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A two-step, two-sample Mendelian randomization analysis investigating the interplay between gut microbiota, immune cells, and melanoma skin cancer

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Abstract

This study aims to rigorously explore the potential causal relationships among gut microbiota (GM), immune cells, and melanoma skin cancer among participants from Europe, where this disease exhibits significant prevalence and profound societal impact. Using the genome-wide association analysis database, a double-sample Mendelian randomization (MR) analysis was drawn upon to investigate GM, immune cells, and melanoma skin cancer. The inverse variance weighted approach was applied to estimate the causal connections among these variables. A two-step MR analysis was employed to quantitatively gauge the impact of immune cells mediated GM on melanoma skin cancer. To address potential sources of bias, such as pleiotropy and heterogeneity, multiple analytical techniques were integrated. The MR analysis pinpointed 6 GM taxa related to either an augmented or declined risk of late-stage melanoma skin cancer. In the same vein, 32 immune cell phenotypes were noticed as correlates with modified risk of melanoma skin cancer. Our study also implies that the probable association between GM and melanoma could be facilitated by 5 immune cell phenotypes. The findings of our study underline certain GM taxa and immune cells as potential influencers on the onset and development of melanoma skin cancer. Importantly, our results spotlight 5 immune cell phenotypes as potential agents mediating this association.

Abbreviations: BAFF-R = B-cell activating factor receptor, CTLs = cytotoxic T lymphocytes, DC = dendritic cells, GM = gut microbiota, GWAS = genome-wide association studies, IVs = instrumental variables, IVW = inverse variance weighted, MDSCs = myeloid derived suppressor cells, MFI = median fluorescence intensities, MR = Mendelian randomization, SNPs = single nucleotide polymorphisms, TME = tumor microenvironment.

Keywords: gut microbiota, immune cells, melanoma skin cancer, Mendelian randomization, two-step analysis

1. Introduction

Cutaneous malignant melanoma, commonly known as melanoma skin cancer, is a highly malignant and invasive solid tumor originating from skin melanocytes.[1,2] Recently, the global incidence of malignant melanoma has been escalating. According to GLOBOCAN's statistical report[3] in 2020, there were over 320,000 new cases and approximately 60,000 deaths were cataloged worldwide. This escalating prevalence underscores the urgent need to enhance melanoma diagnosis, improve treatment modalities, and develop early detection techniques to better patient outcomes.[4] Amidst this backdrop, the scientific community has been exploring alternative pathophysiological pathways that could offer new preventive and therapeutic strategies. Over the past 2 decades, there has been a significant increase in research investigating the

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All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Each study incorporated in the GWAS used in the present study was approved by local research ethics committees or Institutional Review Boards, and all participants had given their informed consent.

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interactions between human diseases and microbial populations.[5] Gut microbiota (GM) has emerged as a critical environmental factor influencing both human metabolism and various pathologies, including melanoma skin cancer. This link is indicative of what is termed as the This association is part of what is known as the gut-skin axis, which potentially plays a crucial role in regulating disease onset and progression, particularly in skin cancer.[6] In recent clinical trials, such as a multicenter, biomarker-stratified, randomized placebo-controlled phase I trial. In a multicenter, biomarker-stratified randomized placebo-controlled phase I trial,[7] GM has shown promise in enhancing responses to immune checkpoint blockade therapies. A recent exploration revealed unique patterns in microbial species level genome bins and pathways correlating with patient progression free survival over 12 months in contrast with those with <12 months progression free survival among 175 patients undergoing immune checkpoint blockade treatment for advanced melanoma, suggesting a complex interplay between GM and melanoma progression.[8] However, the exact causal mechanisms linking GM to melanoma skin cancer, along with the underlying mechanisms, remain uncertain.

In the context of immune dysfunction, the risk of developing malignant skin tumors increases significantly.[9] Despite advancements in immunotherapy, a substantial portion of patients do not respond to current treatment protocols, primarily because these therapies focus on T cell interactions and functions. The immune landscape of melanoma involves a variety of immune cells, which play significant roles in tumor progression and the overall immune response within the melanoma tumor microenvironment (TME).[10] Melanoma cells have developed several mechanisms to evade immune detection, such as immune recognition defects and epithelial–mesenchymal transition,[11] which complicate treatment efforts. Given the variability in GM and the critical roles played by diverse immune cells in influencing disease progression and treatment outcomes,[12] and the potential effects on the efficacy and tolerance of immune checkpoint inhibitors,[13] innovative therapeutic approaches such as fecal microbiota transplantation could potentially enhance the effectiveness of immunotherapy by altering the GM and boosting immune responses.[14,15] This study proposes investigating the probable causal relationships linking GM, immune cells, and melanoma skin cancer, aiming to identify potential early diagnostic markers and therapeutic targets.

To rigorously explore these relationships, we employ Mendelian randomization (MR), a method that uses genetic variants as instrumental variables (IVs) to estimate the causal effects of exposures on disease outcomes. MR is a research approach that utilizes genetic variants to gauge the causal effect of exposures or phenotypes on disease outcomes, serving as a conceptual analogue to the design of randomized controlled trials. This approach, akin to randomized controlled trials, leverages genetic data from separate genome-wide association studies (GWAS) for GM, immune cells, and melanoma to mitigate confounding and explore the intricate interactions among these elements. The strength of MR over conventional trials lies in its use of IVs—genetic variables correlated with exposure but not with confounders of the exposure-outcome relationship. Recent advancements in this field highlights the methodological frameworks and applications of MR in complex trait analysis and its expanding role in understanding the genetic determinants of disease.[16,17] Assuming these IVs possess no direct causal connection to the outcome except through the exposure, they can provide an unbiased estimate of the causal impact of the exposure on the outcome. This unique feature of MR empowers us to extrapolate a causal connection between GM and melanoma skin cancer. Through a two-sample mediation analysis, this study seeks to dissect the multilayered interactions and elucidate the potential pathways through which GM and immune cells influence melanoma skin cancer, providing a clearer understanding of the underlying genetic and environmental mechanisms.

2. Materials and methods

2.1. Ethics statement

All studies contributing data to the GWAS utilized in this research received prior approval from Human Research Ethics Committee of Ningbo No. 2 Hospital. This approval ensures that each study adhered to the highest ethical standards in the treatment and protection of participants. Moreover, informed consent was obtained from all participants involved, affirming their voluntary participation and understanding of the research purposes. These measures collectively uphold the ethical integrity and compliance of our research with international ethical guidelines.

