, the accuracy, safety and effectiveness of radiotherapy are constantly improving. However, due to the unlimited proliferation nature of cancer cells, cancer always becomes resistant to treatments and cancer cells continue to grow. Since immunotherapy was applied to clinical treatment, a milestone breakthrough was made in tumor treatment. However, what is confusing is that many patients still cannot benefit from immunotherapy. It is worth noting that clinical studies have found that the therapeutic effect of combined RT and immunotherapy is better than that of immunotherapy alone, and even some patients who do not respond to immunotherapy can benefit from immunotherapy after receiving combined RT and immunotherapy. Compared with single treatment, combination therapy often achieves better results, and RT, CT and surgery are often used in combination treatment. However, the difference is that the combination of RT, CT and surgery mainly depends on the superposition effect of different treatment methods on the killing of malignant cells, while the promotion of RT and immunotherapy combined therapy may affect the immune response system, which is a direction worthy of exploration. Activation of anti-tumor immune response requires activation of the immune system to recognize tumor antigens and activated immune cells to migrate across the endothelium to the location of cancer cells to exert immune effects. Immunotherapy, such as checkpoint inhibitors, can prevent cancer from escaping the immune system and reactivate the immune system's response to malignant cells. Some patients do not respond to immunotherapy alone, but benefit from the combination of immunotherapy and RT. We speculate that one reason for this phenomenon may be that RT can change the tumor microenvironment (TME) and promote the transendothelial migration (TEM) of immune cells. The TME is one of the most important areas of tumor treatment research, including the aggregation and migration of immune cells, the dysfunction and activation of vascular endothelial cells, and various cytokines such as TNF-α, IFN-γ, IL-6, etc. Therefore, the activation of vascular endothelial cells (ECs) is very important for cancer immunotherapy, and the adhesion molecules such as P-selectin, E-selectin, ICAM1 and VCAM1 on the surface of vascular endothelium play a critical role in the process of TEM of immune cells. In this thesis, I investigated the influence of RT on the TME especially on the regulation of adhesion molecules expressed on the surface of vascular ECs through *in vivo* and *in vitro* experiments. The results showed that RT upregulated the expression levels of E-selectin, P-selectin, ICAM1 and VCAM1 on ECs, and also upregulated the levels of some inflammatory cytokines GM-CSF, IL-1α and IL-1β. The upregulation of vascular endothelial adhesion receptors and inflammatory cytokines may be the targets and new points for the study of combination therapy with RT and immunotherapy.

3. Introduction

3.1. Advances in Cancer Treatment

3.1.1. Brief overview of cancer

Cancer is a collective term for lesions in which normal cells transform into malignant cells that proliferate rapidly beyond their usual boundaries and invade adjacent areas or other organs. According to a survey by the World Health Organization(WHO), malignancies are the second leading cause of death in the world[1]. There were almost 9 million people who died of cancer in 2020, and the proportion of people who died of cancer is about one-sixth of all deaths[1].

The tumor is made of a bunch of cells involving unlimited growth and abnormal division that will shape a lump and have the potential to invade other tissues and organs[2]. The cancer cells are transformed from the normal cells because of the genetic changes, and most genetic mutations are caused by environmental and lifestyle changes[3]. One of the fundamental characteristics is that they have infinitely multiplying capabilities and can spread to other locations of the host via blood and lymphatic[4]. Cancer is hard to cure and causes so many deaths in the world mainly because of this characteristic.

With the development of medical technology, people have been able to use a variety of methods to try to treat cancer. The tumor is classified and staged according to its size, location, cell type and genotype, and treated with surgery, RT, CT, targeted therapy and other treatments[5,6]. Unfortunately, mortality caused by cancer remains high. It was reported that cancer caused nearly 10 million deaths in 2020, which is nearly one in six deaths[7,1]. The treatment of cancer remains a great challenge even now. Surgery, RT and CT are the three most important means of treating cancer[8]. Surgical procedures attempt to remove cancer lesions by excision of tumors. However, the success rate of surgery alone is unfortunately often not sufficient – except for early-stage cancers[9]. In most cases, a small fraction of tumor cells remains due to the inability to remove complete tumor mass, especially in advanced tumors. From the remaining cells, the disease relapses and often metastasizes[10]. Therefore, clinicians require further complementary treatment options to eradicate the tumor. One of these treatment options is RT. RT is based on ionizing radiation to directly damage DNA by creating double-stranded breaks.[11] In this way, the cells, especially rapidly proliferating cells, can't cope with and go under apoptosis[12]. In addition to RT, CT is also a highly successful treatment option. As the name implies, CT is composed of cytotoxic chemical drugs aiming to inhibit the proliferation of the cells[13], but the most prominent effect is on the rapidly dividing of cells such as cancer cells. Unfortunately, even though it is a powerful treatment option, it also has most of the systemic adverse reactions, and cancer cells always develop resistance to the therapy[14]. In the past decades, targeted therapy and immunotherapy have emerged and

attracted scientists' and clinicians' interests. Targeted therapy focuses on specific proteins that cause cancer in the host[15], while immunotherapy aims to kill cancer cells by inducing the patient's autoimmune system[16,17]. However, immunotherapy as a single treatment does not fully eradicate tumors just as surgery, RT and CT. Therefore, patients usually achieve the aim of eradicating tumors and prolonging survival through the combined application of different treatment methods.[18]

3.1.2. Radiotherapy

RT is one of the most essential treatments against tumors, and about 70 percent of the patients suffering from cancer receive RT[19,20]. The application of RT was started with the discovery of X-rays by Wilhelm Röntgen and the isolation of the radioactive element radium in the 19th century by Marie Curie[21]. It was found that X-rays can be used to diagnose and treat diseases, and RT developed cancer treatment. With the advancement of science and technology, RT technology is undergoing continuous reform and innovation. One of the challenges of RT is how to ensure that tumors receive enough radiation doses while normal organs and tissues are not damaged. Simple X-ray tubes and cobalt-60 were initially used for tumor RT in the history of RT, but the disadvantage is that they cannot control the dosage of tumor sites and normal tissues well[22]. Medical linear accelerators are a great development for RT, which began to be put into clinical use in the 1950s[23]. With the development of computer and imaging technology, Three-Dimensional Conformal Radiation Therapy (3D-CRT) is becoming one of the most commonly used RT technology.[24] 3D-CRT gives oncologists a three-dimensional view of tumors and normal organs near the tumor, which helps oncologists map exactly where the tumor is.[25] In this way, 3D-CRT accurately keeps the shape of the radiation field consistent with the tumor shapes and decreases radiation injury to normal tissue. However, it was found that the dose distribution of tumors is very uneven when applying 3D-CRT technology. The dose distribution is not balanced and there is a risk of recurrence for tumors with low doses[26]. Therefore, adjusting the intensity distribution within the radiation field for the average dose within the tumors is necessary. It is dedicated to solving this problem with further developments in technology. Brahme et al. first proposed a model to solve this problem in 1982. This mode is called Intensity-modulated Radiation Therapy (IMRT), which involves sending many small radiation beams of different intensities into the host from many different angles depending on the shape of the tumors[27]. IMRT solves the problem of uneven radiation dose intensity in tumors and has become one of the most widely used RT technologies nowadays. This radiation technology allows tumors to receive sufficient radiation doses while normal organs are adequately protected. Based on the principle of adaptive intensity modulation technology, there are more technical innovations based on the needs of different tumors. Tomotherapy (TOMO) is a new type of IMRT equipment guided by computed tomography scan images. Compared with traditional

IMRT, TOMO has the advantages of higher accuracy, faster computing power, and higher tumor conformity and dose uniformity[28,29]. Volumetric modulated arc therapy (VMAT) is a new technological innovation[30]. VMAT can rotate and irradiate tumors within any angle range by adjusting the high-speed dynamic multi-leaf grating and variable gantry rotation speed[31]. Compared with the traditional RT technology, the irradiation range of VMAT is larger, more flexible, and more precise[32]. Another new RT technology, named stereotactic host radiation therapy (SBRT), uses high-dose fractionated radiotherapy to achieve a radical dose to eliminate tumors in just a few irradiations[33]. In addition to external radiotherapy, brachytherapy has become increasingly common in clinical applications.[34] Brachytherapy places a radioactive source inside or near the target site, and the radiation only affects a very limited area around the radioactive source. Therefore, the amount of radiation exposure to normal tissues far away from the radiation source can be reduced. Brachytherapy is mainly used for cervical cancer, prostate cancer, and breast cancer, etc[35]. In addition, research on proton and heavy ion radiotherapy has developed rapidly in recent years. Proton and heavy ion radiotherapy aims to use the Bragg-peak with high ionization density to adjust the high-dose distribution area to the tumor target area, thereby increasing the tumor dose and reducing damage to normal tissues and organs.[36] Protons and heavy ions are expected to become one of the most important RT methods.

The purpose of continuous innovation in RT technology is to improve the accuracy of RT so that the tumor receives sufficient radiation doses while ensuring the safety of surrounding normal tissues. Radiobiology is another important area of RT. The most important biological mechanism of RT is the damage of nuclear DNA caused by radiation directly or indirectly[37]. In the past decades, research in radiobiology mainly focused on the direct damage of RT on DNA[38]. DNA double-strand breaks induced by RT are the leading cause of cell death[39]. Current research consistently shows that cells in the mitotic phase are most sensitive to radiation[40]. Depending on the important characteristic that tumor cells divide and reproduce faster than normal cells, appropriate RT plans can be formulated based on the radiation damage repair of cells to achieve the best treatment effect[41]. Therefore, one of the focuses of RT research is improving the radiosensitivity of tumor cells. The pathological grade and cell circle of tumor cells impact the tumor radiosensitivity. It was proved that cells that proliferate actively are more sensitive than cells that do not proliferate, and cells with low differentiation are more radiosensitive[42]. In addition, it was also reported that hypoxia reduces the radiosensitivity of tumor cells[43]. Increasing radiosensitivity is also one of the focuses that oncologists have been studying. There are some radiosensitizers such as NBTXR3 and Trans Sodium Crocetinate (TSC) in clinical trials[44,45]. In addition, the impact of RT on the TME, especially on immune cells and other stromal cells, has received stronger attention,

and the combination therapies of RT and immunotherapy have gradually become one of the main researches on enhancing treatment effects[46].

3.1.3. Immunotherapy

Immunotherapy is a milestone in the history of cancer treatment. Surgery, CT and RT are the traditional treatment methods that kill tumor cells directly, while immunotherapy aims to treat tumors by activating the immune system[47]. The host's defense system comprises immune organs, cells, and inflammation-related cytokines[48]. Immune cells are generated, differentiated, developed, and matured in central immune organs, including bone marrow and thymus, and colonized and exerted immune response effects in peripheral immune organs, such as spleen, lymph nodes, and mucosa-associated lymphoid tissue[48]. The immune system primarily protects an organism from microbial infections and malignant transformation and is divided into the innate immune system and the adaptive immune system[49]. The innate immune system quickly responds to pathogens but not antigen-specific. Macrophages, neutrophils, and dendritic cells are the immune cells that primarily respond in the innate immune response, but they cannot produce long-term immunity[50]. The adaptive immune response is antigen-specific, with immune memory and a more robust immune response[51]. Antigen specificity is the key factor for the immune system's response to the specific pathogen of cancer cells[51]. Cancerous cells express tumor-specific antigens on their surfaces that can be identified and eliminated by the host's immune system. T lymphocytes (T cells) and B lymphocytes (B cells) are primary immune cell types and are very important in the immune response to identify and kill tumor cells in the immune system[52,53]. There are two subsets of T cells named $CD8^+$ killer T cells that mainly kill cancer cells and $CD4^+$ helper T cells((THs)) that indirectly activate the immune response by activating other immune cells[54,54]. Unlike CD8+ T cells, which directly remove malignant cells in the host, CD4+ T cells activate memory B cells and cytotoxic T cells, leading to a greater immune response[55]. T lymphocytes, especially their cytotoxicity against antigens, have been the key to research that engages the immune system against cancer depending on the characteristics of this immune system[56]. However, tumor cells escape the immune system's surveillance and develop into cancer mainly because of immune escape[57]. Cancer cells avoid being discovered by killer T cells when the distribution of major histocompatibility complex (MHC) class I molecules on the surface of tumor cells is less than that of normal cells[58]. There are also some cytokines released by some tumor cells that can also suppress immune responses[59]. In addition, immune tolerance may occur in response to tumor antigens by preventing the immune system from continuing to attack tumor cells[60]. Cancer immunotherapy is based on the specific antigens expressed on the surface of cancer cells that the immune system can recognize. Therefore, immunotherapy can stimulate and increase the immune response in the host. For example, Dendritic cell-based pump-priming

or vaccination helps initiate a cytotoxic response against antigen-expressing tumor cells[61]. T-cell adoptive transfer attempts to extract, culture and reinfuse T cells targeting tumor cells to eliminate cancer cells[62]. Checkpoint inhibitors target cancer's immune evasion system to help immune cells recognize and kill cancer cells[63].

Cancer immunotherapy, especially immune checkpoint blockade, has made revolutionary progress[64]. Anti-CTLA-4 and anti-PD-1/PDL-1 antibodies are the two checkpoint inhibitors currently available to patients in clinical[65]. Programmed cell death protein 1 (PD-1) is a protein receptor on the surface of T cells and B cells that suppresses the immune response to prevent autoimmune diseases and prevents the immune system from clearing malignant cells[66]. Programmed death-ligand 1 (PD-L1) is a transmembrane protein that can bind with PD-1 to reduce immune response[67]. Studies have found that PD-L1 is highly expressed in many malignant cells, which may be why many cancers have immune evasion[68]. PD-1/PD-L1 inhibitors can block the combination of PD-L1 and PD-1, thereby limiting the immune system's suppression and allowing the immune system to kill cancer cells [69].

3.1.4. Combination treatment of radiotherapy and immunotherapy

Although immunotherapy has revolutionized the field of cancer treatment, many questions remain unanswered. In addition, immunotherapy is rarely used permanently as a standalone treatment modality, and many patients resist treatment after receiving immunotherapy alone[70]. It is highlighted that it remains unclear why some patients who express the receptor ligand PD-L1 in their tumor tissues and PD-1 in their lymphocytes do not respond to therapy; and some patients who express neither ligand benefit from therapy[71,72]. However, some clinical studies have found that patients combining the treatment of RT with immunotherapy are more effective than those receiving immunotherapy alone. Willemijn S et al. reported that RT may change the tumor microenvironment of non-small cell lung cancer (NSCLC) to relieve immune suppression and enhance the effect of immunotherapy[73]. Tagliaferri L et al. found that combining RT and immunotherapy has positive implications for melanoma patients, especially those with metastatic metastases[74]. Herrera FG et al. found that patients with ovarian cancer also benefit from a combination treatment of immunotherapy and radiation therapy[75]. Altorki N et al. considered that the combination of immunotherapy and RT may be more clinically significant in the treatment of NSCLC[76]. The combination of RT and immunotherapy enhances the efficacy, but the mechanism is still unclear. Researchers are trying to elucidate this mechanism to explore better options for tumor treatment further. There are some explanations for this phenomenon as follows. First, the malignant cell death caused by RT triggers an inflammatory response to activate the immune response system[77]. Second, RT causes changes in the tumor microenvironment, which may produce some cytokines to promote the aggregation and transport of immune cells[78]. It is crucial to further

elucidate the impact of RT on the immune response system and tumor microenvironment to promote the advancement of cancer treatments.

3.2. The tumor microenvironment

3.2.1. The role of tumor microenvironment in tumor growth

Tumor contains a complex ecosystem containing cancer stem cells, blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, various signaling molecules, and extracellular matrix (ECM), which is called Tumor microenvironment (TME)(*Figure 1*)[79,80]. The TME plays an essential role in the cancerization formation and progression[81]. One of the critical factors for tumors to shape their microenvironment is that tumor cells have characteristics such as genetic changes, epigenetic changes, metabolic reprogramming, and abnormal signal release[82]. As a result, The TME supports tumor formation and growth. In addition, immune evasion is another key factor in tumor formation, which prevents immune cells in the TME from functioning normally[83]. Therefore, unlike the microenvironment of normal tissues, the TME has its unique characteristics. In normal tissues, some cancerous cells should be promptly eliminated by the immune system[84]. However, as a tumor-supportive microenvironment, the TME has blood vessels, and ECM remolded for tumor growth that is different from normal tissues and has an inhibitory effect on the immune response[82].

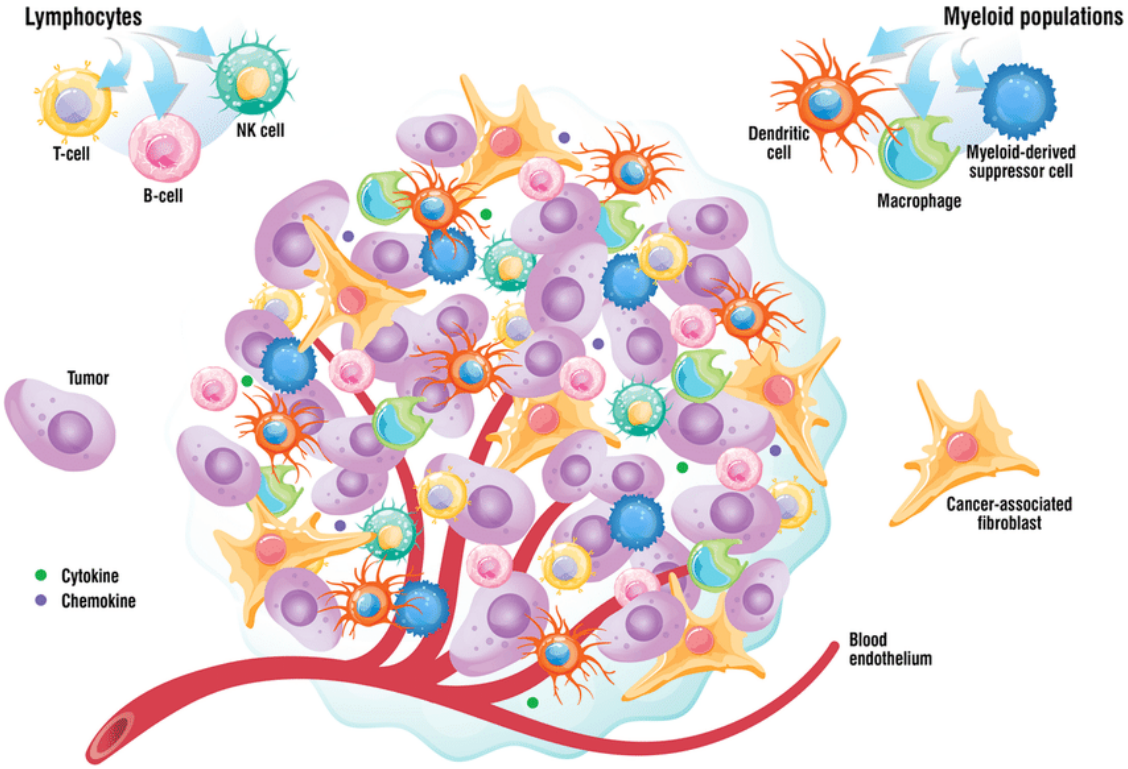


Figure 1: The main components of the tumor microenvironment. The TME contains an extracellular matrix and different types of cells. Cellular components include cancer cells, fibroblasts, endothelial cells, and immune cells. Endothelial cells form blood vessels that

provide nutrients for the growth of cancer cells and transport immune cells, including several subpopulations, such as B cells, T cells, macrophages, dendritic cells and NK cells. The complex tumor microenvironment can affect tumor growth through cell-to-cell contact and cytokines, chemokines, and other interactions[80]*. (Picture from Zhang J. 2022*[80]*)*

3.2.2. Immune cells in the tumor microenvironment

Immune cells are essential for the anti-tumor immune response in the TME. The host has an immune-promoting response and an immune-suppressing system[85]. This may be because the host needs an immune response to clear mutated cells and at the same time, it requires a corresponding immune-suppressing mechanism to avoid autoimmune diseases[86]. Due to the mutation of tumor cells, tumor suppressive mechanisms play a crucial role in helping tumor cells escape surveillance and clearance by the immune system in the TME[87].

T cells

Various kinds of immune cells play different roles in the TME. T cells are born in the bone marrow and mature into different subtypes of T cells in the thymus [88,89]. T helper cells (T_h cells) are T cells that express $CD4^+$ protein on the cell's surface, so they are also called $CD4^+$ T cells[90]. As its name suggests, helper T cells help other immune cells to exert their immune effects. T_h cells express T cell receptors (TCRs) that can bind with class Ⅱ major histocompatibility complex (MHC) expressed on the surface of antigen-presenting cells (APCs) such as macrophages, B cells and DCs to help activate the immune response[91]. There are different subsets of T_h cells, and they play various roles in the immune response. T_h1 helper cells lead to the immune response mainly by activating macrophages and $CD8^+$ T cells[92], and also secrete cytokines such as IFN-γ and IL-2 to trigger the immune response[93]. T_h2 helper cells work on humoral immunity and mainly activate B cells, mast cells and eosinophils[94], and primarily secrete cytokines such as IL-4, IL-5, and IL-13, and inverse triggered by these cytokines[94,95].

$CD8^+$ T cells are a kind of adaptive immune cell and play an important role in the adaptive immune response[96]. They express T cell receptors (TCRs) that can recognize a specific antigen produced by cancer cells and brought to the cell surface by class Ⅰ MHC molecules to prime the immune function and kill cancer cells[97]. $CD8^+$ T cells are mainly activated by APCs and $CD4^+$ T cells, and eliminate cancer cells by killing them directly and secreting cytokines. Some naïve $CD8^+$ T cells differentiate into tissue-resident memory T cells after elimination, and respond rapidly when the host recognizes a pathogen with the same antigen[96], which plays a crucial role in the immune response to cancer[98]. The resident $CD8^+$ T cells with tumor specific antigens are activated and respond to the recognized cancer antigen immediately and start the elimination of cancer cells. The activated memory $CD8^+$ T

cells implement immune function via several paths such as inducing apoptosis of cancer cells and releasing perforins, granzymes and cytokines to destroy tumor cells[99,98]. However, T cell function is considered suppressed in the TME so that the tumors formatted and continue to grow. The suppressive mechanism is complicated. First, regulatory T cells (Tregs) are an important subpopulation in immunosuppression[100]. The role of Tregs in the occurrence and growth of cancer is very complex. The Immunosuppressive function of Tregs can prevent the host from autoimmune disease but give cancer cells opportunities to escape from the immune response. Studies have found that the prognosis of many kinds of cancers is poor when there are many Tregs in the tumor microenvironment, which indicates that Tregs could be one of the critical points in cancer treatment[101]. Tregs can compete with effector T cells for the use of some cytokines, such as IL-2, and inhibit the action of some APCs to prevent the activation of effector T cells[102,103]. In addition, immune checkpoint molecules such as PD-1 and CTLA-4 are also expressed on the cell surface of Tregs, which promotes the occurrence of immunosuppression[104]. Second, some immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF) are secreted by malignant cells and tumor-associated macrophages in the TME, which can inhibit the antigen presenting effect of dendritic cells to suppress the immune response[105,106]. In addition, one subtype of neutrophils also shapes the immunosuppressive microenvironment of tumors[107].