2.2. Study design

MR analysis must satisfy 3 core assumptions. First is the assumption of relevance, where the IVs must be reliably associated with the exposure factor under study. Second is the independence assumption, where the IVs must not be related to known or unknown confounders. Third is the exclusion limitation assumption, where IVs must exclusively affect the outcome through the exposure factor and not through other direct causal paths. Therefore, firstly, the association hypothesis necessitates a robust correlation between IVs, such as single nucleotide polymorphisms (SNPs), and exposure factors, this can be effectively filtered through GWAS *P*-values to identify SNPs that exhibit strong correlations with these exposure factors. Secondly, the independence assumption mandates that there is no dependence between IVs and confounding factors. Consequently, SNPs linked to confounding variables must be excluded during the analytical process to ensure the integrity of the analysis. Finally, the exclusivity assumption posits that IVs influence outcomes solely through exposure factors rather than via alternative pathways. This assertion requires validation through both biological insights and statistical methodologies. To substantiate these hypotheses, we employed statistical techniques including GWAS *P*-value filtering and MR-Egger tests for pleiotropy assessment. These approaches facilitate an evaluation of the relationship between IVs and exposure factors while mitigating potential confounding influences, thereby enhancing the precision of Mendelian randomization analyses.

In our study, we utilized a two-step MR process to probe into the association between GM and the genetic predisposition for melanoma skin cancer, while also inspecting the potential mediating part played by immune cells. The initial step involved using a two-sample MR analysis to estimate the causal effects of e GM and immune cells on the risk of melanoma skin cancer. This allowed us to identify specific GM and immune cells related to the risk of developing melanoma skin cancer. Subsequently, we assessed the causal impact of particular GM taxa on selected immune cells that were identified in the first step. This part of the analysis focused on quantifying how much each mediator contributes to the influence of GM on melanoma skin cancer. It is critical to highlight that our study design ensured no overlap of study subjects across the analysis. The SNPs used to define exposure and outcome were sourced from distinct GWAS datasets, ensuring the independence of our IVs. The procedural flow of our research is illustrated in Figure 1, which outlines the MR analysis steps, and Figure 2 visually represents the overall study design, providing a clear overview of how each component of the study interacts and contributes to our understanding of the complex interactions tween GM immune cells and melanoma skin cancer.

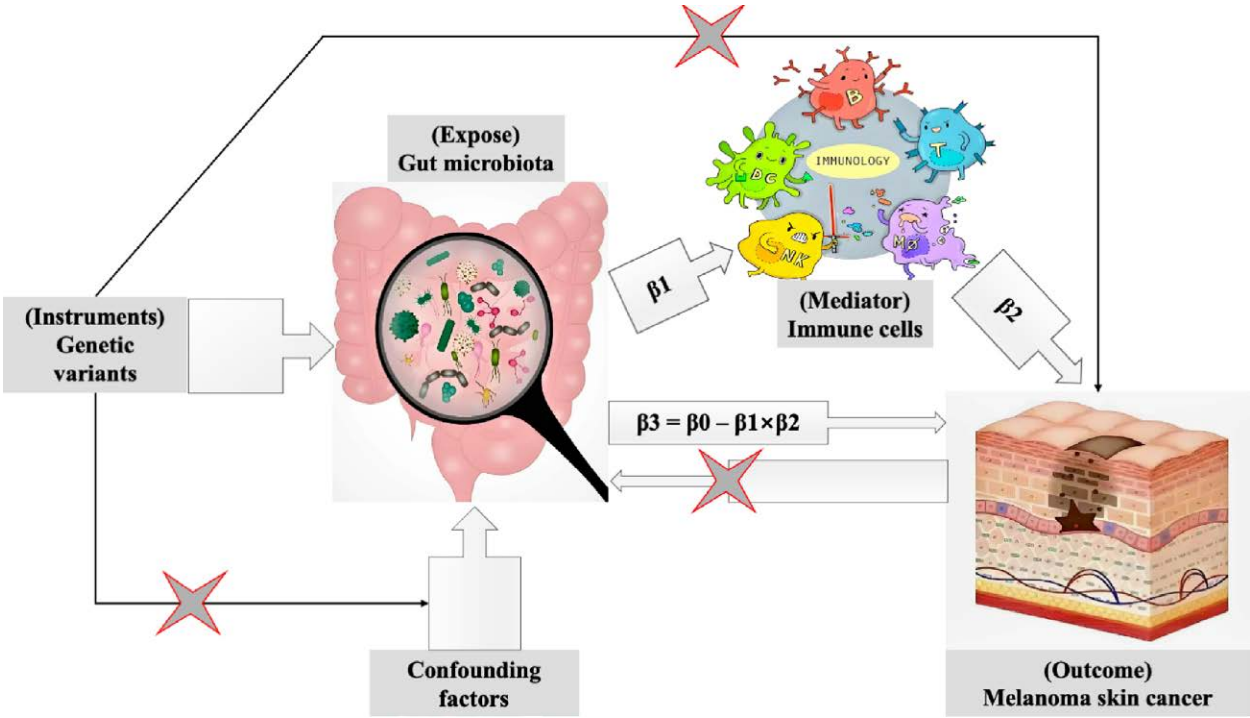

Figure 1. Overview of the Mendelian randomization (MR) analytical framework. This figure illustrates the two-sample MR approach used to investigate the causal relationships between gut microbiota (GM), immune cells, and melanoma skin cancer. The diagram outlines the flow of analysis starting from the selection of genetic instruments from GWAS datasets for GM and immune cells, through to the estimation of their effects on melanoma risk. Key steps include the use of instrumental variables (IVs) to assess causality and the implementation of various statistical methods to address pleiotropy and heterogeneity. GWAS = genome-wide association analysis.

2.3. Data source

The GM data employed to identify relevant variables originated from the MiBioGen Alliance initiative in 2022. This effort carried out all-encompassing genome genotype and 16S fecal microbiome analyses of 18,340 individuals hailing from 24 separate cohorts majorly of European heritage (18 cohorts, equating to 14,306 participants).[17] This meta-analysis of GM was released in 2021, and their study coordinated 16S ribosomal RNA gene sequencing profiles and genotyping data from 18,340 participants in 24 cohorts from the United States, Canada, Israel, South Korea, Germany, Denmark, the Netherlands, Belgium, Sweden, Finland, and the United Kingdom. One of the queue studies is FGFP (Flemish Gut Flora Project), which is a population-based study cohort of 2482 individuals from the Flanders region of Belgium Blood and stool samples of volunteers were collected between June 2013 and April 2016After quality control, 2259 samples had genotype and 16S data (1328 females, 896 males, mean age 52.3 years). Adjustments to the data were made considering gender, age, and primary genetic components. Upon the exclusion of 15 unidentified groups, a total of 196 taxa were recognized as exposure variables. These include 119 genera, 32 families, 20 orders, 16 classes, and 9 phyla. More details regarding these taxa are available in Table S1, Supplemental Digital Content, http://links.lww.com/MD/N863.

The GWAS summary statistics for every immunophenotype used in this study are readily accessible from the GWAS Catalog in 2022, bearing accession numbers ranging from GCST90001391 to GCST9000212117.[18] This data spans a total of 731 immunophenotypes, which are broken down into types such as median fluorescence intensities (reflect surface antigen levels, n = 389), absolute cell counts (n = 118), relative cell counts (n = 192), and morphological parameters (n = 32). It's important to highlight that the median fluorescence intensities, absolute cell counts, and relative cell counts categories envelop various immune cell types. These include B cells, cytotoxic T lymphocytes (CTLs), mature stages of T cells, monocytes, myeloid cells, and the T cells, B cells, natural killer cells group. The morphological parameters category comprises panels relating to CTL and T cells, B cells, natural killer cell sorts. The initial GWAS on the immune signature was conducted using data gathered from 3757 European individuals, with no overlap in cohorts. About 22 million SNPs were genotyped using high-density arrays, followed by imputation employing a Sardinian sequence-based reference panel. Associations were established while factoring in covariates such as age and gender[19] (additional information can be found in Table S2, Supplemental Digital Content, http://links.lww.com/MD/N863).

The GWAS of melanoma were obtained from United Kingdom biobank (https://www.ukbiobank.ac.uk/), with accession number: ieu-b-4969, which included 375,767 samples for (SNPs = 11,396,019) of European ancestry in 2021. All SNPs data of melanoma skin cancer can be found in Table S3, Supplemental Digital Content, http://links.lww.com/MD/N863.

2.4. The selection of instruments

We set filtering conditions for SNPs to act as IVs for GM and immune cells at a *P*-value threshold of $<1 \times 10^{-5}$, which follows standard research procedures.[20] For melanoma skin cancer, we applied even stricter criteria with a *P*-value cutoff of $<1 \times 10^{-5}$. All genetic variants that met these criteria were taken into consideration. To mitigate the potential linkage disequilibrium effects amid SNPs, we established conditions based on an $r^2 < 0.001$ and distance spanning 10 Mb. To ensure a robust correlation between IVs and exposure factors, we employed F-statistics to filter SNPs.[21] The F-statistics entailed calculating the ratio of β to the square of the standard error, with a cutoff set at 10. In addition, to ensure appropriateness of IVs for subsequent analyses, we used a *P*-value $< 1 \times 10^{-5}$ to exclude

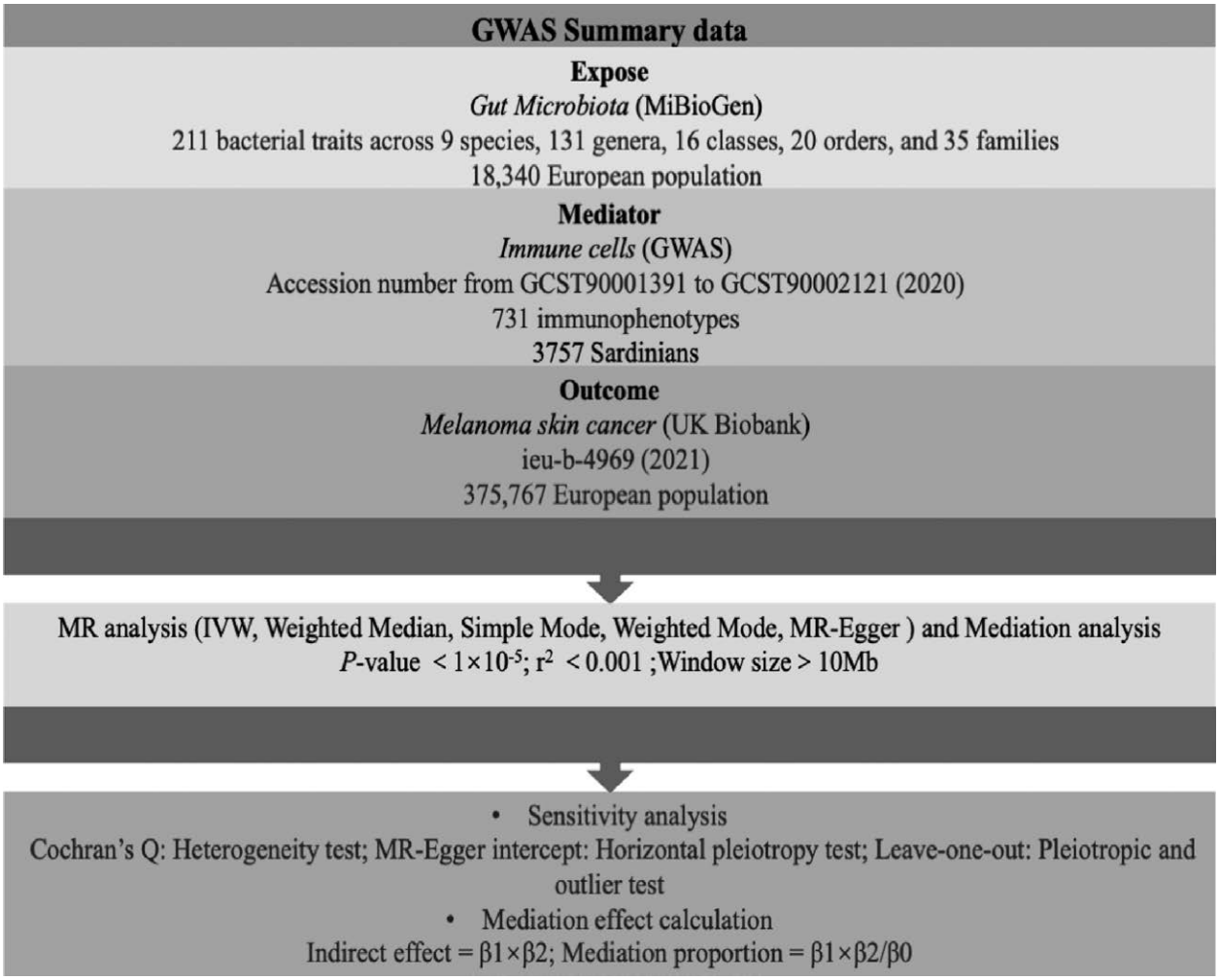

Figure 2. Study design and data sources. The first step involves estimating the impact of GM and immune cells on melanoma using separate GWAS data for exposure and outcome. The second step assesses the mediation effect of immune cells on the relationship between GM and melanoma. The sources of GWAS data for GM, immune cells, and melanoma are also depicted to clarify the separation of data sources, which aids in minimizing confounding. GM = gut microbiota, GWAS = genome-wide association analysis.