B cells

B cells are another subtype of immune cells for humoral immunity. Although most of the topics of immunotherapy research focused on T cells, evidence showed that B cells also have the immune function to inhibit tumor growth[108]. There are two main types of tumor-infiltrating B cells in TME. Effector B cells mainly exert their anti-tumor effects through the following three pathways: first, effector B cells can kill the malignant cells directly with the function of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) to induce tumor cells apoptosis[109]; second, B cells promote T cell response by participating in antigen presentation together with Th1 cells and activating cytotoxic T lymphocyte (CTL)[110]; third, B cells kill cancer cells by secreting immunoglobulins such as antibody-dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC)[111]. The other subtype of B cells is regulatory B cells, which inversely promote tumor growth in TME[112]. Regulatory B cells express transforming growth factor beta (TGF-β) to regulate T cells to suppress the immune response of $CD4^+$ and $CD8^+$ T cells[113]. Another immunosuppressive mechanism that has been discovered is that regulatory B cells produce Interleukin 10 (IL-10) to promote the anti-inflammatory action by activating immunosuppressive myeloid derived suppressor cell (MDSC) and M2-polarized macrophage (M_2)[114] and preventing the anti-tumor effect of natural killer (NK) cells and effector T cells[115].

Tumor-associated macrophages

Macrophages are a type of myeloid immune cells that play a crucial role in the innate immune system and they exert innate immune effects that mainly rely on phagocytosis, which means that they can engulf and digest pathogens such as cancer cells, bacteria, dying cells and foreign particles to protect the host[116]. Macrophages are found in almost all tissues and can colonize strategic organs and tissues that are vulnerable to invasion by pathogens[117]. Tumor-associated macrophages (TAMs) are subpopulations of macrophages resident in the TME and play an important role in tumor-associated inflammation. There are two main subtypes of TAMs in TME depending on their different effects on tumor immunity: classically activated macrophages (M1) and alternatively activated macrophages (M2)[118]. M1 macrophages are responsible for the pro-inflammatory effect to defend against tumorigenesis in the host[119]. In contrast, M2 macrophages are proven to have the inverse function of activating anti-inflammatory response and promoting tumor growth[120]. The anti-tumor effect of M1 macrophages may be exerted by secreting some pro-inflammatory cytokines such as Interferon gamma (IFN-γ), Tumor necrosis factor alpha (TNFα) and Interleukin-1 beta (IL-1β), whereas M2 macrophages can be activated by some biological molecules such as Interleukin-4 (IL-4) and Transforming growth factor beta (TGF-β) to reduce inflammation and promote tumor growth[120].

Dendritic cells

DCs are a group of immune cells that have the function of antigen-presenting, which is not abundant in TME but essential in the tumor-associated immunologic process. Dendritic cells are mainly divided into different subsets according to other cell surface receptors, which play different roles in the immune process[121]. Plasmacytoid dendritic cells (pDCs) secrete abundant immune-associated cytokines mainly type I interferons (IFNs), which are capable of activating other immune cells to promote immune response[122]. Conventional type 1 dendritic cells (cDC1s) are considered to be specialized in antigen cross-presentation and mainly activate $CD8^+$ T cells[123]. Moreover, cDC1s can produce some pro-inflammatory cytokines such as IL-12, type I and type III IFNs [124]. Conventional type 2 dendritic cells (cDC2s) regulate immune response by activating not only different types of Th cells but also Treg cells and $CD8^+$ T cells, and researches demonstrate that cDC2s can produce immune-related cytokines such as IL-12 to stimulate the immune system[125]. There is another subset of DCs named monocyte-derived dendritic cells (MoDCs), and most vitro studies revealed that MoDCs may stimulate $CD8^+$ T cells T regs, and most types of Th cells[126].

Neutrophils

Neutrophils are a type of white phagocyte and the most abundant immune cells in the blood. Neutrophils are among the first responder cell populations in inflammatory conditions to

viruses, bacteria, and tumor cells, and they are an essential component of the TME[127,128]. However, due to the particularity of the TME, tumor-associated neutrophils have both the effect of inhibiting tumor growth and promoting tumor growth[129]. Neutrophils can migrate through blood vessels to sites of inflammation and kill tumor cells directly by phagocytosis[130].

3.2.3. Immune evasion in the tumor microenvironment

The formation of a tumor means that the malignant cells escape from the host's immune system. One reason for immune evasion is that the tumor inhibits the antigens on cancer cells, so the T cells can no longer recognize malignant cells and exert their cytotoxic activity[131]. The success of the immune checkpoint blockade verified this and provided a new direction for cancer treatment to avoid immune evasion. Some pro-tumor factors M_2 phenotypes express immunosuppressive cytokines IL-10 and TGFβ in the TME, promoting immune evasion formation and inhibiting the activation of anti-tumor immune response[132]. In addition, the TEM of immune cells also needs to be noticed. Immunotherapy activates the immune system and helps to increase effective immune cells. Still, the immune cells only play an anti-tumor role when they pass through vascular endothelium from blood to the tumor site[133]. The TEM of leukocytes happens when the ligands on immune cells combine with the receptors on the ECs[134]. Thus, with the development of immunotherapy, research on TEM is also vital.

3.3. Vascular endothelium and leukocyte transendothelial migration

3.3.1. The structure and function of blood vessels

Blood vessels are the circulatory system that transports blood throughout the whole host. Blood travels from the heart to carry oxygen, nutrients, and some molecules to tissues, bringing metabolic waste back to the heart[135]. There are five types of blood vessels according to their different structures and functions. Arteries are the most flexible blood vessels that transport oxygenated blood away from the heart, and arterioles are small branches extending from arteries[136]. Capillaries are the smallest blood vessels in the host and act as a bridge between arteries and veins. Capillaries are where nutrients, oxygen, and wastes are exchanged and are one of the most basic components of the microenvironment of tissues and organs[137]. Venules are the smallest veins that receive deoxygenated blood from capillaries and are the branches of veins. Veins bring deoxygenated blood back to the heart to prepare for the next circulation[138].

There are three basic structures of all the blood vessels except capillaries despite different functions and idiographic structures of varying blood vessels: tunica intima, tunica media and tunica externa (*Figure 2*). Tunica intima mainly comprises a layer of endothelial cells (ECs), the basis of blood vessels found throughout the vascular system. ECs are bonded by a polysaccharide intercellular matrix and are surrounded by a basement membrane to support

the cells. The tunica media mainly comprises polysaccharides, elastic fibers, and connective tissue arranged in a ring, primarily for regulating blood vessels' inner diameter. The tunica externa comprises connective tissue that protects and supports blood vessels. It also contains nerves that provide nutrition to the blood vessels[137]. Unlike other blood vessels, capillaries are the smallest blood vessels in the host. Capillaries comprise only a thin layer of endothelial cells, which serve as a bridge for the exchange of substances between blood and surrounding tissues and cells[138]. Capillaries can provide nutrients to surrounding tissues and cells, transport metabolic waste away, and regulate the local environment. At the same time, the function of ECs is also controlled by the microenvironment[139]. Therefore, ECs play a key and complex role in the TME.

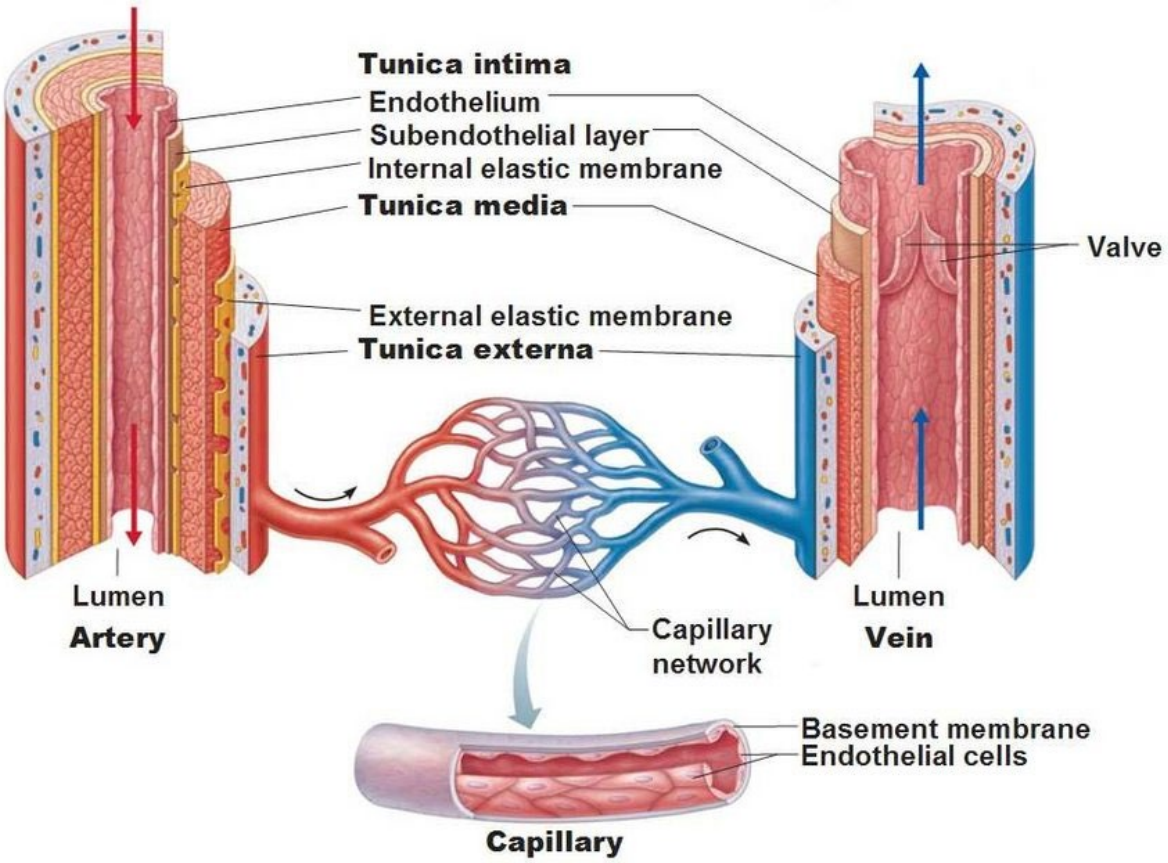


Figure 2: The structure of blood vessels. Blood vessels are mainly divided into arteries, veins, and capillaries. Arteries and veins comprise tunica intima, tunica media, and tunica externa. Tunica intima primarily includes a layer of endothelial cells; tunica media is mainly composed of polysaccharides, elastic fibers, and connective tissue arranged in a ring; tunica externa is composed of connective tissue. Capillaries are primarily composed of endothelial cells and basement membrane[140]. (Picture from Bhuiyan. 2019[140].)

3.3.2. Leukocyte transendothelial migration and adhesion molecules on the endothelial cells

Leukocytes need to migrate across vascular endothelium to enter the tumor site to exert their anti-tumor immune function, so TEM of leukocytes is essential for the effectiveness of immunotherapy. Leukocyte TEM mainly refers to the barrier and transport capacity of ECs[141]. ECs are a single layer of cells located on the inner wall of vessels and the only cell layer of capillary, which facilitate the metabolic exchange of plasma and interstitial fluid and secrete a variety of biologically active substances to maintain the microenvironment of vessels and surrounding tissues[142,143]. Almost all tissues in the host need a blood supply, which provides nutrients and oxygen to the tissue[144]. The ECs form the linings of the blood vessels and regulate exchanges of nutrients and wastes between the blood vessels and tissues[145]. The ECs are involved in many different functions in the microenvironment of tissues, including hemostasis, vascular permeability, vascular and tension regulation, transport of blood cells, and participation in immune response[146].

ECs play an important role in the host, especially during immune response when damage occurs in the tissue[147,148]. The regulation of blood cell fluidity and vascular permeability is closely related to the immune response[134]. The ECs form a semi-permeable barrier between the blood and tissues and regulate the migration of substances into and out of blood vessels through different mechanisms[149]. Fluids and small molecules cross the vascular barrier through the paracellular pathway, while macromolecules cross the blood barrier through the transcellular pathway or paracellular pathway[150]. Small molecules can pass the blood barrier directly through the gaps between the endothelial cells, while normal molecules cannot go directly[141]. However, in some conditions, such as when inflammation, injuries, tumors, and vascular diseases occur, certain cytokines are released from surrounding cells and cause ECs shrinkage and permeability changes[151]. The transcellular pathway mainly depends on the receptors expressed on the surface of blood cells and ECs. Many signaling molecules are presented on the surface of the ECs, pairing with specific receptors on the surface of blood cells, which can help blood cells transmigrate from the blood vascular into the tissues[152].

The immune system is activated when the host is attacked by foreign pathogens or cancer cells are detected. Leukocytes flow in the blood, and they are stimulated and recruited to the inflammatory sites when the host detects damage, foreign pathogens, or cell malignancy and undergoes an inflammatory response[153]. Whether innate immunity or adaptive immunity, the infiltration of immune cells through the blood vessel wall is an essential process. ECs are activated in the microcirculation of inflamed tissues, and there are some cell adhesion molecules and receptors on the cell surface of ECs and leukocytes that interact with each other to help leukocytes migrate into the inflammatory sites[154,146]. The recruitment of

leukocytes is a complex and effective process. There are several steps for leukocytes to pass through the blood vessel wall and reach the target inflammation sites when they are activated: rolling, arrest, crawling, and migration (*Figure 3*).

Leukocyte tethering and rolling

The first step in this process is that the activated ECs capture the flowing leukocytes to slow down, tethering, and then move along the vessel wall rotationally, termed rolling[155]. Leukocyte tethering and rolling are the initial interactions between immune cells and activated ECs in the microcirculation of inflamed tissues, and this process mainly depends on the selectins expressed on ECs[156]. The selectins are a family of Ca^{2+}-dependent cell adhesion molecules containing P-selectin(CD62P) and E-selectin(CD62E) [157]. Rodrigues RM, et al. found that E-selectin plays a crucial role in inducing the recruitment of neutrophils, thereby promoting the occurrence of inflammation[158]. Perkins LA et al. proposed that P-selectin is closely related to endothelial cell activation and the development and progression of different inflammatory diseases. It also proposed its potential as an imaging biomarker and therapeutic target[159]. Thus, leukocyte tethering and rolling mainly depend on the combination of P-selectin glycoprotein ligand-1(PSGL-1) expressed on the surface of leukocytes and selectins such as P-selectin and E-selectin on the surface of stimulated ECs, and this procedure is vital for the inflammation response[160].

P-selectin is a transmembrane protein stored in platelet α-granules and in the Weibel-Palade bodies (WPBs) of ECs[161]. P-selectin comprises three components: a carbohydrate-recognition domain in the N-terminus, an epidermal growth factor-like (EGF-like) domain, and a domain with several short consensus repeats(9 units)[156,162]. When the host is under inflammatory conditions, the endothelial cells in the microcirculation of the inflammatory site are activated, and the P-selectin stored in WPBs is rapidly released to the cell surface of ECs[163]. P-selectin expressed on the surface of activated ECs can bind to PSGL-1 expressed on the surface of leukocytes in the inflamed microcirculation [164]. The binding between P selectin and PSGL-1 causes leukocyte rolling, the initial part of leukocyte TEM. It has been verified that the combination of P-selectin on ECs and PSGL-1 on leukocytes mediates tethering and leads to leukocyte rolling on stimulated ECs[161].

E-selectin is another type of selectin cell adhesion molecule with a structure similar to P-selectin but with a different number of short consensus repeats(6 units)[162]. E-selectin is expressed on the surface of stimulated ECs and can bind to E-selectin ligand complexes such as PSGL-1 expressed on leukocytes[165,166,167]. It is essential for the connections between E-selectin on ECs and specific carbohydrate ligands such as E-selectin ligand-1 (ESL-1), PSGL-1, and CD44 present on leukocytes for regulating the adhesion and recruitment of leukocytes to vascular endothelium under inflammatory conditions[168,169]. The expression level

of E-selectin is increased when the immune system is activated to protect the host from damages[170,171]. Wiese et al. observed the value and importance of E-selectin-E-selectin specific ligands for leukocyte tethering and rolling on the surface of endothelium with parallel-plate flow chamber[172].

In a word, leukocyte rolling is the first step in immune cells' response to inflamed tissues. P-selectin and E-selectin expressed on the cell surface of activated ECs are crucial in this process by combining with their ligands on leukocytes. Therefore, the expression levels of P-selectin and E-selectin are important for activating the TEM of leukocytes.

Leukocyte arrest and adhesion

After a short rolling period along the blood vessel, TEM begins the next phase: arrest. During rolling, leukocytes are tethered by activated endothelial cells and may be induced to flatten their morphology, providing conditions for further interaction between leukocytes and ECs[173]. What's more, stimulated by the microenvironment of inflamed sites and affected by the interactions between selectin adhesion molecules and their ligands, some integrins on leukocytes are activated by chemokines and devoted to leukocyte arrest[174]. Although the mechanism of TEM is still under investigation, it is generally accepted that the interaction of integrins on leukocyte and their receptors on ECs is a crucial process[175]. Integrins, obligate heterodimers composed of eighteen α and eight β subunits in mammals, are a type of membrane protein that plays an important role in cell-extracellular matrix and cell-cell adhesion[176]. According to existing research results, it is known that several subtypes of integrins expressed in different types of leukocytes bind to adhesion molecules on ECs, which leads to slowing down the rolling speed of leukocytes and arresting them to the blood vessel for migration[177]. The integrin lymphocyte function-associated antigen 1 (LFA-1) is a heterodimeric glycoprotein found on lymphocytes, T cells, and monocytes. Intercellular adhesion molecule 1 (ICAM1) is a well-known LFA-1 ligand expressed on the surface of ECs[178]. ICAM1 expressed on ECs is one of the main ligands that affect the TEM of immune cells[179]. Mac-1 can also bind to ICAM-1 present on ECs to slow the rolling of leukocytes and lead to the arrest of leukocytes[180]. The integrin very late antigen-4(VLA-4 or α4β1) is mainly expressed on the cell surface of T cells, B cells, NK cells, and other leukocytes but not neutrophils, and its appropriate ligand on ECs is mainly vascular cell adhesion molecule 1 (VCAM1)[181]. Integrin macrophage-1 antigen (Mac-1 or αMβ2) is mainly found on the cell surface of B cells, T cells, neutrophils, NK cells, and macrophages that bind to ICAM1 to promote the leukocyte recruiment[180,182].

The binding between integrins on leukocytes and adhesion ligands on ECs are crucial in the TEM of leukocytes. Therefore, the expression level of adhesion ligands on the surface of ECs should be another key factor affecting the transmembrane migration of immune cells.

ICAM1, also known as CD54, is an adhesion receptor that is a glycoprotein expressed on the surface of ECs and has been proven to regulate leukocyte recruitment from circulation to the inflammatory sites[183]. ICAM1 consists of the single transmembrane domain, amino-terminus extracellular domain, and carboxy-terminus cytoplasmic domain[184]. It is expressed at a low basal level in endothelial, epithelial, and immune cells but up-regulated on ECs when there is an inflammatory stimulation[185]. VCAM1, also known as CD106, contains six or seven immunoglobulin domains and is another important cell adhesion molecule expressed on the cell surface of ECs. When ECs are stimulated in the inflamed microenvironment, vcam1 expression is upregulated and binds to VLA-4 on immune cells to promote the recruitment and migration of leukocyte[186].

The interaction between adhesion molecules on ECs and integrins on leukocytes leads to the leukocytes' recruitment to the blood vessels. The binding of P-selectin and E-selectin to their receptors on leukocytes allows immune cells flowing in the blood to be captured by activated ECs. The combination of ICAM1 and VCAM1 with integrins allows immune cells to roll along the blood vessel, and their morphology becomes flat and then crawls slowly along the blood vessel. This is the process of recruiting immune cells to the inflamed sites that play a crucial role in the inflammatory response.

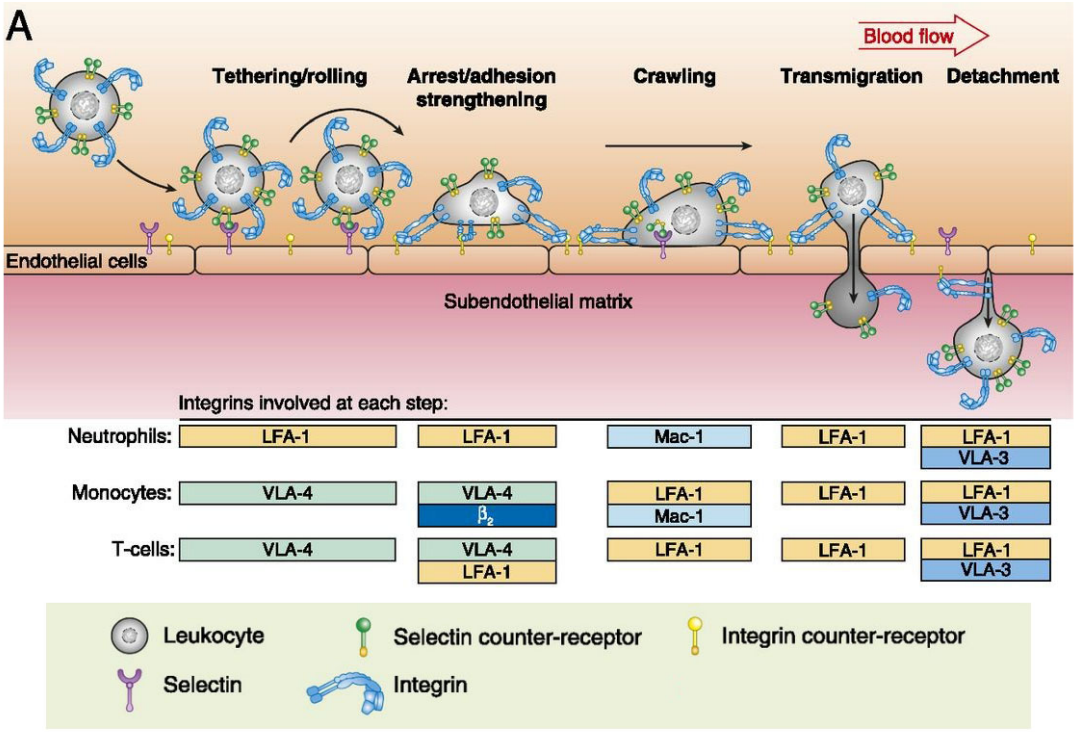


Figure 3, Process of leukocyte recruitment and trans-endothelial migration. Leukocytes recognize selectins and begin to roll downward, then move retrogradely along the endothelial surface to find a suitable transfer point, and then complete the transfer from the endothelium

to the outside through a series of interactions with adhesion molecules of endothelial cells and surrounding cells[148]*. (Picture modified from Herter and Zarbock. 2013.)*

Leukocyte crawling and transendothelial migration

The leukocyte recruited to the blood vessels slowly crawls through the blood vessel walls and reaches the sites of inflammation. Though the mechanism of TEM is complex and still has many problems to solve, it is widely accepted that there are mainly two ways for macromolecules to move across membranes: paracellular and transcellular[187]. Intercellular junctions between adjacent ECs are an important structural basis for maintaining normal vascular barrier function and controlling leukocyte infiltration. The main structure of the adhesion link between ECs is a complex composed of vascular endothelial cadherin (VE-cadherin), p120-catenin, β-catenin, γ-catenin, and α-catenin[188]. It is thought that upon stimulation by a complex inflammatory microenvironment, the connections between endothelial cells loosen and promote immune cell extravasation[133], and catenin primarily connects cadherins to actin filaments[189]. Paracellular migration of immune cells suggests that leukocytes need to pass through in-between adjacent ECs to cross the blood vessel wall. This means that a transient loosening of the connections between endothelial cells is necessary.