palindromic SNPs and incompatible genetic variants strongly associated with melanoma skin cancer.[22]

2.5. MR analysis

To investigate causal factors influencing outcome events while ensuring robust results, we employed 5 methods in two-sample MR: inverse variance weighted (IVW), Weighted Median, Simple Mode, Weighted Mode, and MR-Egger.[23] Upholding the independence and exclusivity assumptions is essential to confirm that IVs do not affect outcomes through unrelated exposure factors.[24] The weighted median method estimates causal effects by weighting the median, without making assumptions about the distribution of IVs. This method is insensitive to horizontal offset and can provide relatively stable estimation results even in the presence of horizontal offset. It is suitable for situations with a large number of IVs and uneven distribution, and can provide more stable estimates of causal effects. The MR-Egger intercept test assesses horizontal pleiotropy and validates the robustness of our findings.[25] The MR Egger method can simultaneously estimate causal effects and horizontal offset, thereby detecting horizontal offset, that is, deviation in the horizontal direction. This method has a high sensitivity to horizontal offset and can identify horizontal deviation issues that traditional MR methods may overlook. It can provide more robust causal inference and is therefore suitable for situations with small sample sizes or the presence of horizontal offset. A *P*-value <.05 indicates the presence of horizontal pleiotropy; otherwise, it is considered absent if above this threshold.[26] In our analysis, we applied the false discovery rate method to adjust IVW *P*-values with a *q*-value threshold of <0.1. Cochran Q statistic and its corresponding *P*-value quantitatively evaluate heterogeneity among selected IVs.[27] Finally, a sensitivity analysis using the "leave-one-out" approach examines each SNP's impact on MR outcomes.[28] Statistical analyses and visualizations were performed using R packages "TwoSampleMR," "VariantAnnotation," and "ieugwasr."

2.6. Analysis of the overall causal effect and the mediation

To evaluate the overall causal impact of GM on melanoma skin cancer, we conducted a two-sample MR analysis using R software, version 4.3.1 (http://www.Rproject.org). Considering heterogeneity and horizontal pleiotropy, we employed the "MendelianRandomization" R package for an initial assessment of the causal relationship between GM and melanoma skin cancer via the IVW method.[29] To validate our findings' robustness, we also applied MR-Egger regression and weighted median analyses.[30,31] Following a two-step MR protocol, we decomposed GM's total impact on melanoma skin

cancer into direct and indirect effects mediated by intermediaries. β0 represents GM's overall influence on melanoma skin cancer. In the first phase, we quantified the causal influence (β1) of GM on mediating variables.[28] In the second phase, we assessed the causal influence (β2) of these mediators on melanoma skin cancer. The proportion of mediated effect relative to total effect was calculated as $R = \beta1 \times \beta2/\beta0$. Finally, the direct impact is represented as $\beta3 = \beta0 - \beta1 \times \beta2$, indicating GM's influence on melanoma skin cancer not mediated by intermediaries.[32]

3. Results

3.1. Total effect of GM on melanoma skin cancer

Our finding reveals that 6 different types of gut bacteria, or taxa, have a significant association with melanoma skin cancer. The MR analyses indicate that certain taxa, identified as genera Veillonella (OR = 1.0028, 95% CI: 1.0004 – 1.0052, *P* = .0238) and Parabacteroides (OR = 1.0037, 95% CI: 1.0004 – 1.0070, *P* = .0259), were found to increase the risk of melanoma skin cancer. In contrast, the other 4 taxa: genera Blautia (OR = 0.9937, 95% CI: 0.9890 – 0.9983, *P* = .0079), Ruminococcaceae UCG013 (OR = 0.9968, 95% CI: 0.9941 – 0.9994, *P* = .0173), Erysipelatoclostridium (OR = 0.9981, 95% CI: 0.9964 – 0.9998, *P* = .0277), and Prevotella7 (OR = 0.9986, 95% CI: 0.9973 – 1.0000, *P* = .0447) appeared to reduce the risk of melanoma skin cancer (Fig. 3). Detailed data can be found in Table S4, Supplemental Digital Content, http://links.lww.com/MD/N863.

3.2. Effect of immune cells on melanoma skin cancer

After examining the causal association between immune cells and melanoma, we found that C-C chemokine receptor type 2 on myeloid Dendritic Cell (OR = 1.0007, 95% CI: 1.0002 – 1.0011, *P* = .0066), CD3 on Central Memory CD8+ T cell (OR = 1.0009, 95% CI: 1.0002 – 1.0015, *P* = .0070), CD4-CD8- T cell %T cell (OR = 1.0014, 95% CI: 1.0003 – 1.0025, *P* = .0107), CD11c+ monocyte %monocyte (OR = 1.0009, 95% CI: 1.0002 – 1.0015, *P* = .0123), CD25++ CD45RA- CD4 not regulatory T cell absolute count (OR = 1.0005, 95% CI: 1.0001 – 1.0008, *P* = .0129), activated CD4 regulatory T cell absolute count (OR = 1.0005, 95% CI: 1.0001 – 1.0009, *P* = .0224), CD14+ CD16- monocyte %monocyte (OR = 1.0004, 95% CI: 1.0001 – 1.0008, *P* = .0254), CD33- HLA DR- absolute count (OR = 1.0006, 95% CI: 1.0001 – 1.0011, *P* = .0275), CD4+/CD8+ T cell (OR = 1.0009, 95% CI: 1.0001 – 1.0017, *P* = .0299), CD39+ CD8+ T cell absolute count (OR = 1.0005, 95% CI: 1.0000 – 1.0010, *P* = .0328), SSC-A on natural killer T (OR = 1.0004, 95% CI: 1.0000 – 1.0008, *P* = .0337), CD28+ CD45RA- CD8+ T cell absolute count (OR = 1.0003, 95% CI: 1.0000 – 1.0006, *P* = .0348), CD127 on CD45RA+ CD4+ T cell (OR = 1.0007, 95% CI: 1.0000 – 1.0013, *P* = .0426) and CD3 on HLA DR+ CD4+ T cell (OR = 1.0007, 95% CI: 1.0000 – 1.0014, *P* = .0475) were significant risk factors in the causal pathway from immune cells to melanoma skin cancer, but on the contrary, interleukin-2 receptor alpha chain (CD25) on transitional B cell (OR = 0.9990, 95% CI: 0.9984 – 0.9997, *P* = .0027), B-cell activating factor receptor (BAFF-R) on IgD+ CD38+ B cell (OR = 0.9995, 95% CI: 0.9991 – 0.9998, *P* = .0043), BAFF-R on IgD- CD38- B cell (OR = 0.9995, 95% CI: 0.9991 – 0.9999, *P* = .0093), CD25 on CD24+ CD27+ B cell (OR = 0.9996, 95% CI: 0.9992 – 0.9999, *P* = .0164), BAFF-R on transitional B cell (OR = 0.9995, 95% CI: 0.9991 – 0.9999, *P* = .0213), CD66b on CD66b++ myeloid cell (OR = 0.9995, 95% CI: 0.9991 – 0.9999, *P* = .0227), BAFF-R on switched memory B cell (OR = 0.9996, 95% CI: 0.9992 – 0.9999, *P* = .0240), CD3 on CD4+ T cell (OR = 0.9994, 95% CI: 0.9988 – 0.9999, *P* = .0263), CD4RA on Terminally Differentiated CD4+ T cell (OR = 0.9996, 95% CI: 0.9993 – 1.0000, *P* = .0282), CD24 on IgD+ CD38- B cell (OR = 0.9995, 95% CI: 0.9991 – 1.0000, *P* = .0316), CD11b on granulocytic myeloid-derived suppressor cells (OR = 0.9996, 95% CI: 0.9992 – 1.0000, *P* = .0361), CD38 on transitional B cell (OR = 0.9994, 95% CI: 0.9988 – 1.0000,

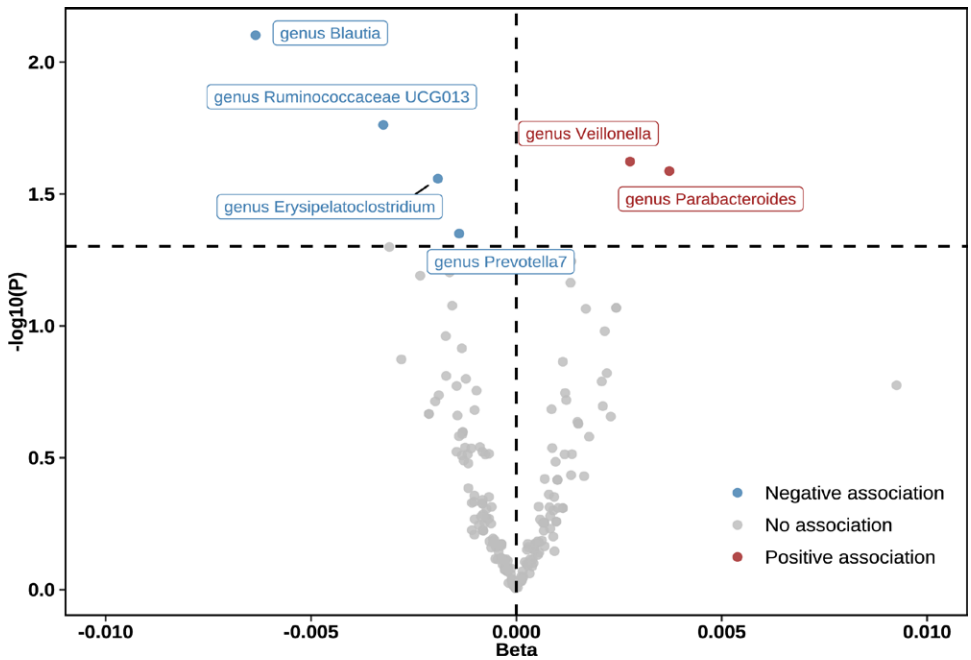

Figure 3. Associations between gut microbiota taxa and melanoma risk. This figure displays the results from the inverse variance weighted (IVW) MR analysis, showing the associations of selected gut microbiota taxa with the risk of developing melanoma skin cancer. Each point represents a different microbial taxon, with their respective effect sizes (odds ratios) and 95% confidence intervals plotted on the *x*-axis. Taxa associated with an increased risk of melanoma are highlighted in red, while those associated with a decreased risk are in green. MR = Mendelian randomization.