It is considered that the binding of integrins and adhesion molecules on ECs, especially ICAM1, can activate numerous intracellular signaling pathways in ECs, which in turn induce the remodeling of the EC actin cytoskeleton and cell junctions[190]. This process weakens the junctions between ECs, thus paving the way for paracellular TEM. The Rho family of GTPases, a family of small signaling G proteins, is a key mediator of actin cytoskeleton remodeling to regulate the formation of cell-cell adhesions[191], and participates in regulating multiple signal transduction pathways in eukaryotic cells[192]. It is confirmed that ICAM-1 can stimulate Rho GTPases and change actin cytoskeletal organization to loosen the intercellular junction[193]. In addition, calcium ions are known as second messengers in intracellular signal transduction[190]. Sandrine et al. reported that ICAM1 can induce an increase in intracellular calcium ion levels, thereby activating the tyrosine phosphorylation of related actin and the rearrangement of the cytoskeleton between endothelial cells, promoting the exudation of molecules out of the vascular wall through the paracellular pathway[194]. VE-cadherin is another major transmembrane protein that acts as a barrier to the transmembrane movement of leukocytes via the paracellular pathway. It is generally believed that tyrosine phosphorylation of cadherin is an important mechanism for inducing the disintegration of endothelial cell-cell junctions[195]. Allingham et al. reported that VE-cadherin-associated tyrosine kinase can be specifically recruited to the site of ICAM-1[196].

Turowski et al. found that ICAM-1-mediated VE-cadherin phosphorylation in brain microvascular cells was also positively correlated with paracellular TEM of lymphocytes[197]. Researchers recently found that VCAM1 has a similar function in promoting paracellular TEM. Reactive oxygen species (ROS) are appreciated as a second messenger in intracellular events such as cell proliferation[198] and inflammation[199]. ROS can activate effector proteins that regulate actin polymerization, such as hsp27, to induce cytoskeletal rearrangement[200]. Researchers found that binding ICAM1 or VCAM1 to leukocytes positively correlates with ROS level[201,202]. Therefore, ICAM1 and VCAM1 are essential players in the paracellular pathway TEM.

Leukocytes can also migrate via the transcellular pathway, meaning that leukocytes do not need to change the cytoskeletal structure of connected endothelial cells but can directly pass through individual endothelial cells. Currently, the widely accepted hypothesis for the mechanism of transcellular TEM is that leukocyte transcellular migration is achieved through the fusion of podosomes with ECs[203]. Although the mechanism of transcellular migration and its impact on inflammation remains controversial, studies have shown that blood vessels in the microcirculation are more supportive of transcellular migration than macrovascular ECs, which means that the research of transcellular TEM is also meaningful in the tumor immune response[204,203]. Yang et al. revealed that the increased expression level of ICAM1 was positively correlated with the occurrence of leukocyte transcellular TEM through an in vitro flow model[205].

From the known mechanisms of TEM, we can conclude that the expression levels of the adhesion receptors on the surface of ECs are important for the migration from blood vessels to the sites of inflammation, which is essential for both the innate and adaptive immune system response to protect the host from damage. Thus, the stimulation of ECs and the expression levels of adhesion molecules on ECs may be critical factors in promoting the effect of immunotherapy.

3.3.3. Cytokines regulate the immune process

From the mechanism of TEM of leukocytes, it is clear that immune cells normally circulate in the blood, migrate through the vessels to the damaged or transformed area, and restore the balance only when the host has an injury, acute inflammation, and transformed malignant cells[206]. During immune cell migration into inflamed tissues, ECs play an essential role. When an injury occurs, endothelial cells are activated and upregulate adhesion molecule expressions like selectins on the surface[207]. E- and P- Selectins can bind to the ligands on the surface of the leukocytes, which results in the leukocytes rolling along the endothelial surface. ICAM1 and VCAM1 are mainly adhesion molecules on the surface of endothelial cells that bind to integrins on leukocytes to slow the leukocyte down further[208]. The immune cells then crawl slowly along the adjacent endothelial cells and cross the basement

membrane into surrounding tissues, and adhesion molecules may also promote the TEM process[209].

Therefore, how to activate and inhibit endothelial cells and regulate the expression levels of ECs adhesion factors is critical for promoting or inhibiting immune responses, and it is also one of the research focuses of tumor treatment in recent years. ECs in the inflamed sites can be activated by inflammation-related cytokines such as interleukins (ILs), interferon gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNFα) to regulate the activation of leukocyte and ECs[210,211,212]. Inflammatory cytokines secreted by various types of cells play an important role in the immune response.

Interleukins

ILs are a family of cytokines that contain more than fifty members and are secreted by leukocytes and many other host cells[213]. They have complex functions in the immune responses. The IL-1 family consists of 11 members(IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ, IL-37 and IL-38) due to their similar gene structure, of which IL-1α and IL-1β are the most important and well-known inflammatory cytokines[214]. IL-1 was first reported in the 1940s because of its relation with fever and was proved to activate lymphocytes in the 1970s[215,216], and has since become a research hotspot in inflammation and immunity. In the 1980s, IL-1 was discovered to consist of two different proteins named IL-1α and IL-1β[217]. Different from IL-1β, the structure of IL-1α is unique because it does not have a complete single peptide fragment before it becomes mature by removing N-terminal amino acids[218]. IL-1 is produced by monocytes, macrophages, B cells, ECs and DCs, and many other cell types[219]. Both IL-1α and IL-1β share the same receptors, such as the Interleukin-1 receptor (IL-1R), and play important roles in promoting the process of inflammation and also have an increased expression in tumors[220]. IL-1 has been known to participate in the differentiation of T_h cells and activate and enhance the function of immune cells such as T cells, B cells, and NK cells[221]. However, there is some difference between IL-1α and IL-1β. IL-1α is more important for T cell priming[222], while IL-1β is preferred to induce fever[223]. Furthermore, Schunk et al. reported that it is IL-1α but not IL-1β helps to promote the adhesion between leukocytes and ECs and upregulate the expression of VCAM1 on ECs[224]. Many other types of IL families also participate in the immune process. IL-6 produced by macrophages, Th cells, B cells, and endothelium can activate T cells, B cells, and plasma cells[225]. IL-17 secreted by T cells works on epitheliums, neutrophils, ECs, and others to promote immune response[226]. On the contrary, some other members of the IL family have an opposite effect on inflammation. IL-10 regulates inflammation by inhibiting the activity of leukocytes such as macrophages, NK cells, and Th1 cells. Thus, IL-10 is considered an anti-inflammatory cytokine[227].

Tumor necrosis factor alpha

Tumor necrosis factor alpha (TNF-α) is a type of transmembrane protein named because scientists discovered that as a cytokine produced by the host, it promotes tumor cell necrosis[228]. TNF-α is produced by various types of cells such as macrophages, monocytes, T cells, and fibroblasts. Two types of receptors have been discovered now, named Tumor necrosis factor receptor 1 (TNFR1) and Tumor necrosis factor receptor 2 (TNFR2)[229]. TNFR1 is expressed on almost all the cells in the host, while TNFR2 is mainly expressed on immune cells and ECs[230]. The binding of TNF-α and TNFR1 results in the signal pathways that induce cell apoptosis and activate immune response[231]. The signal pathway is initiated derived from TNFR1 because the death domain mainly exists in TNFR1, and the TNFR-associated death domain (TRADD) can recruit TNFR2 and receptor interacting protein kinase 1 (RIP1) to form the intracellular signaling complexes. As a result, the intracellular signaling complexes, mainly TNFR2, can activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which can upregulate genes involved in T cell development, maturation, and proliferation[232]. In addition, TNF-α also regulates the expression of adhesion molecules to participate in the regulation of leukocyte recruitment[233].

Interferon gamma

Interferon gamma (IFN-γ) is a cytokine deeply involved in both innate and adaptive immunity by activating Janus activated kinase (JAK)–signal transducer and activator of transcription (STAT) signaling pathway, which is a chain of interactions that participate in various processes such as cell death and immunity[234]. IFN-γ is secreted by many types of immune cells, particularly NK cells, $CD4^+$ Th1 cells, and $CD8^+$ cytotoxic cells, and binds to the receptor that is composed of two subunits: interferon receptor type 1(IFNR1) and interferon receptor type 2 (IFNR2). IFNR1 and IFNR2 are expressed on almost all kinds of cells in the host and separately associate with JAK1-STAT and JAK2-STAT signal pathways to activate immune response by promoting the production of immunity-boosting factors such as antigen-presenting molecules, chemokines, and chemokines[211]. It is proved that IFN-γ can stimulate the immune function of macrophages, Th cells, and Treg cells and inhibit tumor cell proliferation directly by activating APCs and upregulating MHC expression[235,236]. IFN-γ stimulates the ECs as well, Ng et al. reported that IFN-γ increases the expression level of VCAM1 and the leukocyte recruitment[237].

Granulocyte-macrophage colony-stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that acts as a white blood cell growth factor produced by activated T cells, macrophages, NK cells, ECs, mast cells, fibroblasts, and malignant cells. As its name suggests, the primary function of GM-CSF is to promote leukocyte growth by stimulating the stem cells to produce

granulocytes that conclude neutrophils, eosinophils, basophils, and monocytes which migrate into inflamed sites and mature into dendritic cells and macrophages[212]. The granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) is mainly in eosinophils, neutrophils, and monocytes. It comprises two subunits known: a low-affinity binding α-chain and a signaling β-chain that is also expressed on IL-3 and IL-5 receptors[238]. The bind of GM-CSF and GM-CSFR activates four signal pathways as already known: the JAK2/STAT5 signal pathway, the mitogen-activated protein kinase (MAPK) signal pathway, the phosphatidylinositol 3-kinase (PI3K) signal pathway, and the NF-kB signal pathway[239]. In the TME, GM-CSF may stimulate DCs and enhance immune cell recruitment to increase the anti-tumor immune response and suppress tumor growth[240].

3.3.4. The particularity of tumor vascularly environment

The immune system can identify and destroy abnormal substances in our host to protect proper host function[241]. Cancer cells are also abnormal in the host, and the immune system usually eliminates them usually, especially T lymphocyte cells[242]. However, tumors can cause dysfunction in the tumor's vascular environment, leading to immune response failure and escape from immune surveillance (*Figure 4*). Tumor cells can promote angiogenesis, and the newly formed blood vessels provide nutrients for tumor growth. However, the expression of adhesion factors such as ICAM1 on vascular ECs in the TME is inhibited, thereby leading to the adhesion dysfunction of immune cells in the blood to endothelial cells[243].

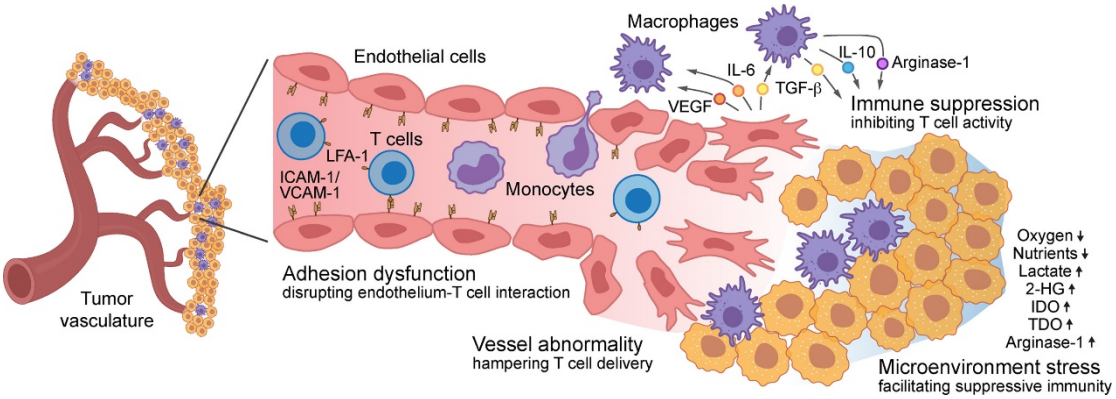


Figure 4, Pro-immunity and anti-immunity factors in TME. Many factors affect the TME and the anti-tumor immune response. Adhesion molecules on the surface of endothelial cells are vital for the transendothelial transport of immune cells. Some cytokines, such as IL-6, can promote immune responses, while other factors, such as IL-10 and hypoxia, can inhibit immune responses[244,245]. (Picture from Lamplugh and Fan. 2021[245].)

Anti-inflammatory cytokines such as IL-10 and TGF-β are produced in the TME to inhibit T cell activity, resulting in immune suppression[246]. Hypoxia and nutrient depletion occur in tumor blood vessels, which may be due to the rapid proliferation and high metabolism of

tumors. The hypoxic environment changes the behavior of cancer cells and drives VEGF upregulation to promote tumor progression further[247]. In addition, the accumulation of some immunosuppressive metabolites and enzymes such as lactate, 2-Hydroxyglutarate (2-HG), and arginase 1 in TME further inhibits the immune response to tumors[248,249,250]. Scientists proposed the immunotherapy concept based on the immune system's function against tumors, which should consider the whole TME. One of the most critical challenges in immunotherapy is destroying tumors' "immune escape" behavior and immunosuppression effect and promoting the recruitment and crossing of immune cells to the tumor[251]. The vascular microenvironment is essential in regulating tumor immune response. The function of vascular endothelial cells is closely related to the migration of T cells. The adhesion molecules, including P-selectin, E-selectin, ICAM1, and VCAM1, are vital to the T cell's recruitment and transport, and the downregulation of adhesion molecules expression will prevent the activation of vascular endothelial cells, which will reduce the recruitment and migration of T cells, resulting in immunotherapy failing to achieve the expected therapeutic effect[244]. What's more, some anti-inflammatory cytokines, including VEGF, IL-6, and IL-10, also change the structure and function of vascular endothelial cells, preventing vascular endothelial cell activation[252].

3.4. Objective of this thesis

The directions of tumor treatment can be roughly divided into two parts: directly killing cancer cells, such as surgery, RT, CT, etc., and promoting the host's immune system to recognize and kill cancer cells through immunotherapy. From the immune response process perspective, the main factors affecting the effect of immunotherapy are the activation of immune cells and the promotion of TEM of immune cells. Immunotherapy that has been used in clinical practice, such as immune checkpoint inhibitors, inhibits the immune escape of tumor cells and increases the immune activity of immune cells against malignant cells. However, it is still unclear why some patients who express the receptor-ligand PD-L1 in their tumor tissue and PD-1 in their lymphocytes do not respond to therapy. Several studies have shown that the combination of RT and immunotherapy has a promoting effect[253,254,255]. Thus, we consider that RT has a positive effect on the recruitment and TEM of T cells. The mechanism may be achieved by regulating adhesion factors on the surface of ECs and the cause of the release of inflammation-associated cytokines in the TME. We hypothesized that RT could directly affect the expression levels of P-selectin, E-selectin, ICAM1 and VCAM1 on ECs or affect the levels of anti-tumor inflammatory cytokines in TME. This experiment aimed to investigate the effects of RT on adhesion molecules on the surface of ECs. In this aim, we used mouse models to explore the impact of RT on the expression levels of P-selectin, E-selectin, ICAM1, and VCAM1 on ECs of vascular and inflammatory cytokines in TME in the

in vivo experiments and analyzed the direct influence of irradiation on ECs using bEND5 cell line in the *in vitro experiments*.

4. Material and Methods

4.1. Materials

4.1.1. Equipment

Equipment	Company
Cell Culture Centrifuge	HERMLE Labortechnik GmbH
CTX Connect Real-Time System	Bio-Rad
Incubator	Axon Labortechnik GmbH
Light microscope CKX41SF	Olympus
Magnetic holder	Miltenyi
Microplate reader	HIDEX Sense
NanoDrop	Thermo Scientific
T100 Thermal Cycler	Bio-Rad
Table Top Centrifuge	LMS
Vortex	LMS
Fluorescence Microscope	Leica Biosystems

Table 1: Equipment

4.1.2. Laboratory and cell culture material

Product	Company	Serial-No.
6-well tissue culture plate uncoated	VWR Collection	734-2777
6-well tissue culture plate TC-treated	VWR Collection	734-2323
12-well tissue culture plate	VWR Collection	734-2324
24-well tissue culture plate	VWR Collection	734-2325
Cell culture centrifuge	HERMLE Labortechnik GmbH	Z 216 MK
Cell culture dishes 100x20 mm	Corning	430167
Cell culture incubator	Axon Labortechnik GmbH	MCO-170AICUVH-PE
Centrifuge tubes Falcon® 15mL	Corning Life Sciences	734-0352
Centrifuge tubes Falcon® 50mL	Corning Life Sciences	734-0343

Coverslips	VWR	631-0153
Cytoflex S	Beckman Coulter	BA52174
Nanodrop 1000	Thermo Scientific	
Magnetic columns	Miltenyi	130-042-401
Magnetic holder	Miltenyi	130-042-303
Magnetic stirrer with heating	Carl Roth	EYE7.1
Neubauer Chamber	Lab medic (Marienfeld)	1110459
Precision X-Ray	Accela	MultiRad 160
Serological pipettes Falcon® 5mL	Corning Life Sciences	734-0350
Serological pipettes 5mL	Sarstedt AG & Co., Nümbrecht	861.253.001
Serological pipettes Falcon® 10mL	Corning Life Sciences	734-0352
Serological pipettes 10mL	Sarstedt AG & Co., Nümbrecht	861.254.001
Serological pipettes Falcon® 25mL	Corning Life Sciences	734-0343
Serological pipettes 25mL	Sarstedt AG & Co., Nümbrecht	861.685.001
Strips of 8 PCR tubes with attached flat individual caps	VWR	211-2613
Thermomixer	Eppendorf	5437

Table 2: Laboratory and cell culture material

4.1.3. Buffers

Solutions	**Ingredients**	**Volume/Concentration**
cDMEM（Bend 5 cells）	DMEM FBS NEAA Sodium pyruvate Penicillin/Streptomycin	435 ml 10% 1% 1% 1%
MACS Buffer	PBS (w/o Ca, Mg)	500 ml

	BSA EDTA	0.5% 2 mM
cDMEM(KP983.3 cells)	DMEM FBS Penicillin/Streptomycin	445 ml 10% 1%
Endothelial growth medium (EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™)	EBM™-2 Basal Medium FBS, Hydrocortisone, hFGF-B VEGF R3-IGF-1 Ascorbic Acid hEGF GA-1000 Heparin	500 mL 10 mL 0.20 mL 2.00 mL 0.50 mL 0.50 mL 0.50 mL 0.50 mL 0.50 mL 0.50 mL 0.50 mL
Mowiol Mounting Medium	TRIS HCL pH 8,5 (2M) Glycerol Mowiol 4-88	24 % 12 % 9,8 %
Tumor Lysis Buffer	PRMI FBS Collegnase 2 DNase	90% 10% 100ul/ml 25 µg/mL

Table 3: Buffers

4.1.4. Solutions and materials

Solution	**Company**
CD31 Microbeads	Miltenyi
CFA	Thermo Fisher
Chloroform	Carl Roth
Collagenase type 2	Sigma
Colloidal commasie blue	Sigma
DMEM	Life Technologies
DMSO	ITW
DNAse I from bovine pancreas	AppliChem
D-PBS, Dulbecco's Phosphate Buffered Saline	Life Technologies

EDTA	Sigma
Ethanol	Carl Roth
FBS (fetal bovine serum)	Life Technologies
Isoflurane	Piramel
Isopropanol	Carl Roth
PBS, DULB (1X)+CA,+MG (CE) 500ML	Life Technologies
Penicillin/Streptomycin	Life Technologies
RPMI	Life Technologies
Sodium Pyruvate	Lonza
TRIzol	Ambion (Life Technologies)

Table 4: Solutions and materials

4.1.5. Antibodies

Target	**Clone**	**Fluorophore**	**Company**
Zombie green		FITC	Biolegend
Zombie NIR		APC/Cy7	Biolegend
CD31	390	PE-Dazzle594	Biolegend

Table 5: Antibodies used in the CD31 positive cells' sorting

4.1.6. Antibodies for immunofluorescence

Product	**Host**	**Company**	**Clone**
AlexaFluor555 anti rat	Donkey	Abcam	ab150153
AlexaFluor647 anti mouse	Goat	BioLegend	Poly4053
AlexaFluor488 anti rabbit	Goat	Abcam	Polyclonal
AlexaFluor647 anti rabbit	Donkey	BioLegend	406414
E-selectin	Rat	BioXCell	9A9
ICAM-1	Mouse	Santa Cruz	G-5
P-selectin	Rat	BD Biosciences	RB40.34
VCAM-1	Rat	BioXCell	M/K-2.7
CD31	Rabbit	Abcam	Ab28364

Table 6: Antibodies used in the immunofluorescence experiments.

4.1.7. Primers

Gene	Forward primer	Reverse primer
E-selectin	AGCTACCCATGGAACACGAC	AAGTGGTGTTCGGACCAAAG
GAPDH	TCACCACCATGGAGAAGG	GCTAAGCAGTTGGTGGTGCA
ICAM-1	TTCACACTGAATGCCAGCTC	GTCTGCTGAGACCCCTCTTG
ICAM-2	ATCAACTGCAGCACCAACTG	ACTTGAGCTGGAGGCTGGTA
P-selectin	GTCCACGGAGAGTTTGGTGT	AAGTGGTGTTCGGACCAAAG
VCAM-1	CCCAGGTGGAGGTCTACTCA	CAGGATTTTGGGAGCTGGTA

Table 7: Real-time primers used in the qPCR assays

4.1.8. Reagents

Reagents	Company
Accutase® Cell Detachment Solution	BioLegend
Bovine serum albumin (BSA)	Sigma
DAPI (4', 6-diamidino-2-phenylindole)	Thermo Fisher
Glycerol	Carl Roth
iScript™ cDNA Synthesis Kit, 100 stored at -20°C	Bio-Rad
Iraq Universal SYBR Green Supermix, 100 (stored at -20°C)	Bio-Rad
Mowiol 4-88	Sigma
Paraformaldehyde (PFA) 4% PFA in PBS, stored at -20°C	Merck
Phosphate buffered saline (PBS) Dulbecco	Life technologies
TRIS-HCL	Carl Roth
Triton X-100 0,1% in PBS	Sigma
Trypsin-EDTA Solution 10X	Sigma

Table 8: Reagents

4.1.9. Kits

Products	company
mouse inflammatory panel	Legendplex Biolegend

4.1.10. Software

BD FACS Diva
Biorad CFX Manager

Cytexpert
Graph Pad Prism 6
GSEA/Cytoscape
Illustrator
Image J
Microsoft Office
PANTHER
R studio

Table 9: Software

4.2. Methods

4.2.1. Cell culture

bEND5 cells

bEnd5 cell (Mouse-brain-derived endothelial cells) has been established from brain endothelial cells of Balb/c mice. For the following experiments, bEnd5 cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 % fetal calf serum (Gibco), 1% Penicillin/Streptomycin (Lonza), 11 mmol/L sodium pyruvate, 1 % nonessential amino acids were used. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

KP983.3 cell line

KP 983.3 cell line is a non-small cell lung cancer (NSCLC) cell line with TRP53 deletion and KrasG12D mutation. KP983.3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% fetal calf serum (Gibco) and 1% Penicillin/Streptomycin (Lonza). Cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

4.2.2. bEND5 cells irradiation

$5X10^4$ bEND5 cells were seeded in the 24 well plastic plates with a cover glass one day before and sealed with the sealing strip. Then, plates were transferred on ice in a cooler box to the facility where the radiation machine was located. The plates were put in the radiation accelerator, set as 160Kv and 25mA. The plate was placed into the radiation accelerator, and the cells were irradiated with 0.5Gy, 2Gy, 4Gy, 6Gy, and 10Gy. After the irradiation, the medium was changed under the hood, and the plate was placed back in the incubator.