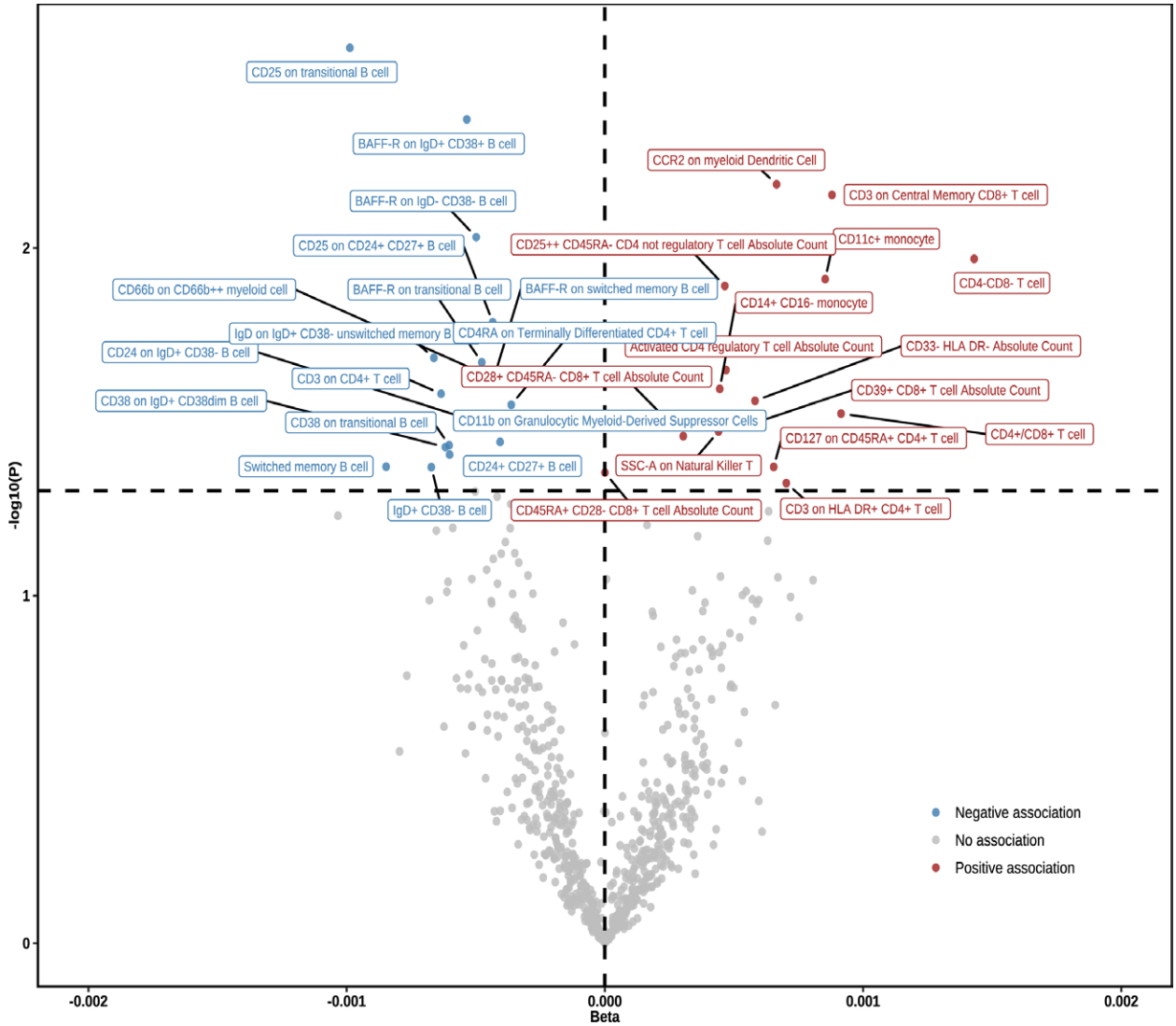

Figure 4. Impact of immune cell phenotypes on melanoma risk. This visualizes the findings from the MR analysis regarding the role of various immune cell phenotypes in modifying the risk of melanoma, the effect sizes and confidence intervals are plotted for each immune cell phenotype. Phenotypes that elevate the risk of melanoma are marked in red, whereas those that mitigate the risk are shown in blue. This figure underscores the complex interplay between immune regulation and melanoma pathogenesis. BAFF-R = B cell activating factor receptor, HLA-DR = human leukocyte antigen DR, MR = Mendelian randomization.

P = .0369), CD38 on IgD+ CD38dim B cell (OR = 0.9994, 95% CI: 0.9988 – 1.0000, *P* = .0374), CD24+ CD27+ B cell %B cell (OR = 0.9994, 95% CI: 0.9988 – 1.0000, *P* = .0393), switched memory B cell %lymphocyte (OR = 0.9992, 95% CI: 0.9983 – 1.0000, *P* = .0426), IgD+ CD38- B cell %B cell (OR = 0.9993, 95% CI: 0.9987 – 1.0000, *P* = .0427) were protective factors (Fig. 4) (Table S5, Supplemental Digital Content, http://links.lww.com/MD/N863).

3.3. Effect of GM on immune cells

The MR analysis revealed that genus Erysipelatoclostridium was highly associated with CD4- CD8- T cell %T cell (OR = 0.7738, 95% CI [0.6410, 0.9344], *P* = .0077), genus Prevotella7 was highly associated with CD25 on transitional B cell (OR = 0.8239, 95% CI [0.6934, 0.9789], *P* = .0276) and SSC-A on natural killer T (OR = 0.8630, 95% CI [0.7516, 0.9910], *P* = .0368), genus Blautia was highly associated with CD24 on IgD+ CD38- B cell (OR = 2.0823, 95% CI [1.0324, 4.2001], *P* = .0405) and CD38 on IgD+ CD38dim B cell (OR = 2.0380, 95% CI [1.0078, 4.1213], *P* = .0475) (Table S12, Supplemental Digital Content, http://links.lww.com/MD/N863). No heterogeneity and horizontal pleiotropy were observed, and a particular SNP did not drive causal estimates (Tables S13, S14, Supplemental Digital Content, http://links.lww.com/MD/N863).

3.4. Sensitivity analyses

Our sensitivity analysis followed a systematic procedure designed to uphold the credibility and sturdiness of the MR results. As a preliminary step, we executed Cochran Q test to examine for any signs of heterogeneity among the chosen IVs associated with the gut microbial taxa. This test revealed no significant heterogeneity (*P* > .05) (refer to Table S6, Supplemental Digital Content, http://links.lww.com/MD/N863 and Table S7, Supplemental Digital Content, http://links.lww.com/MD/N863). The MR-Egger regression intercepts consistently aligned with the null hypothesis, effectively ruling out the presence of horizontal pleiotropy (refer to Table S8, Supplemental Digital Content, http://links.lww.com/MD/N863 and Table S9, Supplemental Digital Content, http://links.lww.com/MD/N863). To further fortify the robustness of the identified associations, we conducted a ``leave-one-out" sensitivity analysis, the

results of which serve as supporting evidence (refer to Table S10, Supplemental Digital Content, http://links.lww.com/MD/N863 and Table S11, Supplemental Digital Content, http://links.lww.com/MD/N863).

3.5. Mediation effect of GM on melanoma skin cancer

We excluded mediating factors that were not causally affected by GM and those that did not causally influence melanoma skin cancer. Five candidate mediators met the criteria. The overall effect can be separated into direct effect (via mediators) and indirect effect (without mediators). Our results demonstrated that CD4- CD8- T cell %T cell levels accounted for 1.15% in the causal pathway from genus Erysipelatoclostridium to melanoma, CD25 on transitional B cell and SSC-A on natural killer T cell levels accounted for 46.70% and 1.41% from genus Prevotella7 to melanoma, CD24 on IgD+ CD38- B cell and CD38 on IgD+ CD38dim B cell levels accounted for 5.00% and 2.81% from genus Blautia to melanoma (Table 1). Our findings demonstrate a causative link between specific GM and melanoma, highlighting 5 immune cell phenotypes as potential mediators in this association.