4.2.3. Immunofluorescence staining of bEND5 cells

To perform immunofluorescence (IF) staining of bEND5 cells, the irradiated cells were fixed with PFA at 4 hours, 24 hours, and 48 hours after irradiation. Briefly, the cells were washed one time with 1x PBS, then fixed with 4%PFA at 37°C incubator for 10 minutes, then washed

3 times with 1x PBS for 5 minutes. After the fixation, the cells were kept at 4°C for up to 2 weeks before further steps.
The fixed cells were permeabilized with 0,1% Triton X-100 in 1× PBS for 10 min at room temperature, washed once with 1 ml 1x PBS for 5 minutes, and blocked with 0,75 ml 5% BSA-PBS for 2 hours at room temperature. Primary antibodies of P-selectin (clone RB40.34, dilution 1:50, BD Bioscience), E-selectin (clone 9A9, dilution 1:50, BioXCell), ICAM1(clone G-5, dilution 1:50, Santa Cruz) and VCAM1(clone BioXCell, dilution 1:50, M/K-2.7) were diluted in 1% BSA-PBS. Cells were incubated overnight at 4°C with the target primary antibody. After the incubation, the samples were washed 3 times with 1% BSA-PBS for 5 minutes. Secondary antibodies anti-rat A555 (clone ab150153, dilution 1:250, Abcam) for P-selectin, E-selectin, and VCAM1 and anti-mouse A647 (clone Poly4053, dilution 1:100, BioLegend) for ICAM1 were diluted in 1% BSA-PBS, and the cells were incubated with secondary anti host for 2 hours at 4°C. Later, the cells were washed 2 times with 1ml PBS for 5 minutes and stained with 500 µl DAPI for 5 minutes. Then, they were washed once with 1ml PBS for 5 minutes and once with ddH2O. Finally, the cells were mounted with 25µl Mowiol Mounting Medium, and slides were stored at 4°C until the pictures were taken with the microscope. The experiment was repeated three times.

4.3. Animal experiments

4.3.1. Experimental mice

In this thesis, 8-20-week-old wild-type mice with a C57BL/6 background were used for the animal experiments. All animal experiments were conducted under the animal license TVA 81-02-04-2017-A511, which was approved by the State Office of North Rhine Westphalia.

4.3.2. Flank tumors injection

To harvest KP983.3 cells from the cell culture plate, cells were washed with 1× PBS and incubated in 1ml of 1% trypsin at 37°C for 5 minutes. The cells were collected with 5ml DMEM and centrifuged at 500g for 5 minutes at 4°C. Then, the supernatant was discarded, and the cells were counted using a hemocytometer. Afterward, the cells were washed with 20 ml PBS and centrifuged at 500g for 5 minutes at 4°C. The pellet was resuspended in PBS with a $5x10^6$ cells/100µl concentration. Cells were kept on the ice before injection.
The mice were anesthetized intraperitoneally (i.p.) with a mixture of ketamine (100 mg/kg) and xylazine (10mg/kg). KP983.3 cells were injected 100µl cell suspension per side subcutaneously into bilateral flanks with a syringe. After the subcutaneous injection, the mice were put on the heating pad until they were awake. They were placed back in the cages where they wholly recovered from anesthesia. Tumors were determined every three days.

4.3.3. Tumor irradiation

The flank tumors were irradiated when their volume reached about 300mm³. Initially, mice were anesthetized with a mixture of ketamine at 100mg/kg and xylazine at 10mg/kg. When mice reached the appropriate depth of anesthesia, they were fixed on the radiation accelerator, and other organs were shielded with stereotypes except tumors. In this way, only the tumors were exposed to the radiation. The radiation accelerator was set as 160Kv and 25mA, and the tumors were irradiated with 2Gy and 10Gy. After RT, the mice were placed in a heating pad in a quiet environment until they fully recovered from anesthesia. The mice were returned to their cages and observed until they became conscious and regained activity.

4.3.4. Tumor endothelial cells (TECs) isolation

TECs were isolated at 24 hours after the RT. Mice were sacrificed by inhalation of isoflurane, and the blood was cleared by transcardiac perfusion with 1× sterile PBS for 10 minutes. The right and left flank tumors were isolated with surgical scissors and surgical tweezers carefully. The dissected tumors were placed in DMEM with 2%FBS and were cut into small pieces (less than 1mm) with a scissor on a sterile dish. Later, the minced tumors were digested in 10mg/ml Collagenase Type 2 (100µl/ml) and 25µg/mL DNase I(5µl/ml) in RPMI with 10% FBS containing at 37 °C with vigorous shaking for 40 minutes. After the incubation time, digestion was stopped with 2%FBS/PBS, and the tumors were mashed with a cannula about 15 times for further separation. The cell suspension was filtered through a 100-µm cell strainer placed on the top of a 50 mL conical tube to collect the single cell suspension. Cells were centrifuged at 280g for 5 minutes at 4℃. The pellet was resuspended in 10ml PBS. Cells were again filtered through a 100-µm cell strainer and centrifuged at 300g for 5 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 3ml PBS. Then, 3 ml of 60% percoll was added carefully and slowly to the bottom of the falcon tube with a needle. The sample tube was centrifuged at 400g for 20 minutes at 22 °C. The interface between the upper and lower layers was carefully transferred to a new 50 mL falcon, and cells were washed with 30 ml PBS and centrifuged at 400 g for 10 minutes at 4 °C. The supernatant was discarded, and the cells were resuspended with 1×10^7 cells per 90 µL MACS buffer and 10µl CD31 microbeads (Miltenyi Biotec, Bergisch Gladbach). The cells were incubated with the CD31 beads for 15 minutes at 4 °C. The cells were washed with 1 mL of MACS buffer per 1×10^7 cells and centrifuged at 300×g for 5 minutes at 4 °C. Then, the cell pellet was resuspended in 5 mL MACS buffer, and the cell suspension was applied to MACS LS columns (Miltenyi Biotec). After initial flow through with cells, LS columns were washed with 3 mL of MACS buffer 3 times. LS columns were transferred into the new 15 ml conical tube, and the CD31 positive cells were flushed out by applying the plunger firmly. The

CD31-positive cells were collected by centrifuging at 280g for 5 min at 4 °C and used for further analysis.

4.3.5. Supernatant collection

The supernatant for cytokine assay was collected simultaneously with TEC isolation. Mice were sacrificed by inhalation of isoflurane, and the blood was cleared by transcardiac perfusion with 1× sterile PBS for 10 minutes. Then, the right and left flank tumors were isolated with surgical scissors and surgical tweezers carefully. The dissected tumors were placed in DMEM with 2%FBS and were cut into small pieces (less than 1mm) with a scissor on a sterile dish. Later, the minced tumors were digested in 10mg/ml Collagenase Type 2 (100ul/ml) and 25 µg/mL DNase I (Desoxyribonuklease) (5ul/ml) in RPMI with 10% FBS containing at 37 °C with vigorous shaking for 40 min. After the incubation time, digestion was stopped with 2% FBS/PBS, and the tumors were mashed with a cannula about 15 times for further separation. The cell suspension was filtered through a 100-µm cell strainer placed on the top of a 50 mL conical tube to collect the single cell suspension. Cells were centrifuged at 280g for 5 minutes at 4°C. The supernatant was aliquoted into 2ml EP tubes and stored at -20°C until the cytokine assay.

4.3.6. Preparation of Tumor Immunofluorescence Specimens

The tumors were harvested at 24 hours after RT. Mice were sacrificed by inhalation of isoflurane, and the blood was cleared by transcardiac perfusion with 1× sterile PBS for 10 minutes. The right and left flank tumors were isolated with surgical scissors and surgical tweezers carefully. The dissected tumors were saved in the 20% sucrose solution immediately. Then, they were cut into small slides and embedded in the OCT in dissected tissue blocks with no more than 5mm thickness. The base mold containing the tissue blocks was slowly placed into liquid nitrogen till the entire tissue block was submerged into liquid nitrogen to ensure the tissue was frozen completely and saved at -80°C until section.

4.4. Molecular biology techniques

4.4.1. Fluorescence-activated cell sorting of TECs

Following TEC isolation, the cell pellet from the last step was resuspended in PBS containing CD31/PE-Dazzle antibody (1:100) and Zombie NIR (1:500) as live/death marker and incubated for 20 minutes at 4 °C. Then the CD31 positive cells were purified via BD FACS Aria and Sony MA900 using a 100µm nozzle at the Laboratory of Experimental Immunology, Institute of Virology (Kleins Lab, Cologne) and the Department of Pathology (Hillmers Lab, Cologne). The further purified endothelial cells were resuspended in 1mL Trizol and saved at -20°C.

4.4.2. RNA isolation

The frozen samples in Trizol were thawed at room temperature, and 200µl chloroform was added to the samples to help isolate nucleic acid. Then, samples were centrifuged at 12000g for 12min at 4°C. After the centrifugation, three phases appeared, and the RNA phase (upper phase) was carefully transferred to a new tube. Then RNA was precipitated with 2 times the volume of isopropanol and 3µl glycogen, centrifuged at full speed for 12 minutes at 4°C. Afterward, RNA was washed briefly in 1ml 80% Ethanol (EtOH) with centrifugation at full speed. The pellet was resuspended in 25µl Sterile Double Distilled Water (ddH_2O), and the optical density (OD) was measured with NanoDrop at a wavelength of 260 nm.

4.4.3. cDNA synthesis

The RNA concentrations were adjusted to 200ng/µl depending on the sample's concentration. The iScript cDNA Synthesis kit from Bio-Rad synthesized cDNA from RNA. This kit contains nuclease-free water, iScript reverse transcriptase, a Ribonuclease H (RNase H+), and the iScript reaction mix with oligo(dT) and random hexamer primers. The reaction mix was prepared as follows: 1µl reverse transcriptase and 4 µl reaction mix were added in the tubes containing 200 ng/µl RNA and finally reached a volume of 20 µl by adding the nuclease-free water. The thermal cycling protocol was set as *Table 10*. After the complication of the synthesis, the cDNA samples were stored at -20 °C.

Step	Temperature(°C)	Time(minutes)
Priming	25	5
Reverse transcription	46	20
RT inactivation	95	1

Table 10: Settings for thermal cycling protocol of cDNA synthesis

4.4.4. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (q-PCR) was performed to investigate the gene expression of endothelial adhesion markers. The q-PCR is based on the SYBR Green system (iTaqTM Universal SYBR Green supermix from Bio-Rad). The reaction master mixture was prepared according to *Table 11* as follows:

Buffer	µl
ddH20	8
Reverse primer	0.5
Forward primer	0.5
cDNA	1
SYBR	10
Total volume	20

Table 11 Reaction mixture for cDNA synthesis.

Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) was used as a housekeeping gene. The plate was sealed with a transparent film and vortexed for 30 seconds. Before starting the qRT-PCR, the plate was centrifuged for 5 minutes at 500 g at 4°C. Thermal cycling conditions were used. The fluorescence intensity was measured after each PCR amplification cycle and was used to quantify the amount of template DNA, as shown in *Table 12*. The experiment was repeated three times.

95°C	30 seconds
95 °C	5 seconds
60 °C	30 seconds
repeat the second and third steps	40 times

Table 12 Settings for q-PCR assays

4.4.5. IF staining for tumors

The samples were sectioned as 5 μm thickness, placed onto glass slides suitably, and dried at room temperature overnight. The prepared sections were fixed with 4% PFA(Paraformaldehyde) for 10 minutes at room temperature and then washed twice with 300 ml PBS at a neutral pH for 5 minutes. The sections were put on a plank, and circles were drawn around the tissues with a hydrophobic pen. Then, they were blocked with 5% BSA/PBS in a humidified chamber for 1 hour at room temperature. Primary antibodies of P-selectin (clone RB40.34, dilution 1:100, BD Bioscience), E-selectin (clone 9A9, dilution 1:100, BioXCell), ICAM1(clone G-5, dilution 1:50, Santa Cruz), VCAM1(clone BioXCell, dilution 1:50, M/K-2.7) and CD31 (clone Ab28364, dilution 1:50, Abcam) were diluted in 1% BSA-PBS. 100 μl of appropriately diluted primary antibodies of target adhesion molecular gathered with CD31 in 1% BSA/ PBS were applied to the sections on the slides and incubated in a humidified chamber overnight at 4°C. Slides incubated overnight with primary antibodies were rinsed for 5 minutes twice. Secondary antibodies anti-rat A555 (clone ab150153, dilution 1:250, Abcam) for P-selectin, E-selectin, and VCAM1, anti-mouse A647 (clone Poly4053, dilution 1:100, BioLegend) for ICAM1 and AlexaFluor488 anti-rabbit (clone Polyclonal, dilution 1:250, Abcam) for CD31 were diluted in 1% BSA-PBS. 100 μl secondary antibodies with an appropriate dilution in 1% BSA/PBS and incubated in a humidified chamber for 1 hour at room temperature. The slides were rinsed in 300 ml PBS for 2 changes and then incubated in 100μl DAPI for 5 minutes at room temperature. The slides were washed with 300ml PBS for 2 changes at the end, and a drop of mounting media was put onto the tissue. Then, the dry coverslip was carefully placed onto the mounting media without bubbles between the slide and the coverslip. The sealed slides were carefully cleaned, observed, and photographed under a microscope. The immunofluorescence photos were analyzed with ImageJ. The experiment was repeated three times.

4.4.6. Cytokine experiment

The supernatant from the tumor was used for the cytokine experiment. Reagents were prepared as per the instructions in the Kit. The Pre-mixed Beads bottle was vortexed for 1 minute to resuspend the beads to prepare for the Antibody-Immobilized Beads thoroughly, and the 20× Wash Buffer was diluted to 1× Wash Buffer with deionized water. Lyophilized Mouse Inflammation Panel Standard Mixture containing 250 µL of Assay Buffer was reconstituted before use and allowed to stay at room temperature for 10 minutes before transferring the standard to an appropriately labeled polypropylene microcentrifuge as the top standard C7. Six additional polypropylene microcentrifuge tubes were labeled as C6, C5, C4, C3, C2, and C1, and 75 µL of assay buffer was added to each of the six tubes to make a 1:4 dilution, 25 µL of top Standard C7 was transferred to C6 tube and mixed well as C6 standard. C5, C4, C3, C2, and C1 standards were serially diluted 1:4 in the same manner, and assay buffer was used as C0 (0 pg/mL standard) as the *Table 12* below:

Tube/ Standard ID	Serial Dilution	Assay Buffer to add (µL)	Standard to add	Final Conc. (pg/mL) *	Final Conc. (pg/mL) **
C7	--	--	--	10,000	50,000
C6	1:4	75	25 µL of C7	2,500	12,500
C5	1:16	75	25 µL of C6	625	3,125
C4	1:64	75	25 µL of C5	156.3	781.3
C3	1:256	75	25 µL of C4	39.1	195.3
C2	1:1024	75	25 µL of C3	9.8	48.8
C1	1:4096	75	25 µL of C2	2.4	12.2
C0	--	75	25 µL of C1	0	0

Table 13 Buffer settings for cytokine assay

The V-bottom plate is used for cytokine assays. All reagents were brought to room temperature for preparation, 25µL of assay buffer was added to all wells, 25 µL of each standard was added to standard wells, and then 25µL of samples were added to the sample wells, and 25 µL of the mixed beads were added to each well after vortexing for 30 seconds. The total liquid volume per well now reached 75µL. The V-bottom plate was sealed with plate sealant and wrapped in aluminum foil to protect the plate from light. The plate was shaken on a plate shaker at 800 rpm for 2 hours at room temperature to ensure the beads remained in suspension during incubation. the V-bottom plate was centrifuged at 150 rpm/4°C for 5 minutes after the incubation was completed, and the supernatant was removed quickly; 200µL of wash buffer was dispensed into each well to wash, the plate was shaken at 800 rpm for 1 minute, and centrifuged at 150 rpm/4°C for 5 minutes, the supernatant was

discarded. The same procedure for washing was repeated one more time. Then, the V-bottom plate was added with 25μL of detection antibody per well and sealed with a new plate sealant, wrapped in aluminum foil to protect the plate from light, and shaken on a plate shaker at 800 rpm for 1 hour at room temperature. Afterward, 25μL SA-PE was directly added to each well without washing and shaken on a plate shaker at 800 rpm at room temperature for 30 minutes. Every well was washed with 200 μL of Wash Buffer once, and 150 μL of 1X Wash Buffer was used to resuspend the beads, and the samples were read on the flow cytometer. The experiment was repeated three times.

4.5. Statistics

Statistical analyses were performed by one-way ANOVA. Error bars represented the standard error. Statistically significant P values are abbreviated as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

5. Results

5.1. P-selectin regulation after irradiation in vivo

To reveal the effect of RT on the adhesion molecules represented on the surface of ECs in TME and on the expression levels of inflammatory cytokines in TME, I performed in vivo experiments with flank tumors (*Figure 5*). Briefly, The KP983.3 cells were injected in the ventral side of the mice, and the tumors were irradiated when their volume reached 200mm^3 and harvested 24h after irradiation. Next, endothelial cells were sorted from half of the cancer for RNA extraction, and the other half was preserved for IF staining. The supernatant of the cancer is stored for inflammation-related cytokines test. I initially analyzed the amplification of corresponding deoxyribonucleic acid (DNA) with the real-time polymerase chain reaction (qPCR) and the expression of adhesion molecules with immunofluorescent (IF) staining.

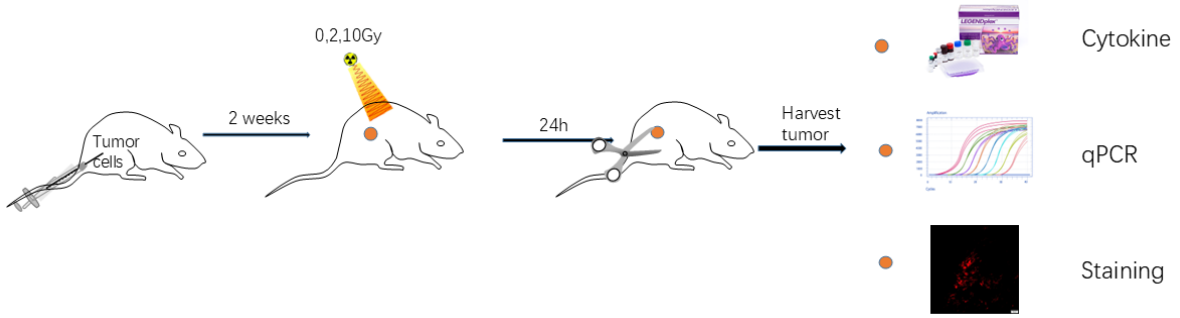


Figure 5: The procedure of the mice experiments. Tumor cells were implanted in the ventral side of mice. 14 days after the tumor cell implantation, the tumor was irradiated and collected 24 hours after RT.

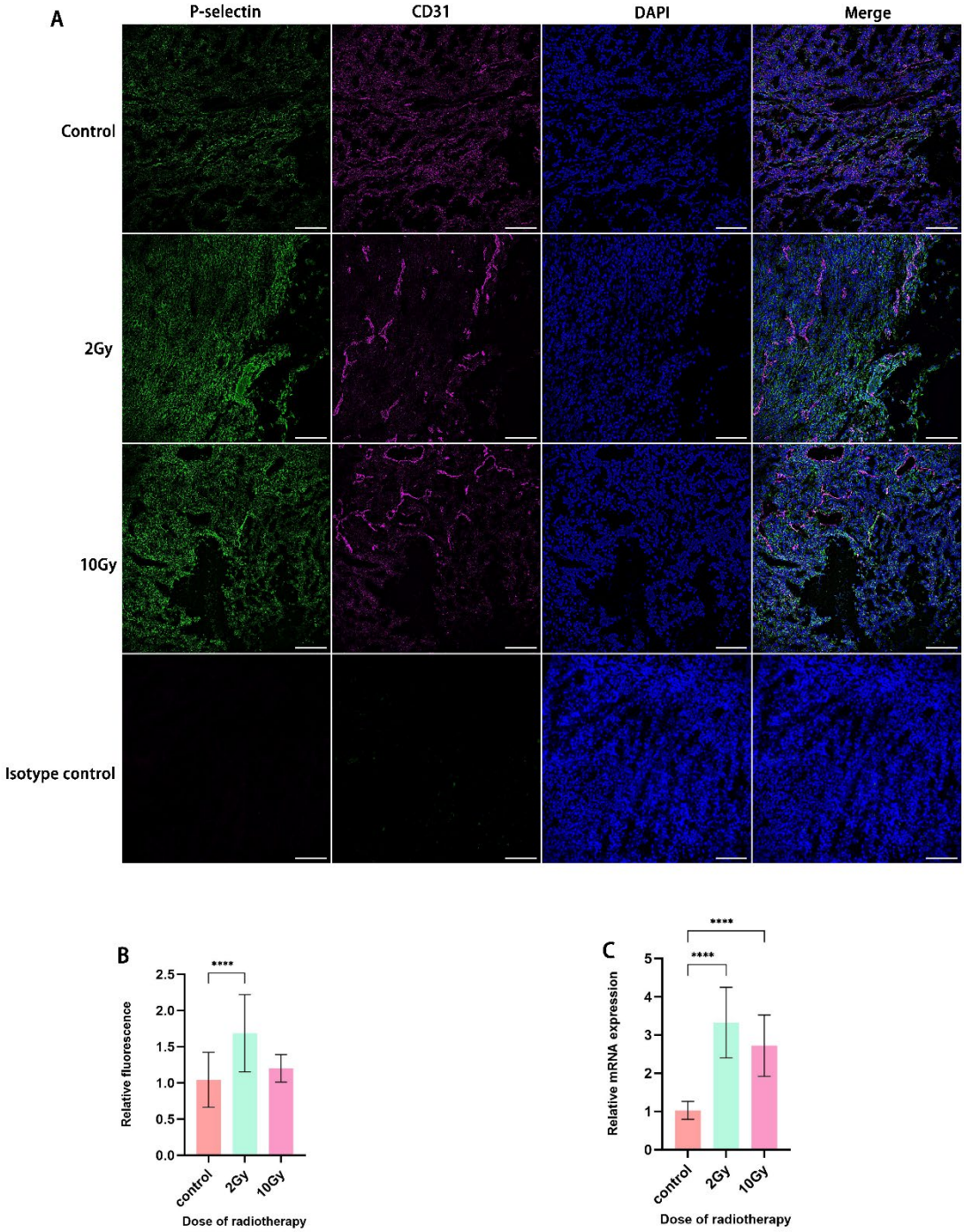


Figure 6： P-selectin was regulated by radiotherapy in vivo. A: Immunofluorescence images with different staining channels: P-selectin(green), CD31(magenta), and DAPI (blue). Samples of the control, 2Gy, and 10Gy groups, respectively, represent tumors that did not accept any treatment, were irradiated with 2Gy and 10Gy, and were collected 24 hours after irradiation. Isotype control was only stained with secondary antibodies. DAPI identified cells. B: Quantification of the immunofluorescence staining of P-selectin at 24h after irradiation. C: q-PCR of P-selectin of tumor endothelial cells at 24h after irradiation. Statistically significant

*differences are shown as * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.005; **** = p ≤ 0.001, scale bar=100μm.*

The regulation of P selectin with and without RT was examined at 24 hours after RT by two experimental methods: IF staining and qPCR. We evaluated the impact of RT on the expression levels of P-selectin on TECs using IF staining. P-selectin is expressed on the surfaces of ECs and other cells, such as platelets[159]. To distinguish the target P-selectin molecular expressed on TECs, we combined the IF staining with P-selectin and PECAM1(CD31) as a marker for TECs. According to the localization of CD31 on blood vessels, it can be found that P-selectin was expressed on the vascular ECs in the tumor tissues (*Figure 6*-A). The quantification of IF staining showed that the expression of P-selectin on the TECs was significantly upregulated at 2Gy (****p<0.0001) but without any significance at 10Gy (*Figure 6*-B). To evaluate the impact of RT on the gene abundance of P-selectin, we isolated TECs from the tumor. The gene expression of P-selectin was observed by qPCR, and the result showed that the expression of the P-selectin gene was upregulated at 2Gy(****p<0.0001) and 10Gy(****p<0.0001) (*Figure 6*-C).

5.2. E-selectin regulation after irradiation in vivo

The expression of E-selectin on the surface of ECs in the TME was also observed by IF staining and qPCR at 24 hours after RT with 2Gy and 10Gy. We stained P-selectin with PECAM1(CD31) to verify the location of E-selectin. E-selectin was expressed on the vascular ECs in the tumor tissues (*Figure 7*-A). The quantitative analysis of IF staining showed that E-selectin expression level was upregulated at 24h after RT at 2Gy(****p<0.0001) and 10Gy(****p<0.0001) (*Figure 7*-B). The gene expression of E-selectin of isolated TECs was tested with qPCR. Consistent with the results of IF, the result of qPCR showed that the expression of the E-selectin gene was also upregulated at 2Gy(****p<0.0001) and 10Gy(****p<0.0001) (*Figure 7*-C).

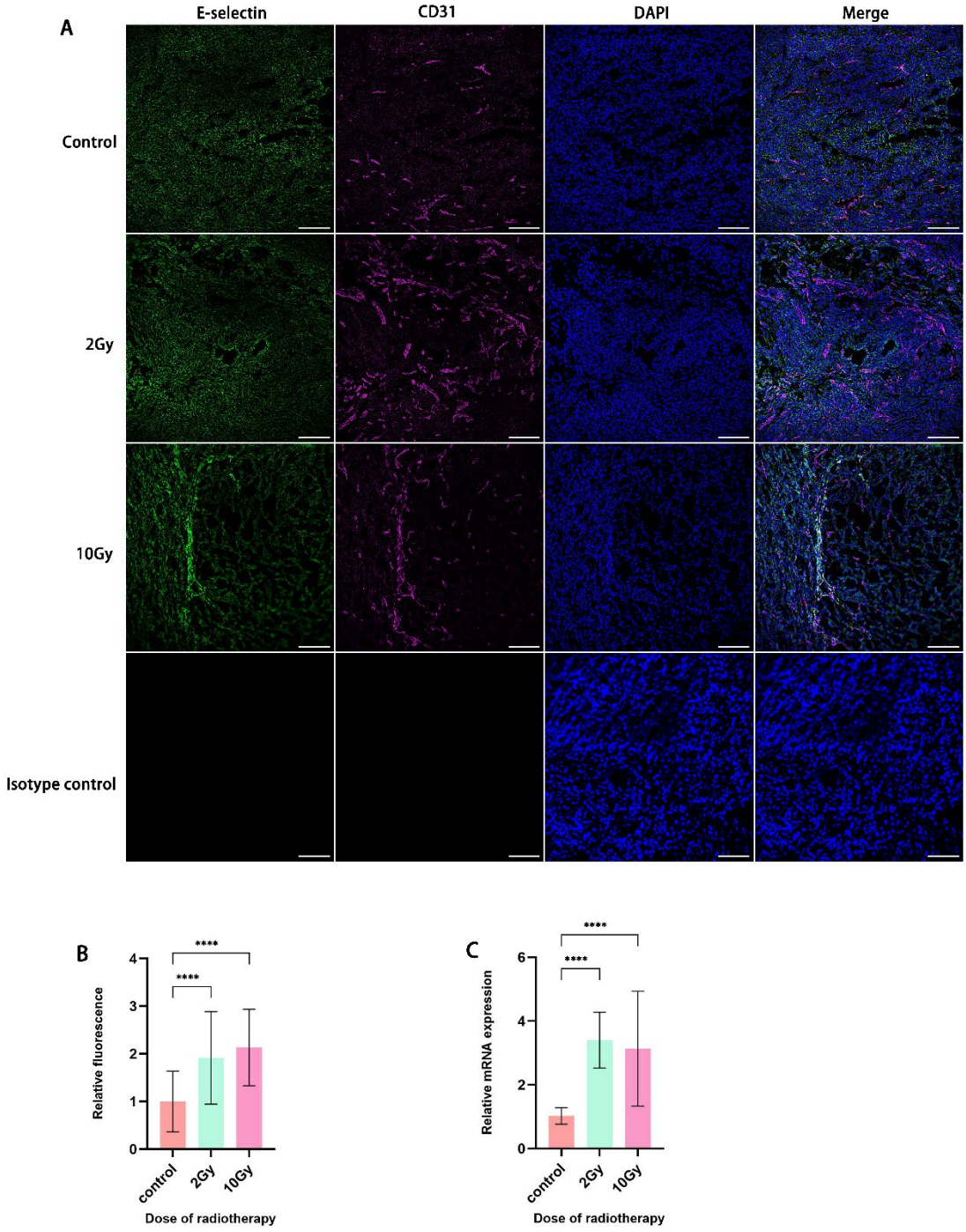


Figure 7： E-selectin was regulated by radiotherapy in vivo. A: Immunofluorescence images with different staining channels: E-selectin(green), CD31(magenta), and DAPI (blue). Samples of control, 2Gy, and 10Gy groups, respectively, represent tumors that did not accept any treatment, irradiated with 2Gy and 10Gy, and collected at 24 hours after irradiation. Isotype control was only stained with secondary antibodies. DAPI identified cells. B: Quantification of the immunofluorescence staining of E-selectin at 24h after irradiation. C: q-PCR of E-selectin of tumor endothelial cells at 24h after irradiation. Statistically significant

*differences are shown as * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.005; **** = p ≤ 0.001, scale bar=100µm*

5.3. ICAM1 regulation after irradiation in vivo

The expression level of ICAM1 in response to different doses of RT was also observed with IF staining and qPCR. The IF staining of ICAM1 and CD31 showed that ICAM1 was expressed on the ECs in tumors (*Figure 8*-A). The quantification of the IF staining indicated that compared to unirradiated tumors, tumors with irradiation at 2Gy(**p<0.01) and 10Gy(**p<0.01) have a significant upregulation (*Figure 8*-B). At the same time, the qPCR of ECs ($CD31^+$ cells) isolated from the tumors also showed a noticeable increase at the dose of 2Gy(**p<0.01) and 10Gy(****p<0.0001) (*Figure 8*-C).

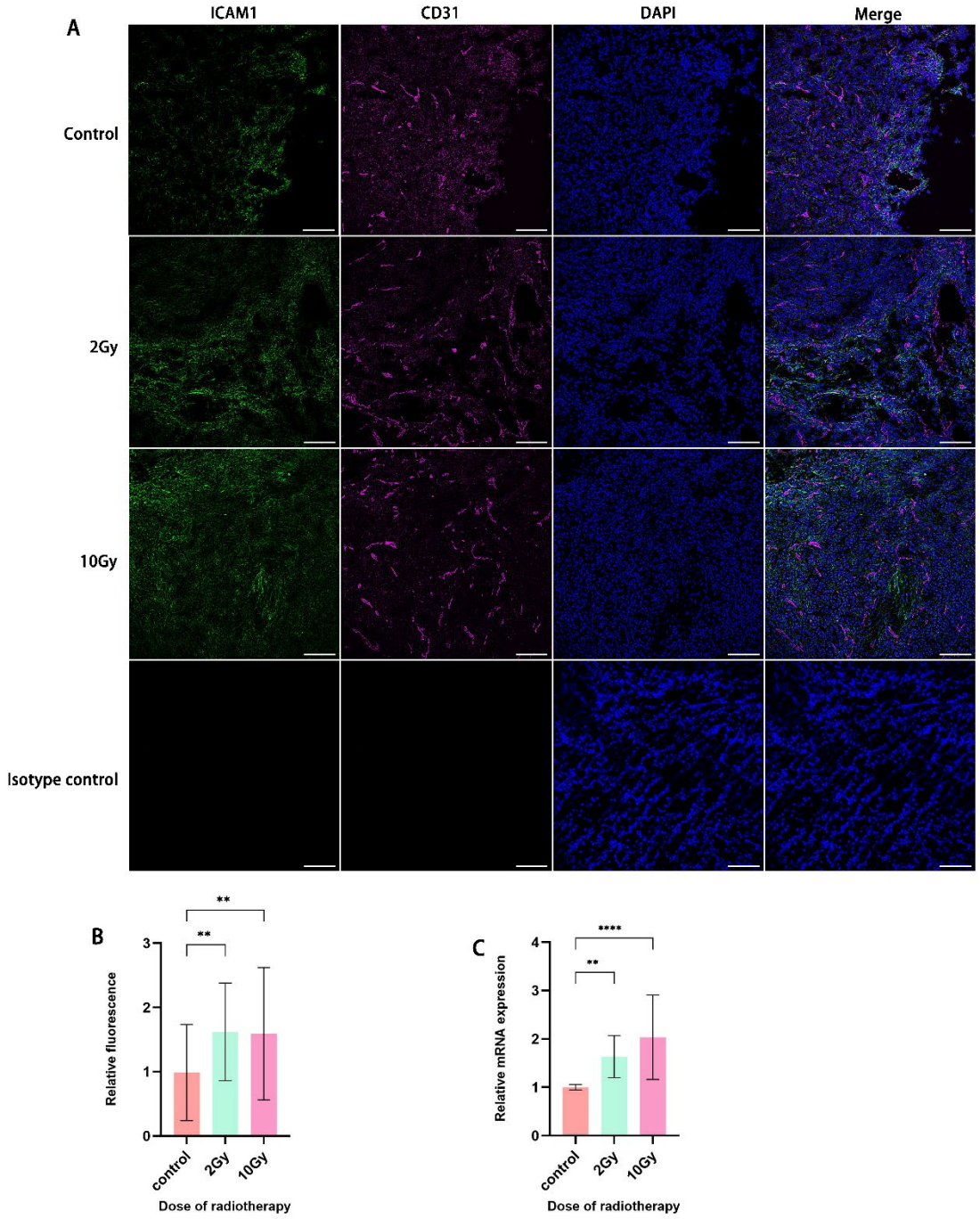


*Figure 8： ICAM1 was regulated by radiotherapy in vivo. A: Immunofluorescence images with different staining channels: ICAM1 (green), CD31(magenta) and DAPI (blue). Samples of control, 2Gy and 10Gy groups respectively represent tumors that did not accept any treatment, irradiated with 2Gy and 10Gy and collected at 24 hours after irradiation. Isotype control was only stained with secondary antibodies. DAPI identified cells. B: Quantification of the immunofluorescence staining of ICAM1 at 24h after irradiation. C: q-PCR of ICAM1 at 24h after irradiation. Statistically significant differences are shown as * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.005; **** = p ≤ 0.001, scale bar=100μm*

5.4. VCAM1 regulation after irradiation in vivo

VCAM1 expressions in tumors also had upregulated changes 24 hours after RT. We stained VCAM1 with CD31 to locate the target molecular on the blood vessel endothelium (*Figure 9*-A). VCAM1 molecules were observed to be expressed in the tumors, and the quantification of the IF staining shows that VCAM1 significantly upregulated after RT at 2Gy(**$p<0.01$) and 10Gy(****$p<0.0001$) (*Figure 9*-B). The qPCR tested the gene abundance of TECs, and the result showed that VCAM1 significantly upregulated after RT at 2Gy (*$p<0.05$) and 10Gy (*$p<0.05$) (*Figure 9*-C).

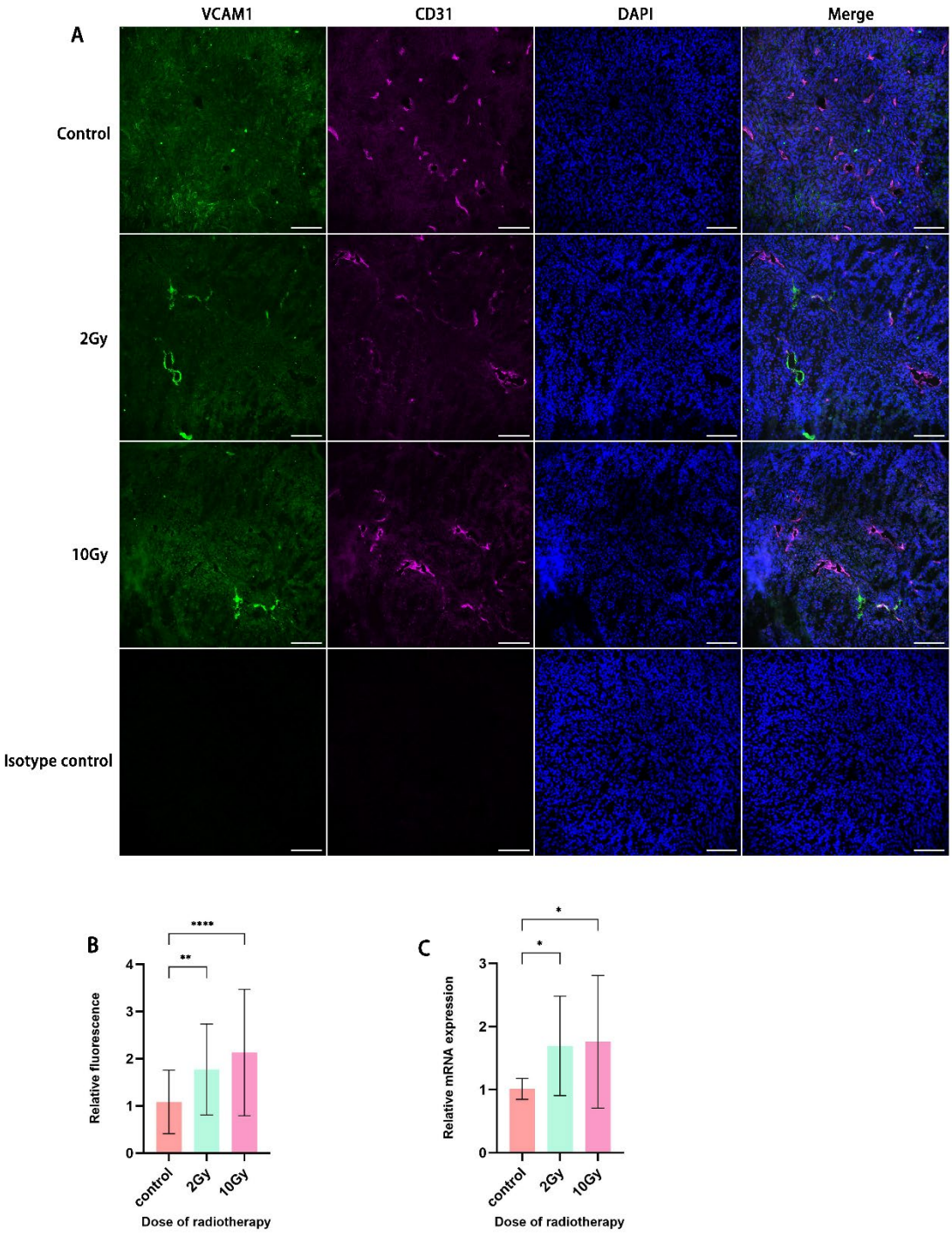


Figure 9: VCAM1 regulated by radiotherapy in vivo. A: Immunofluorescence images with different staining channels: VCAM1 (green), CD31(magenta) and DAPI (blue). Samples of control, 2Gy, and 10Gy groups, respectively, represent tumors that did not accept any treatment, irradiated with 2Gy and 10Gy, and collected 24 hours after irradiation. Isotype control was only stained with secondary antibodies. DAPI identified cells. B: Quantification of the immunofluorescence staining of VCAM1 at 24h after irradiation. C: q-PCR of VCAM1 at

*24h after irradiation. Statistically significant differences are shown as * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.005; **** = p ≤ 0.001, scale bar=100µm*

5.5. P-selectin regulation directly after irradiation *in vitro*

To analyze whether RT regulates the adhesion molecules directly, we analyzed the changes in DNA amplification and adhesion molecules expression of the mouse-brain-derived endothelial cells (bEnd5) cells after different doses of RT at various time points. Briefly, the End5 were irradiated with increased dosage of radiation (0.5Gy, 2Gy, 4Gy, 6Gy, 10Gy), and samples were collected at the time points 1.5 hours, 4 hours, 24 hours after the irradiation for RNA levels analysis. For testing the expression levels of adhesion molecules regulated by RT, the bEND5 cells were fixed for IF staining without RT and at the time points of 4 hours, 24 hours, and 48 hours after RT with 0.5Gy, 2Gy, 4Gy, 6Gy, 10Gy.

The expression of P-selectin on bEND5 cells was observed by IF staining (*Figure 10*). The quantification results show that the P-selectin molecule expressed on bEND5 cells was upregulated at various dosages and times after RT. At 4 hours after RT, the expression level of P-selectin on the surface of the bEND5 cell was increased at 2Gy (**p<0.01) and 10Gy (*p<0.05)(*Figure 11*-A). At 24 hours after RT, the expression level of P-selectin on the surface of the bEND5 cell was upregulated at the dosages of 0.5Gy (*p<0.05), 2Gy (****p<0.0001), 4Gy (*p<0.05) and 10Gy (*p<0.05)(*Figure 11*-A). At 48 hours after radiotherapy, the expression level of P-selectin on the surface of bEND5 cells no longer changed significantly (*Figure 11*-A). The qPCR results showed that the P-selectin gene expression levels of bEND5 cells were upregulated considerably at different doses and times after RT. At 1.5 hours after irradiation, the gene expression of P-selectin did not have any significance(*Figure 11*-B). At 4 hours after RT, the gene expression level was upregulated at the dose of 2Gy (**p<0.01)(*Figure 11*-B). At 24 hours after irradiation, the gene expression of P-selectin on bEND5 cell was increased at the doses of 0.5Gy (*p<0.05), 2Gy (****p<0.0001), 4Gy (****p<0.0001) and 6Gy (****p<0.0001), 10Gy (****p<0.0001) (*Figure 11*-B).

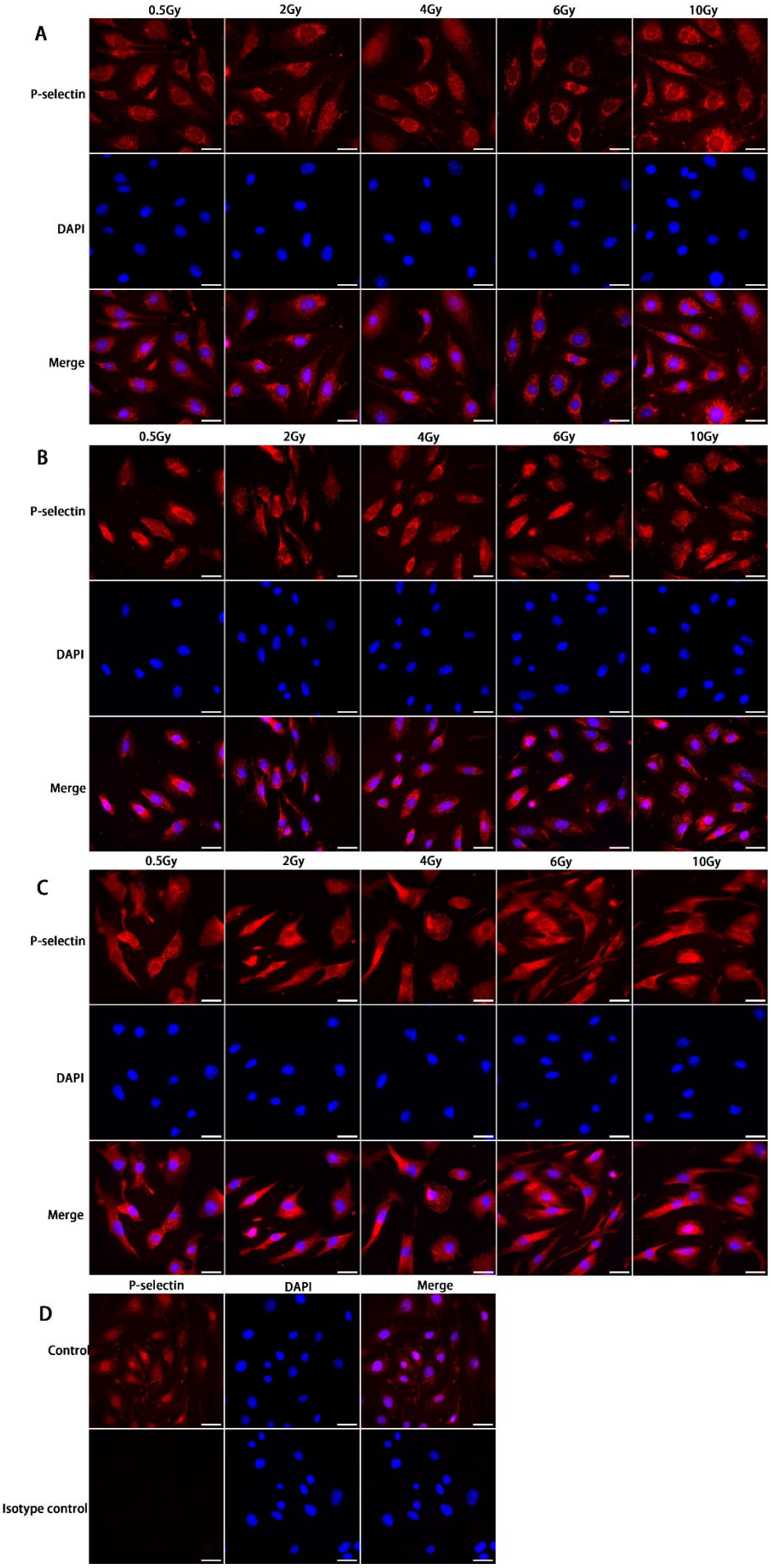

0.5Gy
2Gy
4Gy
6Gy
10Gy
A
P-selectin
DAPI
Merge
B
C
D
Control
Isotype control

Figure 10: Immunofluorescence images of P-selectin on bEND5 cells. P-selectin channel (red) was stained to show the expression of the P-selectin molecule on the surface of bEND5 cells. DAPI channel (blue) was stained to identify the cell nuclei of bEND5 cells. Samples of 0.5G, 2Gy, 4Gy, 6Gy and 10Gy groups respectively represent bEND5 cells were irradiated with 0.5G, 2Gy, 4Gy, 6Gy and 10Gy. A: bEND5 cells were collected and stained at 4 hours after irradiation. B: bEND5 cells were collected and stained at 24 hours after irradiation. C: bEND5 cells were collected and stained at 48 hours after irradiation. D: Samples in the control group were bEND5 cells without any treatment and stained with P-selectin channel and DAPI channel. Samples in the isotype control group only stained with secondary antibody. Scale Bar=30μm.