4. Discussion

Skin malignant melanoma ranks among the most lethal malignancies in humans due to its high rates of metastasis and mortality. Despite advancements in therapies such as immune checkpoint inhibitors, adoptive cell therapy, and tumor vaccines[33] showing promise for treating skin melanoma patients, reliable prognostic biomarkers remain scarce. Current evidence suggests a correlation between GM composition and patient prognosis during immunotherapy for various cancers including melanoma,[34] lung cancer,[35] and renal cancer,[36] supporting a potential role for GM in modulating host immunity, stabilizing the immune system, and influencing tumorigenesis.[37] Additionally, the infiltration of immune cells within tumors holds promise as novel prognostic markers, making it a focal point of research interest.[38,39] The precise mechanisms through which GM impacts melanoma occurrence via immunoregulation remain elusive. This study is the first to use two-stage, two-sample Mendelian randomization analysis to explore the causal relationship between GM, immune cell phenotype, and malignant melanoma from a genetic perspective, and to evaluate the mediating role of immune cells.

MR studies, particularly those investigating the intricate relationships among GM, immune cells, and melanoma skin cancer, are inherently complex and susceptible to various confounding factors that can significantly influence the outcomes. Environmental confounders such as dietary patterns, exposure to ultraviolet radiation, and lifestyle choices are pivotal, as they can directly affect both the composition of the GM and the immune response, thereby potentially altering susceptibility to melanoma. Additionally, genetic confounders play a crucial role; genetic variations that influence GM composition or immune function may also be linked to melanoma risk, independently of the pathways being studied. These genetic factors can create pleiotropy, where a single genetic variant influences multiple traits, complicating the interpretation of causal inferences.[40] Properly addressing these confounders in MR analyses involves using advanced statistical techniques such as MR-Egger regression to test for and adjust pleiotropic effects, thus helping to ensure that the observed associations are not merely artifacts of unmeasured confounding.[41] By carefully considering these environmental and genetic factors, researchers can better elucidate the potential causal pathways linking GM and immune responses to melanoma development, leading to more robust and reliable conclusions. Overall, we found that higher richness of genus Veillonella, genus Parabacteroides levels and 15 immune cell phenotypes were positively associated with the risk of melanoma skin cancer, and genus Blautia, genus Ruminococcaceae UCG013, genus Erysipelatoclostridium, genus Prevotella7 and 17 immune cell phenotypes were negatively associated with the risk of melanoma skin cancer. The use of appropriate genetic IVs (F-statistics > 10 and $r^2 < 0.001$) in this study led to the absence of significant SNP detection using the retention method, yielding highly consistent results across 5 MR analyses. Consequently, we consider the findings of this study to be reasonably robust.

A recent study[42] found through 16S ribosomal RNA amplification sequencing and gas chromatography/mass spectrometry that the proportion of Veillonella in the feces of patients who did not respond to targeted therapy or immunotherapy increased in a cohort of 31 patients with unresectable IIIC–IV stage cut throat melanoma. Similarly, in the study by Wu et al,[43] the abundance of Parabacterioids in fecal samples of cancer patients who responded to PD-1 and chemotherapy combination therapy was higher, while Ruminococcus lactis was more enriched in the feces of nonresponsive patients, that was consistent with the research results of Bao et al.[44] However, there were also conflicting data on Veillonella recorded in other literature.[45] Nevertheless, some still believed that the increased relative abundance of Veillonella was a favorable feature of immunotherapy. In addition, although there has been no research on the relationship between genus Erysipelotocolstridium and

Table 1

Mediation effect of genus Erysipelatoclostridium, genus Prevotella7, and genus Blautia on melanoma skin cancer via immune cell traits.

GM	Mediator	Total effect β (95% CI)	Direct effect A β (95% CI)	Direct effect B β (95% CI)	Mediation effect β (95% CI)	*P*	Mediated proportion (%) (95% CI)
Genus Erysipelatoclostridium	CD4- CD8- T cell %T cell	1.0018 (0.9996, 1.0039)	1.2621 (1.0467, 1.5220)	1.0001 (0.9995, 1.0006)	1.0000 (0.9999, 1.0002)	<.0001	1.15% (-0.0692, 0.0922)
Genus Prevotella7	CD25 on transitional B cell	1.0010 (0.9992, 1.0029)	0.7681 (0.6111, 0.9656)	0.9982 (0.9960, 1.0003)	1.0005 (0.9997, 1.0013)	.005	46.70% (-0.2688, 1.2028)
	SSC-A on natural killer T	1.0010 (0.9992, 1.0029)	1.3967 (1.0521, 1.8540)	1.0000 (0.9997, 1.0004)	1.0000 (0.9999, 1.0002)	<.0001	1.41% (-0.1178, 0.1459)
Genus Blautia	CD24 on IgD+ CD38- B cell	0.9997 (0.9985, 1.0009)	1.2332 (1.0130, 1.5014)	1.0001 (0.9994, 1.0008)	1.0000 (0.9999, 1.0002)	<.0001	-5.00% (0.5395, 0.4396)
	CD38 on IgD+ CD38dim B cell	0.9997 (0.9985, 1.0009)	1.1884 (1.0086, 1.4003)	0.9999 (0.9992, 1.0006)	1.0000 (0.9985, 1.00029)	<.0001	2.81% (-0.3888, 0.4450)

Total effect: the causal role of GM on melanoma; direct effect A: the causal role of GM on immune cell traits; direct effect B: the causal role of immune cell traits on melanoma; β(indirect effect) = β(direct effect A) * β(direct effect B); the mediated proportion = β(indirect effect)/β(total effect).

CI = confidence interval, GM = gut microbiota, SSC = side scatter.

melanoma, other studies had found a correlation between genus Erysipelotocolstridium and cancer progression, including oral cancer,[46] diffuse large B-cell lymphoma,[47] hepatocellular carcinoma,[48] etc. In recent years, research on Gut brain skin axis has linked GM with skin health, which has immunological significance for understanding the occurrence of skin cancer. A higher abundance of Erysipelatocolstridium was found in the feces of adult atopic dermatitis patients than in normal individuals,[49] suggesting a potential mechanism by which structural changes in the gut microbiome and metabolic dysfunction may cause skin inflammation. Finally, some studies[50–54] have found a correlation between the clinical outcomes of Prevotella and melanoma patients, but there is also opposing evidence.[55] Therefore, our research provides genomic evidence for the changes of microbiota in the occurrence and development of melanoma to regulate the body's immune response and response to immunotherapy, and further demonstrates the potential of these microbiota as blocking reaction markers of immune checkpoints.