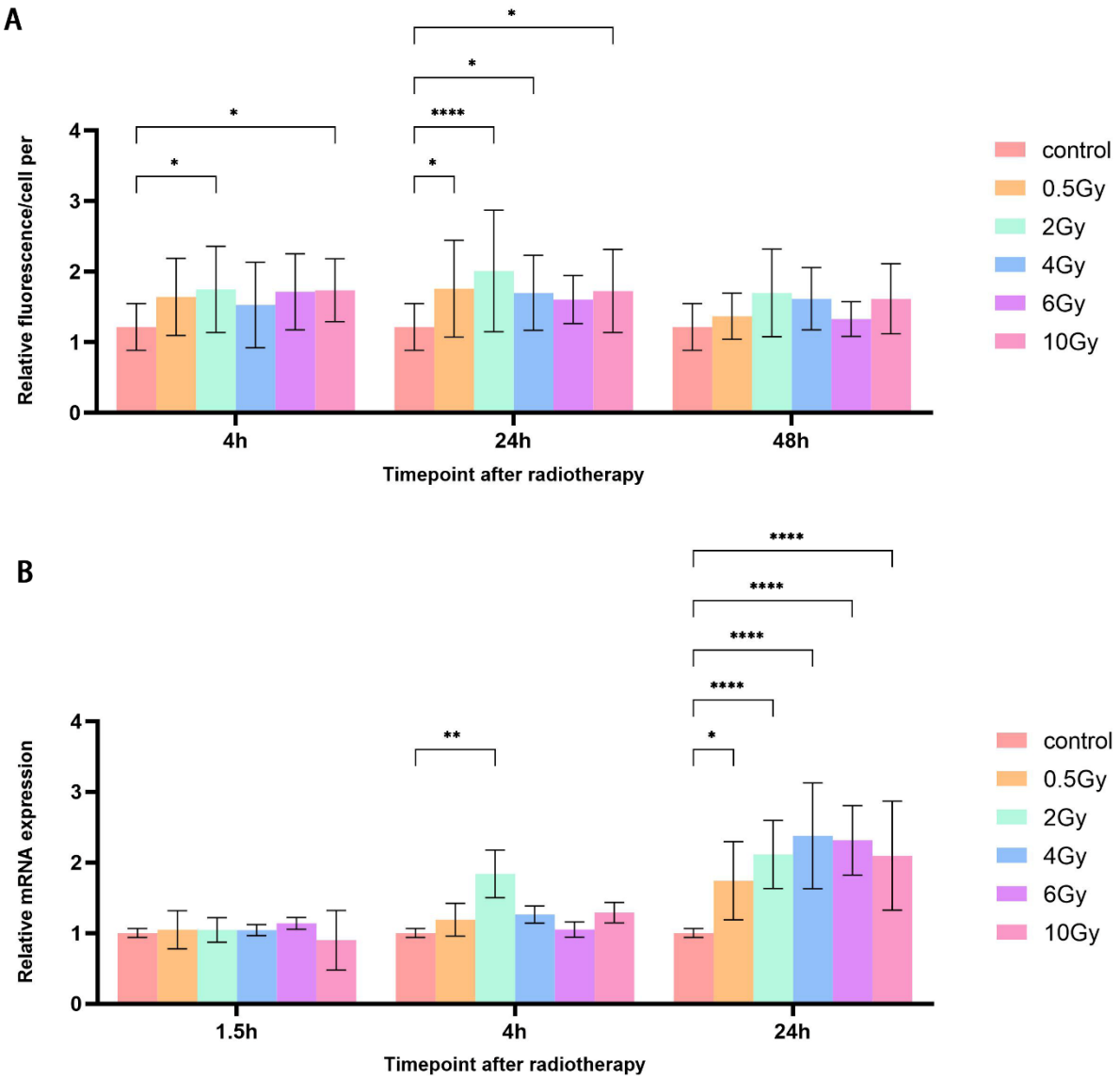


Figure 11: P-selectin was regulated by radiotherapy in vitro. A: Quantitative immunofluorescence data illustrating the effect of different doses at 0.5 Gy, 2 Gy, 4 Gy, 6 Gy, and 10 Gy of radiotherapy on bEND5 cells. B: qPCR confirmation of the gene expressions was

*performed using GAPDH as the reference control, and bEND5 cells irradiated with 0.5 Gy, 2 Gy, 4 Gy, 6 Gy, and 10 Gy were compared with untreated cells. Results were expressed as Mean±SD. Statistically significant differences are shown as * = p ⩽ 0.05; ** = p ⩽ 0.01; *** = p ⩽ 0.005; **** = p ⩽ 0.001.*

5.6. E-selectin regulation directly after irradiation *in vitro*

We stained the bEND5 cells with E-selectin to analyze the impact of RT on the expression level of E-selectin on ECs. The results of IF staining revealed that E-selectin levels were significantly upregulated after RT(*Figure 12*). At 4 hours after irradiation, the expression level of the E-selectin molecule was upregulated with the dose of 2Gy (**p<0.01) and 6Gy (*p<0.05) (*Figure 13*-A). At 24 hours after irradiation, the expression level of the E-selectin molecule was upregulated at 2Gy (**p<0.01), 4Gy (*p<0.05), 6Gy (***p<0.001), 10Gy (**p<0.01) (*Figure 13*-A). At 48 hours after irradiation, the expression level of the E-selectin molecule with 2Gy (****p<0.0001), 4Gy (*p<0.05), 6Gy (*p<0.05), 10Gy (****p<0.0001)(*Figure 13*-A). The results of qPCR revealed the impact of RT on the gene abundance of E-selectin of bEND5 cells. At 1.5 hours after the irradiation, RT did not influence the gene expression of E-selectin(*Figure 13*-B). At 4 hours after the irradiation, RT upregulated the gene expression level of bEND5 cells at 2Gy (**p<0.01)(*Figure 13*-B). At 24 hours timepoint after irradiation, the gene expression level of bEND5 cells was increased at the doses of 0.5Gy (*p<0.05), 2Gy (****p<0.0001), 4Gy (****p<0.0001) and 6Gy (*p<0.05), 10Gy (*p<0.05)(*Figure 13*-B).

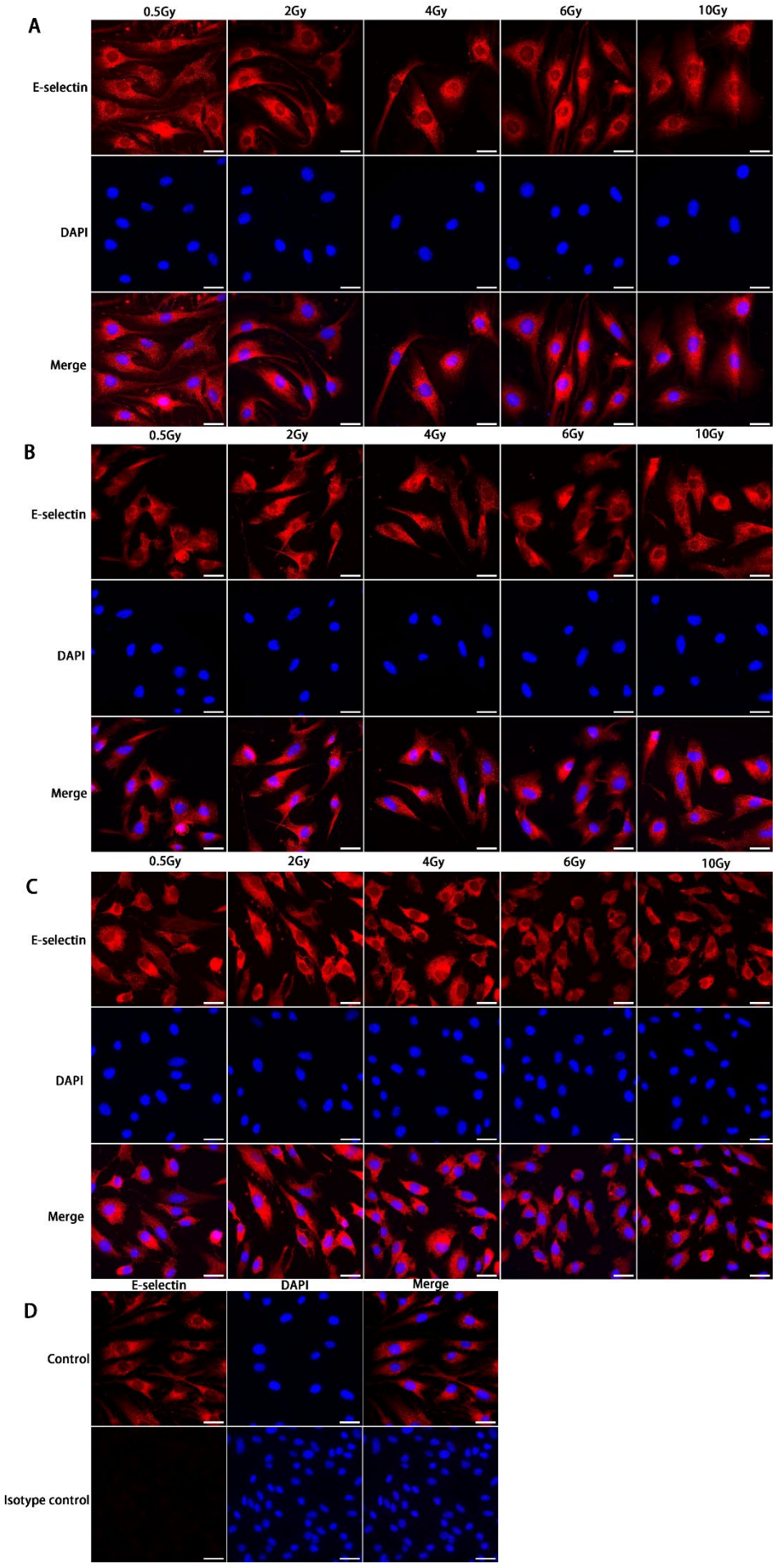

A
0.5Gy
2Gy
4Gy
6Gy
10Gy
E-selectin
DAPI
Merge
B
0.5Gy
2Gy
4Gy
6Gy
10Gy
E-selectin
DAPI
Merge
C
0.5Gy
2Gy
4Gy
6Gy
10Gy
E-selectin
DAPI
Merge
D
E-selectin
DAPI
Merge
Control
Isotype control

Figure 12: Immunofluorescence images of E-selectin on bEND5 cells. The E-selectin channel (red) was stained to show the expression of the E-selectin molecule on the surface of bEND5 cells. DAPI channel (blue) was stained to identify the cell nuclei of bEND5 cells. Samples of 0.5G, 2Gy, 4Gy, 6Gy and 10Gy groups respectively represent bEND5 cells were irradiated with 0.5G, 2Gy, 4Gy, 6Gy and 10Gy. A: bEND5 cells were collected and stained at 4 hours after irradiation. B: bEND5 cells were collected and stained at 24 hours after irradiation. C: bEND5 cells were collected and stained at 48 hours after irradiation. D: Samples in the control group were bEND5 cells without any treatment and stained with an E-selectin channel and DAPI channel. Samples in the isotype control group were only stained with secondary antibodies. Scale Bar=30μm.

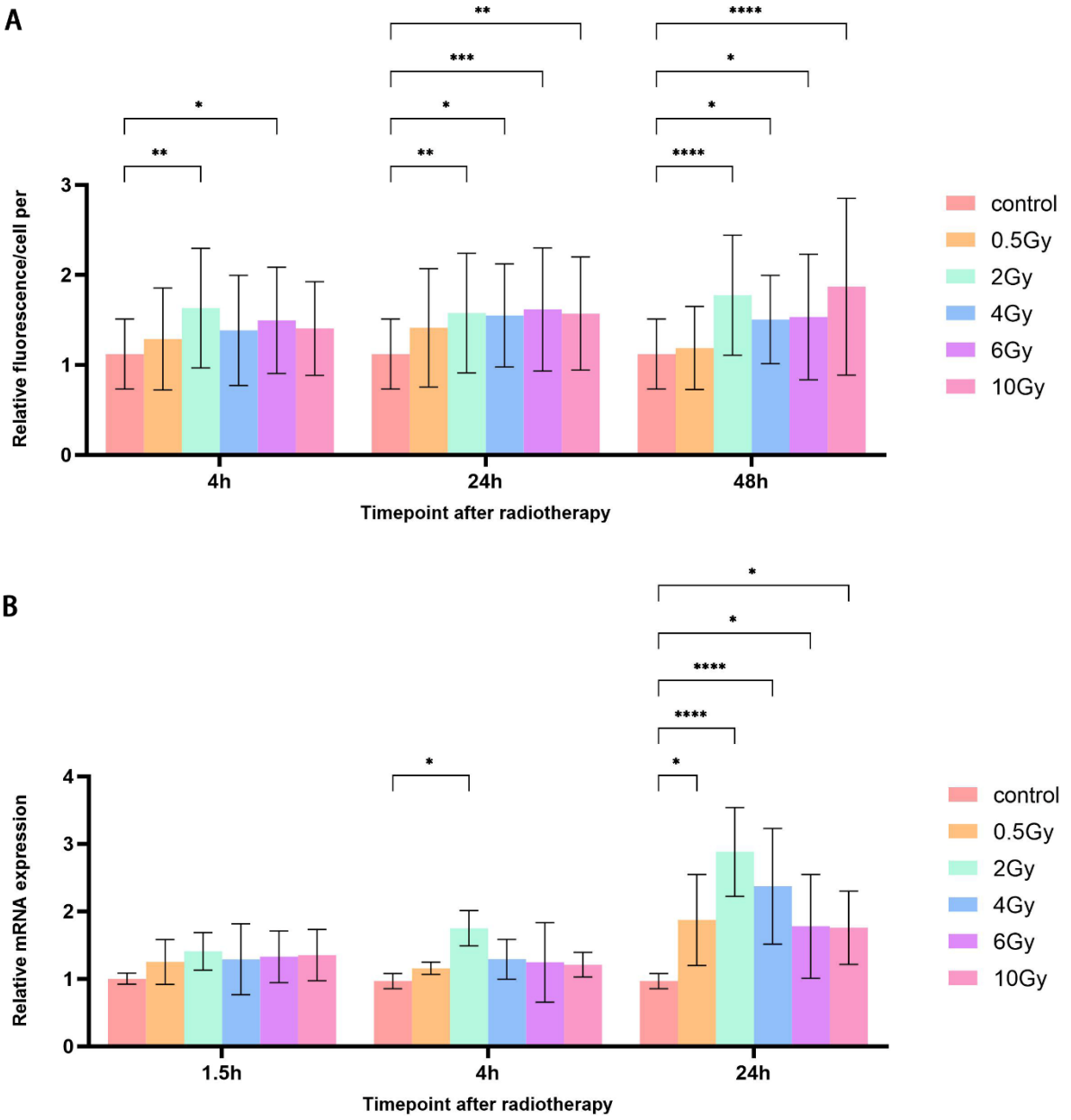
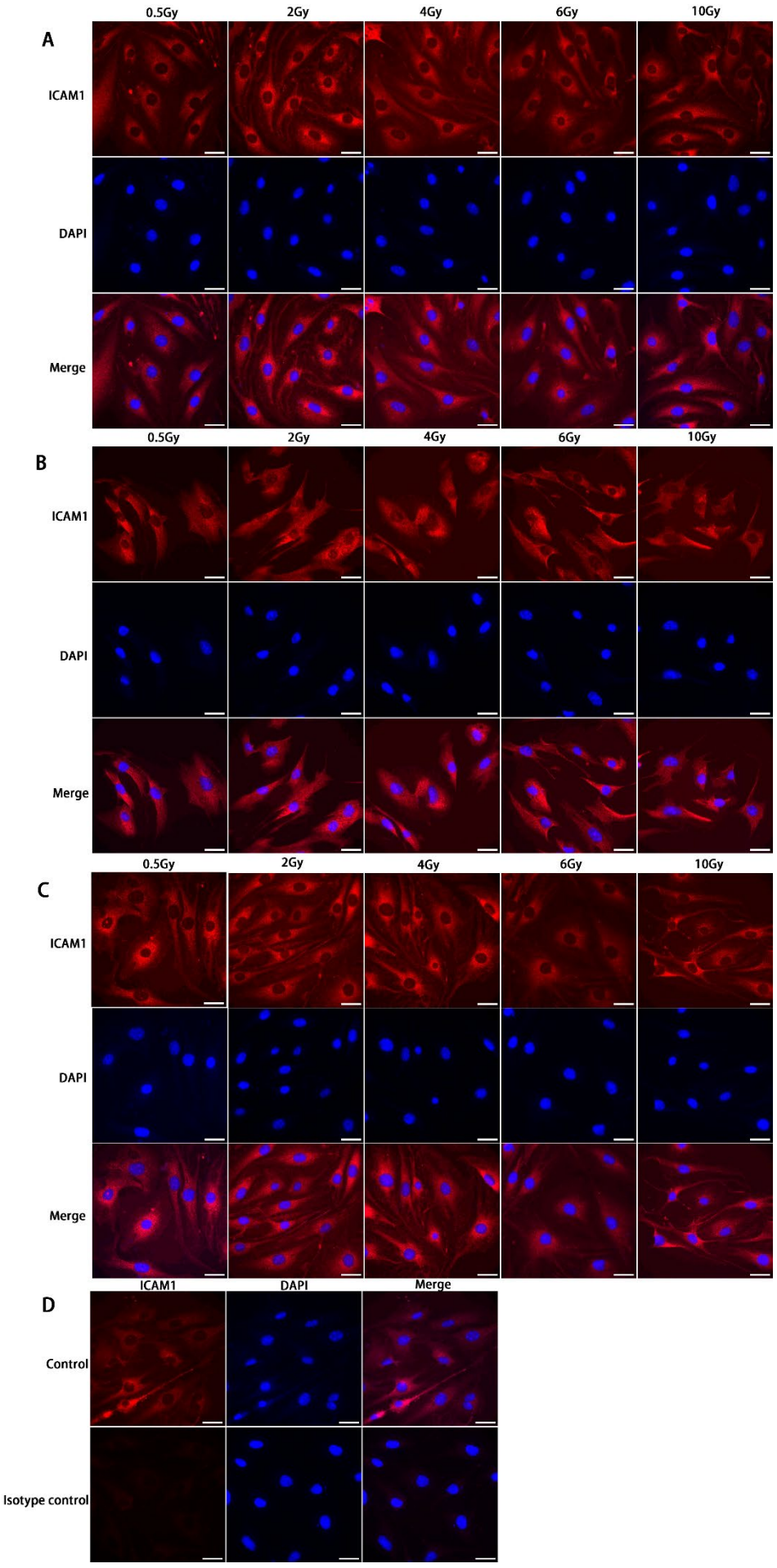


*Figure 13: The regulation of E-selectin on bEND5 cells after irradiation in vitro. A: Quantitative immunofluorescence data illustrating the effect of different doses of radiotherapy on bEND5 cells. B: qPCR confirmation of the gene expressions was performed using GAPDH as the reference control, and bEND5 cells irradiated with 0.5 Gy, 2 Gy, 4 Gy, 6 Gy, and 10 Gy were compared with untreated cells. Results were expressed as Mean±SD. Statistically significant differences are shown as * = p ⩽ 0.05; ** = p ⩽ 0.01; *** = p ⩽ 0.005; **** = p ⩽ 0.001.*

5.7. ICAM1 regulation directly after irradiation *in vitro*

We tested the direct effect of RT on ICAM1 on bEND5 cells using IF staining and qPCR in the *in vitro* experiments. IF staining showed that ICAM1 was clearly expressed on the surface of bEND5 cells (*Figure 14*), and the quantification of the IF staining results showed that the ICAM1 on the cell surface significantly upregulated after RT. At 4 hours after RT, we did not see any significance at 4 hours after irradiation (*Figure 15*-A). At 24 hours after RT, we found that the expression level of ICAM1 molecule on the surface of bEND5 cells was significantly upregulated at the doses of 2Gy (**p<0.01), 4Gy (**p<0.01) and 6Gy (***p<0.001) (*Figure 15*-A). At 48 hours after irradiation, the expression level of ICAM1 molecule on the surface of bEND5 cells was increased at the doses of 2Gy (**p<0.01), 4Gy (**p<0.01), and 10Gy (**p<0.01) (*Figure 15*-A). We analyzed qPCR to investigate the impact of RT on ICAM1 gene expression. The result showed that at 1.5 hours after RT, the gene abundance of ICAM1 showed no significant difference (*Figure 15*-B). At 4 hours after RT, it showed that there is an upregulation of ICAM1 gene abundance with 2Gy (*p<0.05) (*Figure 15*-B). At 24 hours after irradiation, the gene abundance of ICAM1 showed substantial upregulation at 0.5Gy (***p<0.001), 2Gy (****p<0.0001), 4Gy (****p<0.0001), 6Gy (*p<0.05) and 10Gy (**p<0.01) (*Figure 15*-B).


A
0.5Gy
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ICAM1
DAPI
Merge
B
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2Gy
4Gy
6Gy
10Gy
ICAM1
DAPI
Merge
C
0.5Gy
2Gy
4Gy
6Gy
10Gy
ICAM1
DAPI
Merge
D
ICAM1
DAPI
Merge
Control
Isotype control

Figure 14: Immunofluorescence images of ICAM1 on bEND5 cells. ICAM1 channel (red) was stained to show the expression of the ICAM1 molecule on the surface of bEND5 cells. DAPI channel (blue) was stained to identify the cell nuclei of bEND5 cells. Samples of 0.5G, 2Gy, 4Gy, 6Gy and 10Gy groups respectively represent bEND5 cells were irradiated with 0.5G, 2Gy, 4Gy, 6Gy and 10Gy. A: bEND5 cells were collected and stained at 4 hours after irradiation. B: bEND5 cells were collected and stained at 24 hours after irradiation. C: bEND5 cells were collected and stained at 48 hours after irradiation. D: Samples in the control group were bEND5 cells without treatment and stained with ICAM1 and DAPI channels. Samples in the isotype control group were only stained with secondary antibodies. Scale Bar=30μm.

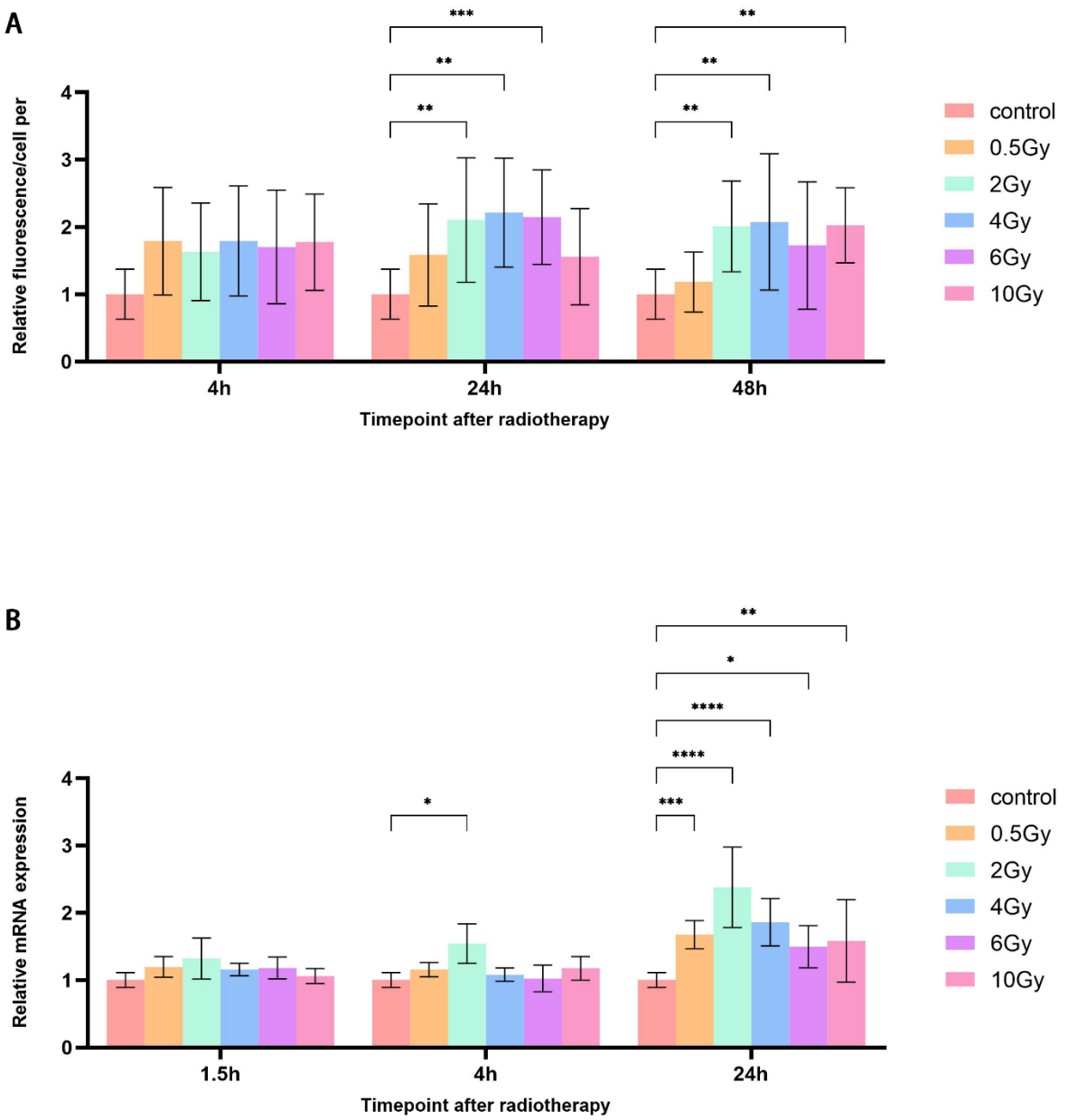
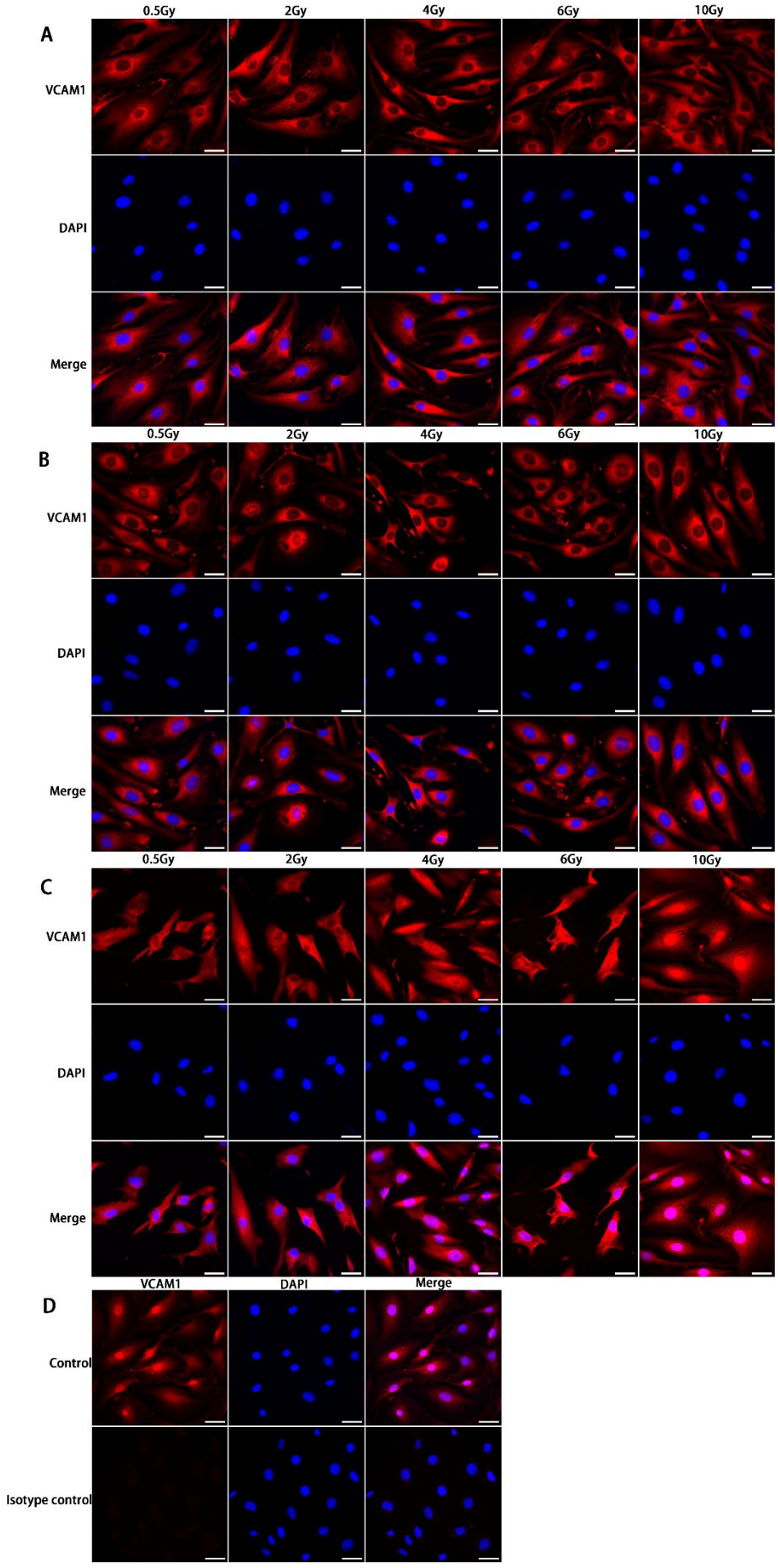


*Figure 15: The regulation of ICAM1 on bEND5 cells after irradiation in vitro. A: Quantitative immunofluorescence data illustrating the effect of different doses of radiotherapy on bEND5 cells. B: qPCR confirmation of the gene expressions was performed using GAPDH as the reference control, and bEND5 cells irradiated with 0.5 Gy, 2 Gy, 4 Gy, 6 Gy, and 10 Gy were compared with untreated cells. Results were expressed as Mean±SD. Statistically significant differences are shown as * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.005; **** = p ≤ 0.001.*

5.8. VCAM1 regulation directly after irradiation in vitro

Next, we revealed the impact of RT on the molecule expression level changes and gene abundance level changes of VCAM1 ligand on the bEND5 cells (*Figure 16*). The IF staining showed VCAM1 on TECs in the tumor was upregulated after irradiation at different time points and dosages. From the quantification of the IF staining, we can see that at 4 hours

after RT, the VCAM1 molecule was upregulated with 2Gy (*p<0.05) and 10Gy (**p<0.01) (*Figure 17*-A). At 24h after RT, the expression level of the VCAM1 molecule was significantly increased with 2Gy (***p<0.001), 4Gy(****p<0.0001), 6Gy(****p<0.0001), and 10Gy (****p<0.0001) (*Figure 17*-A). At 48h after irradiation, the expression level of the VCAM1 molecule was still upregulated with 2Gy (****p<0.0001), 4Gy (**p<0.01), and 10Gy (***p<0.001) (*Figure 17*-A). The gene abundance of VCAM1 had a similar trend with the molecule expression. The qPCR results showed that VCAM1 gene abundance had no significant change at 1.5 hours after irradiation (*Figure 17*-B). However, at 4 hours timepoint after irradiation, the gene expression of VCAM1 was upregulated with 2Gy (***p<0.001) (*Figure 17*-B). At 24h after irradiation, the gene abundance level of VCAM1 was increased with 0.5Gy (****p<0.0001), 2Gy (****p<0.0001), 4Gy (****p<0.0001), 6Gy (****p<0.0001) and 10Gy (**p<0.01) (*Figure 17*-B).


A
0.5Gy
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6Gy
10Gy
VCAM1
DAPI
Merge
B
0.5Gy
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6Gy
10Gy
VCAM1
DAPI
Merge
C
0.5Gy
2Gy
4Gy
6Gy
10Gy
VCAM1
DAPI
Merge
D
VCAM1
DAPI
Merge
Control
Isotype control

Figure 16: Immunofluorescence images of VCAM1 on bEND5 cells. VCAM1 channel (red) was stained to show the expression of the VCAM1 molecule on the surface of bEND5 cells. DAPI channel (blue) was stained to identify the cell nuclei of bEND5 cells. Samples of 0.5G, 2Gy, 4Gy, 6Gy and 10Gy groups respectively represent bEND5 cells were irradiated with 0.5G, 2Gy, 4Gy, 6Gy and 10Gy. A: bEND5 cells were collected and stained at 4 hours after irradiation. B: bEND5 cells were collected and stained at 24 hours after irradiation. C: bEND5 cells were collected and stained at 48 hours after irradiation. D: Samples in the control group were bEND5 cells without treatment and stained with VCAM1 and DAPI channels. Samples in the isotype control group were only stained with secondary antibodies. Scale Bar=30μm

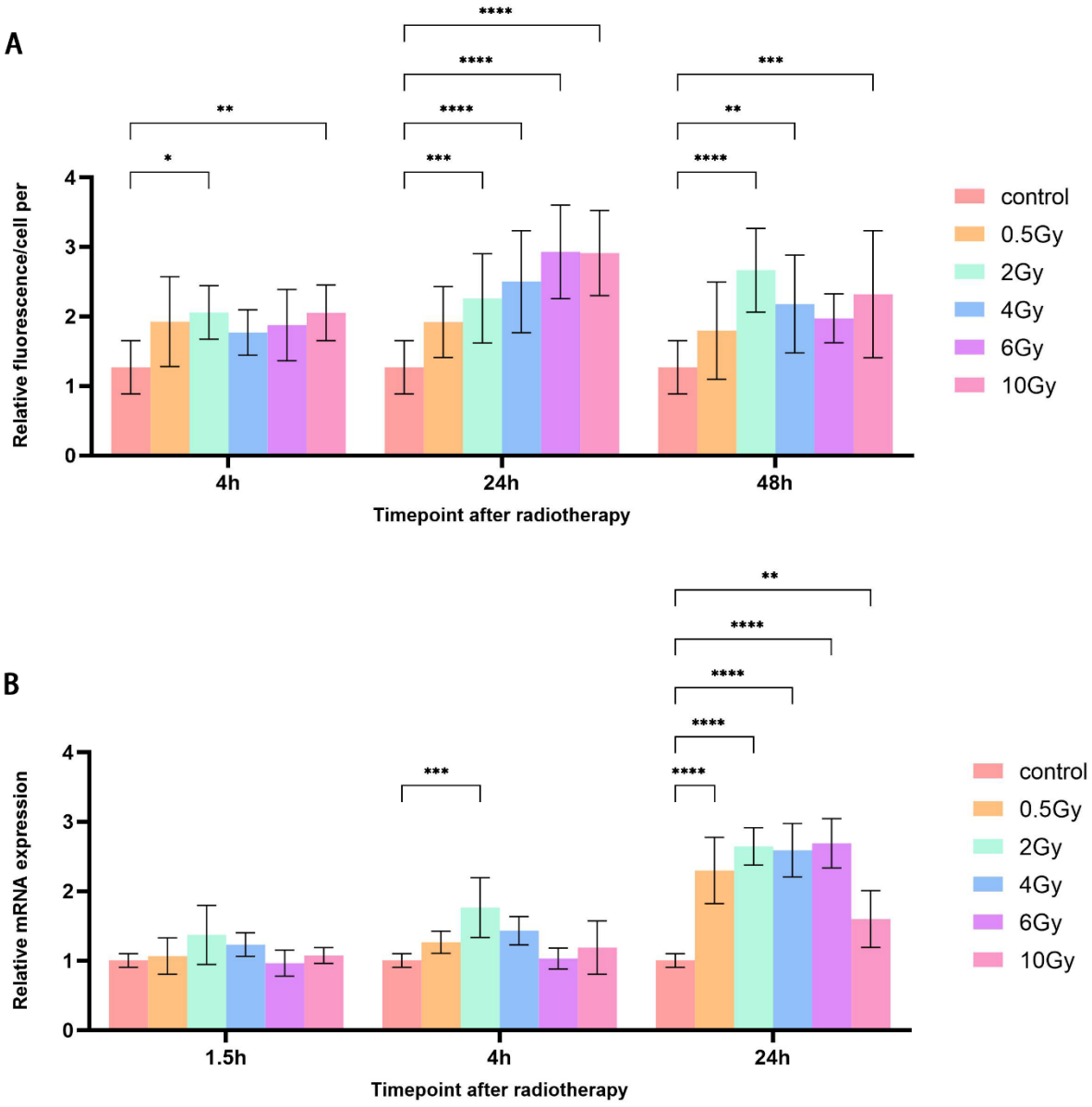

Figure 17: The regulation of VCAM1 on bEND5 cells after irradiation in vitro. A: Quantitative immunofluorescence data illustrating the effect of different doses of radiotherapy on bEND5 cells. B: qPCR confirmation of the gene expressions was performed using GAPDH as the reference control, and bEND5 cells irradiated with 0.5 Gy, 2 Gy, 4 Gy, 6 Gy, and 10 Gy were

*compared with untreated cells. Results were expressed as Mean±SD. Statistically significant differences are shown as * = p ⩽ 0.05; ** = p ⩽ 0.01; *** = p ⩽ 0.005; **** = p ⩽ 0.001.*

5.9. Mouse Inflammation Cytokine

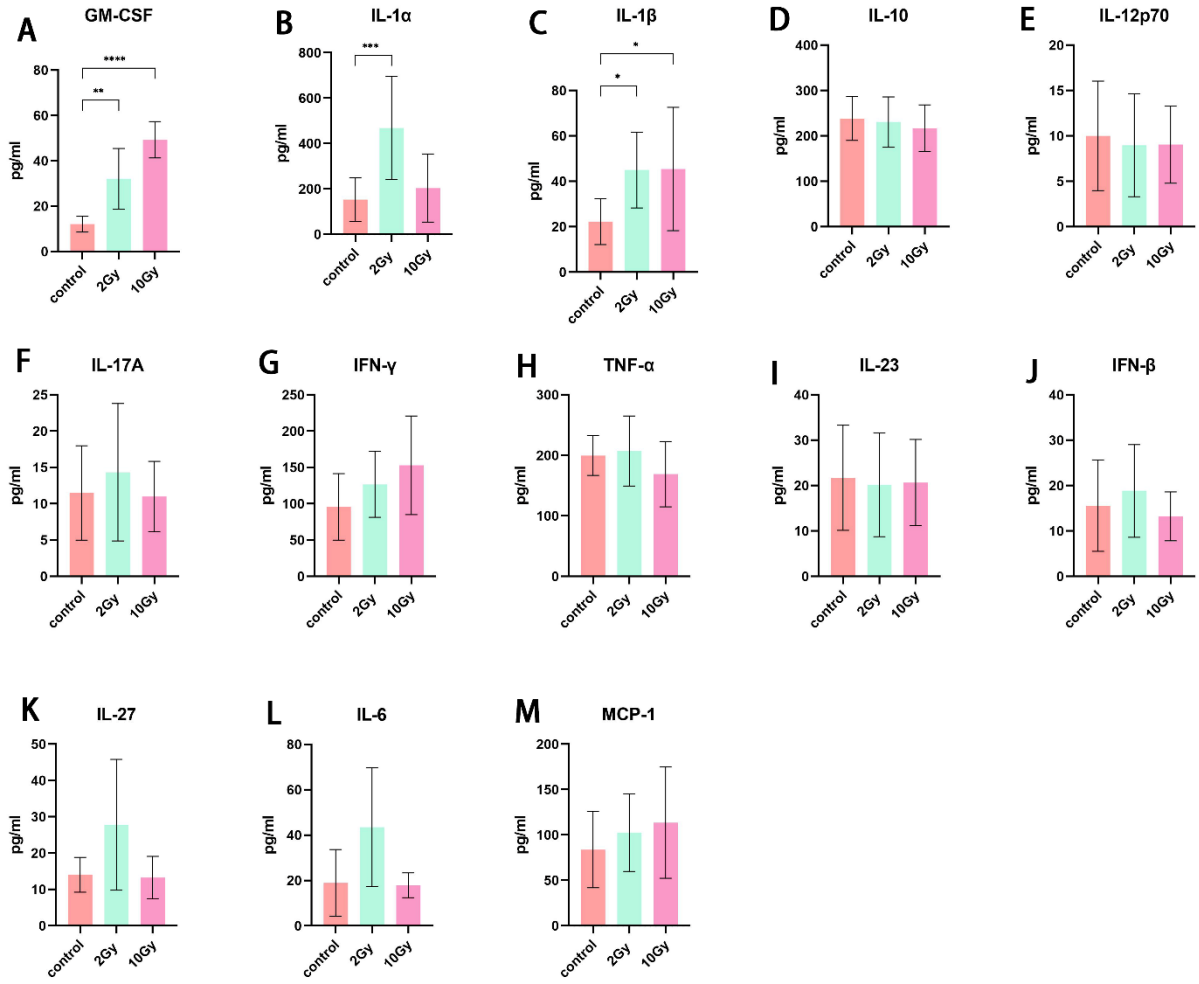

*Figure 18: A-M: Regulations on levels of cytokines in the tumor supernatant at 24h after RT. Results were expressed as Mean±SD. Statistically significant differences are shown as * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.005; **** = p ≤ 0.001.*

To extend the detection of the underlying influence of RT on the inflammatory environment of the TME, we measured the inflammation-related cytokines released into the tumor supernatant 24 hours after RT (*Figure 18*). The LEGENDplex™ Mouse Inflammation Panel allows simultaneous quantification of 13 mouse cytokines, including IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF. We tested the supernatant of the tumor at 24 hours after RT with the Mouse Inflammation Panel. The results show that GM-CSF was significantly upregulated at 2Gy (**p<0.01) and 10Gy (****p<0.0001), and it was worth noting that changes in GM-CSF levels seemed correlated with dose (*Figure 18*-A). IL-1α was upregulated at 2Gy (***p<0.001) (*Figure 18*-B), and IL-1β

was also significantly upregulated after RT at 2Gy (*p<0.05) and 10Gy (*p<0.05) (*Figure 18*-C), but the other cytokines, such as IL-23, IFN-γ, TNF-α, CCL2 (MCP-1), IL-12p70, IL-10, IL-6, IL-27, IL-17A, IFN-β seem unaffected by radiation therapy(*Figure 18*-D-M).

6. Discussion

6.1. Radiotherapy regulates the expression levels of vascular adhesion molecules

Our study explored whether radiotherapy affects the expression of P-selectin, E-selectin, ICAM1, and VCAM1 on the surface of vascular ECs. The combination of RT and immunotherapy has become one of the focuses of cancer treatment research in recent years[256]. The study of the mechanism of RT-promoting immunotherapy can provide direction and a basis for further research on the efficacy of combined therapy. The TEM of leukocytes is a crucial process for immunotherapy to exert its immune effect, and the related adhesion molecules P-selectin, E-selectin, ICAM1, and VCAM1 on the surface of ECs play a vital role in the TEM of leukocytes[257]. In this study, we tried to investigate the potential possibilities and mechanisms for the synergetic effect of RT and immunotherapy by observing the effect of RT on the expression of adhesion molecules P-selectin, E-selectin, ICAM1, and VCAM1 on ECs in the tumor and on bEND5 cells *in vitro* directly, and by testing the level of inflammatory-related cytokines in TME before and after RT. qPCR is one of the most frequently used techniques to quantify gene expression in biological samples[258]. IF staining is a crucial immunochemical technique that combines specific antibodies labeled with fluorophores, which can detect and localize multiple antigens visually in various cells and different types of tissues with significant advantages, such as excellent sensitivity and signal amplification capabilities[259]. Therefore, we designed experiments to observe the effect of RT on adhesion molecules with these two methods. In the animal experiment, we planted KP983.3 cells on the mice plants and irradiated the flank tumors with 2Gy and 10Gy. 24 hours after irradiation, the tumors were collected, and the adhesion molecules were detected.

For P-selectin, we can see that in the *in vivo* experiment, IF staining showed that the signal expression was upregulated at 2Gy after RT. Although P-selectin also seemed to have an upward trend at 10Gy, this was not significantly different. From the result of qPCR, we can see an increase in P-selectin gene expression at not only 2Gy but also 10Gy. We can conclude from the consistent gene expression and molecule staining that the RT can induce P-selectin expression at 24h after irradiation in TME. It should be noticed that qPCR showed that P-selectin gene expression was upregulated after irradiation with 10Gy. In contrast, the IF staining showed that the P-selectin molecule's expression level did not significantly increase. First, this may be because there is still a translation process from mRNA to protein[260]. The high radiation dose of 10Gy may affect the expression of P-selectin. Unfortunately, our experiment did not reach the time when the protein began to show significant differences, or the complex procedures from mRNA to protein, such as translation and translation post-translational modification (PTM), were inhibited, so the protein

expression was not upregulated with mRNA. Second, the P-selectin could be stored in the Weibel–Palade bodies (WPBs)[261]. RT might also have a certain effect on the release of P-selectin. However, the specific reasons need further exploration.

To investigate whether RT can directly induce P-selectin expression, we irradiated bEND5 cells *in vitro* with dose gradients from 0.5Gy to 10Gy. Considering the time difference between gene expression and protein expression, we set the time points of the qPCR experiment earlier than IF staining. We can see that at 0.5Gy, both gene expression and molecule expression of P-selectin on bEND5 cells were significantly upregulated at 24h after irradiation. At the dose of 2Gy, the result of IF staining showed that P-selectin was upregulated as early as 4 hours after irradiation, and there was still a significant upregulation at 24 hours after irradiation, but without any difference of 48 hours after RT. The gene expression level was also upregulated at 4 and 24 hours after RT but without any difference at 1.5 hours after RT at 2Gy. At the dose of 4Gy, both gene expression and molecule expression of P-selectin on bEND5 cells were significantly upregulated at 24h after irradiation. At the dose of 6Gy, gene expression was significantly upregulated at 24h after irradiation, while molecule expression did not have any significance. At 10Gy, the result of IF staining was upregulated as early as 4 hours after irradiation, while gene expression only started to have an upregulation at 24 hours after irradiation.

There is some unconformity between gene expression and molecular expression. At the radiation dose of 10Gy, molecular expression showed significant differences 4 hours after RT, while gene expression did not change significantly yet at this time point after RT. There was an increase in gene expression at the dose of 6Gy at 24 hours after RT, but we did not see any changes in molecular expression. These phenomena indicated a discrepancy between gene expression levels and protein expression levels. The complex correlation between mRNA and protein levels has long been debated. Theoretically, mRNA abundance should be consistent with the protein expression level because protein is translated from mRNA. However, many studies showed no apparent correlation between mRNA and protein expression levels. Chen et al. reported that only 17% of protein spots were associated with the corresponding mRNA levels in lung adenocarcinomas in their study[262]. Gu et al. considered that the upregulation of protein levels could be regulated at the translational level[263]. Prabahar et al. found that there was no correlation between specific mRNA and protein expression under different conditions[264]. It is speculated that the reasons for this phenomenon may be as follows: First, the analytical technology needs to be further improved, and there is a time lag between changes in gene and protein levels[265,266]; Second, protein expression and stability are regulated by PTM, so there will be inconsistencies with mRNA changes[267]; Third, the buffering effect of the gene proximity effect may buffer the expression of protein or mRNA[268]. Therefore, in the *in vitro* experiment, the inconsistency

between the changes in gene expression abundance and protein expression levels under high-dose RT may be due to the regulatory effect of high-dose radiation on PTM. This issue needs further verification. We can conclude that irradiation can upregulate the expression of P-selectin not only on ECs in the TME *in vivo* but also on bEND5 cells directly. However, we did not see a clear correlation with radiotherapy doses, which may require further exploration.

For E-selectin, we can see that the molecule expression on the ECs in the tumor after RT was upregulated with the doses of 2Gy and 10Gy in both gene abundance and molecule level results. Thus, we can conclude that RT induces E-selectin expression on the ECs in the TME *in vivo*. The cell experiment was designed to investigate if RT stimulates ECs directly *in vitro*. From the results of the qPCR experiment *in vitro*, we can see that at the dose of 0.5Gy, gene expression of E-selectin was upregulated at 24 hours after RT. Still, the E-selectin molecule did not have any significance. With the irradiated dose of 2Gy, both gene expression and protein expression levels increased at 4 hours and 24 hours after RT. Still, the E-selectin molecule was also upregulated at 48 hours after RT. At 4Gy, gene abundance was increased 24 hours after RT, and the molecule expression level was upregulated at 24 and 48 hours after RT. For 6Gy, the gene expression was upregulated at 24 hours, and the E-selectin level was increased as early as 4 hours after RT and lasted until 48 hours after RT. For 10Gy of irradiation, gene abundance was increased 24 hours after RT, and the molecule expression level was upregulated at 24 and 48 hours after RT. We can see that the gene expression of E-selectin was upregulated, but there was no difference in relative immunofluorescence with the dosage of 0.5Gy. In addition, at 4 hours after RT, we can see significant upregulation of IF staining, but there was no significant result of qPCR at the dosage of 6Gy. The reason for this result may also be complicated. First, it was probably because the time of increased gene amplification was inconsistent with the time of increased expression of protein molecules, and my experiment did not capture the correct time point of gene amplification. Second, RT might have inconsistent effects on gene transcription into mRNA and translation into protein. Kraiss et al. reported that, under certain conditions, the E-selectin protein level was regulated while the E-selectin mRNA did not change because the translation was inhibited[269]. So, just as the inconsistency of gene and molecule expression results in P-selectin in the *in vitro* experiments, perhaps RT affects the process of translation and PTM. Multiple genes regulate protein expression and gene transcription, and the impact of RT on these processes needs to be further verified.

Therefore, RT upregulated E-selectin on the surface of TECs in the TME *in vivo* and on the surface of bEnd5 cells *in vitro*. Changes in the expression level of E-selectin on the surface of bEND5 cells reflect the direct effect of RT on ECs. We can see that E-selectin was upregulated as early as 4 hours after RT at the dose of 2Gy, and 24 hours after RT seems to

be the optimal time point because almost all doses of radiotherapy in the dose gradient we set up regulated E-selectin's expression level.

The mouse experiment indicated that both gene abundance and molecule expression were significantly upregulated for ICAM1 at 2Gy and 10Gy. Thus, we could conclude that RT induced the expression of ICAM1 on the ECs in the TME at the radiation doses 2Gy and 10Gy 24h after RT. In the cell experiment, for 0.5Gy of RT, the gene abundance of ICAM1 on bEND5 cells showed a significant upregulation 24 hours after RT, but the protein level did not change. At 2Gy, the ICAM1 mRNA level was increased as early as 4 hours after RT, and the upregulation lasted till 24 hours after RT; the protein level on the surface of bEND5 cells began upregulating at 24 hours after RT and still increased at 48 hours after RT. At 4Gy and 6Gy, the ICAM1 mRNA level was increased 24 hours after RT, and the protein level was upregulated 24 and 48 hours after RT. For 10Gy, the ICAM1 mRNA level was increased 24 hours after RT, and the protein level was upregulated 48 hours after RT.

It should be noted that gene abundance significantly increased when receiving 0.5Gy, but IF staining did not show significant changes. The unconformity between gene and molecule expression in bEND5 cells may be because unknown regulatory mechanisms regulate PTM or protein expression with the RT dose of 0.5Gy, or we missed the right time when the protein translation was completed. In addition, ICAM1 gene abundance was upregulated 24 hours after RT, and the protein level did not increase until 48 hours after RT. This phenomenon may be because RT upregulated the expression level of ICAM1, but the mRNA translation was not completed at 24 hours after RT. We can conclude that irradiation directly influenced the ICAM1 expression on the surface of bEND5 cells. We searched some relevant studies and found that Kiyohara et al. reported that ICAM1 molecule expression on the surface of HUVECs increased at 48 hours after RT with the radiation doses of 5Gy and 10Gy in their experiment.[270] Though it was an experiment with a cell line from a different origin, it might be possible to provide some evidence for our inferences regarding the effect of direct irradiation on ICAM1 expression on the surface of ECs.

Furthermore, the changes of ICAM1 before and after RT have a certain consistency in the results of *in vivo* and *in vitro* experiments. At 24 hours after RT with the dose of 2Gy, the ICAM1 expression on the surface of ECs in tumor and Bend5 cells increased significantly, which showed that RT can not only directly activate ECs but also increase the expression level of ICAM1 under the joint action of multiple influencing factors in the complex TME. However, the effect of 10Gy RT on endothelial cells is more complicated. In the *in vitro* experiments, IF staining did not show any significant changes yet at 24 hours after RT, but in the *in vivo* experiments, both gene abundance and molecular expression were significantly improved at the dose of 10Gy at 24 hours after RT. This phenomenon indicates that the effect of 10Gy radiation on ICAM1 expression in the TME is more stable than in vitro. And it

might be because that ICAM1 was also affected by other factors in TME. It was reported that IL-1β induced the expression of ICAM1[271]. Our experiment demonstrated that the IL-1β level in the TME was upregulated after RT. Therefore, RT promoted the secreting of IL-1β that induces the expression of ICAM1 in the TME, which should contribute to the difference between *in vivo* and *in vitro* experiments.

For VCAM1, in the animal experiments, the gene abundance and molecular expression showed significant increases after the tumor received 2Gy and 10Gy of radiation simultaneously. This animal experiment first determined that the expression of VCAM1 on the surface of ECs in the TME was significantly upregulated after receiving 2Gy and 10Gy of RT. To further explore the effect of radiation on the expression of VCAM1 on the surface of ECs, bEND5 cells were irradiated with different doses and analyzed *in vitro*. The result showed that when receiving 0.5Gy of RT, the VCAM1 mRNA level of bEND5 cells was upregulated 24 hours after RT, but the VCAM1 protein expression level was insignificant. At 2Gy, the mRNA level of VCAM1 was upregulated at 4 and 24 hours after RT, and the protein level was upregulated at 4, 24, and 48 hours after RT. For 4Gy, the results showed that the gene abundance was increased 24 hours after RT, and the protein expression on the surface of bEND5 was upregulated 24 and 48 hours after RT. At the dose of 6Gy, the mRNA and the protein levels of VCAM1 were increased at 24 hours after RT. For 10Gy of irradiation, the gene abundance was increased 24 hours after RT, and the protein expression on the surface of bEND5 was upregulated at 4, 24, and 48 hours after RT. There was also some dissimilarity between gene abundance and protein expression. The qPCR result was upregulated but without significance for the IF staining result at 0.5Gy. This means that when receiving an RT dose of 0.5Gy, the translation process of the VCAM1 molecule may be affected so that the IF staining of protein molecules detects no corresponding changes. At the RT dose of 10Gy, the protein level increased as early as 4 hours after RT, but the gene abundance did not significantly change until 24 hours after RT. Though studies reported that many factors disrupt the coordination between mRNA and protein expression[272,273,274], we have not found related studies about the transcription and translation of the VCAM1 gene yet. The cell experiment demonstrated that RT directly upregulates the VCAM1 mRNA and protein levels, but the mRNA translation may be worth researching. Mollà et al. also reported that 24 hours after receiving 10Gy of RT, the expression of VCAM1 in mice was significantly upregulated, and the upregulation of VCAM1 occurred later than ICAM1[275]. This is similar to our results, and we found that 2Gy of RT already causes the upregulation of the molecular expression.

Our results from the comprehensive analysis verified that the expression levels of P-selectin, E-selectin, ICAM1, and VCAM1 on the surface of ECs are upregulated after irradiation. In *in vivo* experiments, our study aimed to verify whether RT affects the expression levels of P-

selectin, E-selecting, ICAM1, and VCAM1 in the complex TME. We set a low radiation dose of 2Gy and a high radiation dose of 10Gy to study the dose-dependent effects on target molecules. IF staining showed that P-selectin, E-selectin, ICAM1, and VCAM1 expressed on the surface of ECs of vascular in TME. The fluorescence intensity of P-selectin, E-selectin, ICAM1, and VCAM1 was significantly increased at 2Gy of RT, and the fluorescence intensity of E-selectin, ICAM1, and VCAM1 was also significantly increased at 10Gy of RT. However, there was no difference between 2Gy and 10Gy. The result of qPCR showed that gene abundance of P-selectin, E-selectin, ICAM1, and VCAM1 on the surface of ECs of vascular in TME was also upregulated, and there was also not any difference between 2Gy and 10Gy. Therefore, our study verified that RT increased the expression levels of P-selectin, E-selectin, ICAM1, and VCAM1 ligands on the surface of ECs. It has been known that P-selectin and E-selectin on ECs are receptors that bind to specific ligands on immune cells to promote leukocyte recruiment[276], and ICAM1 and VCAM1 on ECs mainly promote the adhesion between leukocyte and ECs[277]. Thus, the expression levels of P-selectin, E-selectin, ICAM1, and VCAM1 should affect TEM, and many researches have confirmed the importance of selectins and adhesion molecules on leukocyte recruitment and TEM from the microvasculature to the site of inflammation[278,279,134,280]. Zuchtriegel et al. verified that P-selectin, together with P-selectin glycoprotein ligand-1, initiates the TEM of neutrophils, and E-selectin launches the recruitment of monocytes in the inflammatory response[281]. Jesus et al. reported that neutrophil adhesion and vascular TEM were promoted when the level of ICAM1 expression on ECs was induced to increase[282]. VCAM1 was also identified to regulate inflammatory vascular adhesion and to help the TEM of leukocyte[283]. Our study demonstrated that when the tumor received 2Gy of RT, the expression levels of P-selectin, E-selectin, ICAM1, and VCAM1 on the surface of ECs of vascular in TME increased significantly, and when the tumor received 10Gy of RT, the expression levels of E-selectin, ICAM1, and VCAM1 *in vivo* also increased significantly. This may promote the vascular recruitment and TEM of immune cells and stimulate an anti-tumor immune response, which may be the mechanism of the synergistic effect of combined RT and immunotherapy. There are some publications related to RT and adhesion molecules. Mollà et al. irradiated the abdomen of mice with 4Gy and 10Gy, and found that P-selectin on ECs in the abdomen increased at as early as 2 hours after RT. Though the dose and irradiated tissue differ from our experiment, it is still evidence that RT upregulates the expression of P-selectin *in vivo*. Sławiński reported that the levels of ICAM1 and VCAM1 in the blood of lung cancer patients who received RT were upregulated[284]. The difference is that our experiments tested tumor tissue in animal models, but the common ground is that RT regulates expression levels of adhesion molecules.

To further investigate the direct effects of RT on ECs and try to find out the best dose and time point after RT for activating ECs, we irradiated bEND5 cells *in vitro* with different doses and stained and abstract RNA at various time points. Considering the time lag from gene amplification to protein translation, we set the time points of qPCR earlier than IF staining. At first, it was verified that RT upregulated the expression levels of P-selectin, E-selectin, ICAM1, and VCAM1 on the surface of bEND5 cells. Christopher et al. gamma irradiated a human brain endothelial cell line with 50Gy and found that E-selectin was increased at 24 hours after irradiation, and VCAM1 was elevated slightly but decreased at 48 hours, and ICAM1 was not altered significantly[285]. Schröder et al. irradiated human EC EA.hy926 cells with doses between 0.1Gy and 6Gy and concluded that ICAM1 and VCAM1 were upregulated at almost all of the doses they set, while E-selectin did not change significantly[151]. However, some other studies reported that E-selectin was upregulated as early as 4 hours after RT[286,287] or started to increase after 48 hours[288]. There are some differences between these studies on ECs, which may be because the experiment conditions and cell lines are different. Our results demonstrated that the gene and protein levels of P-selectin, E-selectin, and VCAM1 on ECs were increased as early as 4 hours after RT with 2Gy. The gene expression of ICAM1 was upregulated as early as 4 hours after RT. The time after radiotherapy was prolonged until 24 hours after RT, and the expression levels of adhesion molecules increased over time at almost all radiation doses. Moreover, the expression levels of E-selectin, ICAM1, and VCAM1 increased at 48 hours after RT. The cells receiving 2Gy of RT seemed activated earlier, but we did not see any significance between doses 24 hours after RT.

6.2. Radiotherapy regulates the level of inflammation-related cytokines in TME

TME is a complex system, and RT affects ECs and may change other components of the TME. And ECs may also be stimulated by different factors in TME. Our previous research confirms that TNFα and IFNγ stimulated ECs to increase the expression of adhesion molecules[289]. We assumed that RT may change the TME and thus affect the inflammatory response. We detected the changes in inflammatory-related factors in the TME before and after radiotherapy. To evaluate the inflammation-related changes in the TME, changes in inflammatory cytokines levels in the TME before and after RT were detected. At the dose of 2Gy, we found that the levels of IL-1α, IL-1β, and GM-CSF were upregulated. At the RT dose of 10Gy, the levels of IL-1β and GM-CSF were upregulated, while IL-1α did not have any difference anymore, though it was slightly elevated. GM -CSF is widely considered to play an important role in inflammatory response. GM-CSF stimulates stem cells to produce granulocytes (neutrophils, eosinophils, and basophils) and monocytes, which migrate into

tissues and mature into macrophages and dendritic cells, and the immune response may be effectively activated by this process[290]. Many studies reported that GM-CSF treatment combined with RT or combined with immunotherapy improved the therapeutic effect of cancer patients[291,292,293]. Our study also found that RT increased the secretion of GM-CSF, and this is similar to other publications in that the level of GM-CSF was also upregulated in normal tissues of mice[294]. The interleukin family is a group of cytokines that play a key role in regulating innate and adaptive immune responses during the development of inflammation in the host[295]. IL-1α is present in many types of cells and can be released when cells undergo necrosis and act as an alarmin, while IL-1β can be expressed when myeloid monocytes are stimulated[296]. IL-1α and IL-1β are important pro-inflammatory active factors[297]. Therefore, detecting changes in their levels is important for evaluating the inflammatory microenvironment of the TME. Rübe et al. irradiated the thoraces of mice with mice and found that TNF-α, IL-1α, and IL-6 in the lung tissue were increased at 6 hours after RT[298]. Chen et al. also reported that the levels of IL-1α and IL-6 in the blood of cancer patients increased after RT[299]. Han et al. found that s RT upregulates the level of IL-1β in the tumor of mice[300], while Chang et al. irradiated mesothelioma cell lines and found that the levels of IFN-γ and IL-6 were increased[301]. Our study has some consistency with the publications that IL-1α, IL-1β, and GM-CSF have been proven to have a synergistic effect with immunotherapy and play an important role in inflammation, which means that IL-1α, IL-1β, and GM-CSF may become targets for combined RT and immunotherapy. In addition, it was reported that the expression of ICAM1 can be upregulated by many inflammatory mediators, concluding TNF α, IFNγ, and IL-1β[302], IL-1 can upregulate the expression level of E-selectin[303]. So the secretion of IL-1α and IL-1β helps to stimulate ECs and upregulate E-selectin and ICAM-1 expression in the TME after RT.

Therefore, we can assume that in addition to direct damage to tumor cells and stimulating ECs to promote leukocyte recruitment, RT also regulates the TME to promote the inflammatory response. The release of inflammatory factors indicates that the immune response of the TME may be reactivated and also promotes the ability of vascular ECs to transport immune cells, providing a theoretical basis for the combined treatment of RT and immunotherapy. There are two core elements of the TME: the immune environment and chronic inflammation, and inflammatory response is an important part of the tumor microenvironment[304,305]. Compared with normal tissues, the tumor microenvironment is in a state of chronic inflammation and has reached a balanced state that evades immunity. Therefore, in the TME, the immune system cannot play an immune role in killing cancer cells. Immunotherapy promotes the treatment of tumors by suppressing immune escape and improving immune response[16]. There are also problems to be solved: many patients who express the receptor ligand PD-L1 in their tumor tissue and PD-1 on their lymphocytes do not

benefit from immunotherapy alone[70,71,72]. In addition, based on clinical cases, many studies have found that immunotherapy and RT synergize in promoting the therapeutic effect[306,307,308]. Our experimental results provided a direction for the mechanism of combined treatment of RT and immunotherapy. In addition to directly killing cancer cells, RT may also help change the TME by increasing the release of inflammation-related cytokines to activate the system's immune response and increasing the expression levels of vascular ECs adhesion factors to promote the migration of immune cells across the endothelium to eliminate tumor cells.

6.3. Conclusion

This study verified that RT directly upregulates the expression levels of adhesion receptors of vascular ECs, concluding P-selectin, E-selectin, ICAM1, and VCAM1, and increases the levels of inflammatory cytokines IL-1α, IL-1β, and GM-CSF in TME, and this may help explain why some patients who received combined therapy of RT and immunotherapy had better therapeutic effects. It has been reported that the upregulation of P-selectin, E-selectin, ICAM1, and VCAM1 promotes the recruitment and TEM of leukocytes in microvascular to tumor sites, which provides a direction for further research to improve the efficacy of combined RT and immunotherapy for cancer treatment. However, there are still many questions that are worth further research. It is necessary to determine the optimal RT dose and time point after RT to promote the TEM of leukocytes the most effectively and help patients achieve the best therapeutic effect. Our experimental results show that the expression levels of P-selectin, E-selectin, ICAM1, and VCAM1 on the surface of vascular endothelium in the TME were significantly increased at 24 hours after receiving 2Gy and 10Gy of RT. The expression levels of P-selectin, E-selectin, and VCAM1 on bEND5 cells *in vitro* were significantly increased as early as 4 hours after receiving 2Gy of RT. However, clinical RT is a process of fractionated dose accumulation. Many clinical cases have shown that the therapeutic effect of immunotherapy in patients receiving a total radiation dose is significantly better than that of immunotherapy alone[306]. Therefore, in the process of tumors receiving a full RT dose, the regulatory changes of vascular endothelial adhesion receptors in the tumor microenvironment and adhesion factors related to the TEM of immune cells are worthy of further exploration to find the best combined RT and immunotherapy treatment plan. In addition, we also found that RT increased the levels of inflammation-related cytokines IL-1α, IL-1β, and GM-CSF in TME. The upregulation of inflammation-related cytokines after RT means that RT changed the inflammation environment in TME and may activate an anti-tumor inflammatory response. Therefore, regulating inflammation-related cytokines should be another target of the study of combination therapy with RT and immunotherapy.

6.4. Outlook

RT has existed independently as a treatment modality for a long time, and inflammation is a double-edged sword for cancer patients. Although studies have proved that inflammatory factors are higher than in ordinary people in the blood of cancer patients, the success of immunotherapy informs us that it is also important to help more T cells enter tumor tissues in the treatment of cancer, which can help stimulate inflammatory responses and eliminate tumors through the host's immune response. In this thesis, we can see that RT can increase the expression of adhesion factors P-selectin, E-selectin, ICAM1, and VCAM1 on the surface of ECs, which shows that RT has the potential to promote the TEM of leukocytes from the blood to the tumor. The increase of inflammatory cytokines IL-1α, IL-1β, and GM-CSF in the irradiated TME may further encourage and strengthen the translocation of leukocytes. This provides new evidence, ideas, and potential for treating tumors, especially regarding the direction of RT combined with immunotherapy. Of course, how to design radiation therapy to maximize the immune response is still a problem that needs to be solved and studied.

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8. Appendix

8.1. List of figures

8.2. List of tables

9. Advance publication of results

Wagner E, Niu L, Kiljan M, Ibruli O, Hesselmann I, Sahbaz Y, Cai J, Hettich M, Mayer M, Kamp F, Baues C, Marnitz S, Herter JM, Herter-Sprie GS. Impact of radiation therapy on vascular endothelial adhesion receptor expression. Experimental Biology (2021). [R3429, abstract pending publication in FASEB J].

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