As one of the most immunogenic tumors, melanoma skin cancer has great potential to respond to immunotherapy. However, the various inhibitory mechanisms obtained during the development of melanoma still form its ability to evade innate and adaptive immunity.[56] Therefore, elucidating the cellular and molecular events related to immune suppression during melanoma development is beneficial for us to better explore new therapeutic targets and more effective synergistic combinations of immunotherapy, targeted therapy, and chemotherapy. In our study, a total of 32 different immune cell phenotypes were identified as genetic evidence for promoting or reducing the risk of melanoma.

The role of B cells in antitumor immunity is controversial, however, it is known that B cells contribute to antitumor immune responses in immunogenic tumors such as melanoma.[57] Previous Mendelian randomization studies[58,59] found an increase in the abundance of CD25 on IgD+ CD24- B cells and CD25 on IgD- CD38dim B cells that were negatively correlated with the risk. These studies found that higher levels of CD25 expression on these B cell subsets were inversely correlated with the risk of melanoma, suggesting a protective role. CD25 (interleukin-2 receptor alpha chain) plays a critical role in the immune system by regulating the activities of white blood cells. BAFF-R, another important immune marker mentioned in our study, is integral for B cell development and survival. This receptor is particularly significant in the context of its interaction with its ligand BAFF a crucial survival factor for B cells. Our study also found high levels of CD25 on transitional B cells, BAFF-R on IgD+ CD38+ B cell, BAFF-R on IgD- CD38- B cell, CD25 on CD24+ CD27+ B cell, BAFF-R on transitional B cell, BAFF-R on switched memory B cell, CD24 on IgD+ CD38- B cell, CD38 on transitional B cell, CD38 on IgD+ CD38dim B cell, CD24+ CD27+ B cell% B cell, switched memory B cell% lymphocyte and IgD+ CD38- B cell% B cell are associated with reduced risk of melanoma, but no evidence has been found for B-cell related immune cell phenotypes in promoting melanoma risk. This suggests that B cells may play a potential protective role in the development of melanoma. Rodgers et al[60] proposed that tumor infiltrating B cells are associated with conflicting clinical prognoses of tumors, B cells and their subpopulations may have different roles in different time and space,[61] which may also reflect a lack of consistency between cell subpopulation classification studies and the differences in markers used, especially when a single marker is often used instead of distinguishing multiple subpopulations. There is a correlation between the low number of CD20+ B lymphocytes and the progression of melanoma.[62] Study[63] has shown that high expression levels of CD38 are a favorable diagnostic factor for melanoma skin cancer and a major mechanism for acquired resistance to PD-1/PD-L1 blockade, as it leads to CD8+ T cell suppression. The absence of CD38 can also increase cell death and reduce the number of cancer-related fibroblasts and blood vessels,[64] therefore, CD38 can work together with PD-L1 to improve antitumor immune response.[65] BAFF-R is a membrane protein that recognizes the tumor necrosis factor receptor superfamily of BAFF. BAFF-R is a key receptor for B cell survival and an effective co stimulatory factor for B cell and T cell activation. BAFF/BAFF-R activates the NF-κ B2 signaling pathway, which is crucial for the survival, differentiation, and homeostasis of primary B cells.[66] The role of BAFF derived from dendritic cells (DC) and A proliferation-inducing ligand in inducing antitumor immunity in vivo has been confirmed.[67] BAFF levels were also found to be significantly higher in the serum of patients with uveal melanoma metastasis than in patients without metastasis and the control group.[68] In addition, it was found in animal experiments[69] that BAFF cytokines regulate monocytes in the melanoma microenvironment to inhibit tumor growth, highlighting the importance of BAFF in antitumor immunity in melanoma.

Unlike the unclear role of B cells and their subsets in melanoma, current tumor immunotherapy focuses on targeting T cells and natural killer cells to inhibit tumor growth. Our study found that C-C chemokine receptor type 2 on myeloid DCs, CD3 on Central Memory CD8+ T cell, CD4- CD8- T cell %T cell, CD11c+ monocyte %monocyte, CD25++ CD45RA- CD4 not regulatory T cell Absolute Count, CD14+ CD16- monocyte% monocyte, CD33- HLA DR- absolute count, CD4+/CD8+ T cell, CD39+ CD8+ T cell absolute count, SSC-A on natural killer T, CD28+ CD45RA- CD8+ T cell absolute count, CD127 on CD45RA+ CD4+ T cell and CD3 on HLA DR+ CD4+ T cell were significant risk factors in the causal pathway from immune cells to melanoma skin cancer. Disrupting T cell exhaustion to combat cancer immune evasion and the immunogenicity of melanoma is key to the effectiveness of anti-PD-1.[70] In a melanoma mouse model, changes in immune cell infiltration were found to lead to the accumulation of myeloid derived suppressor cells (MDSCs) and regulatory T cells, and reduce the infiltration of DC, CD8+, and CD4+ T cells in the TME,[71] while MDSCs were downregulated after lymphocyte immunotherapy.[72] Tumor associated macrophages produced by monocytes develop into functional, fully activated macrophages, which subsequently acquire various immunosuppressive functions to maintain the TME.[73] Human monocyte chemoattractant protein-1 (MCP-1/CCL2) is the same chemokine derived from tumor cells as previously described. This chemokine is believed to be responsible for the accumulation of tumor associated macrophages and has become a candidate target for clinical intervention.[74] High levels of classical monocytes were also observed in peripheral blood of patients with metastatic melanoma who responded to CPI. These monocytes preferentially transitioned to macrophages expressing chemokine (C-X-C motif) ligand 9 in the tumor, and the trajectory of tumor infiltrating CD8+ T cells was found to differentiate at the effector memory/stem cell like T cell level,[75] indicating that monocytes and their derived subsets induce an immunosuppressive TME together with other immunosuppressive cells.

HLA DR is a component of major histocompatibility complex II and is abnormally expressed in melanoma,[76] but the cause is unknown. In patients undergoing immunotherapy for meta melanoma, the location and degree of T cell and DC infiltration, as well as the expression of tumor cell HLA DR, were observed to be associated with the clinical response of melanoma immunotherapy.[77] Although HLA DRs can induce signaling cascades leading to cell proliferation[78] and have been emphasized for their foreseeable role in targeted therapy for human melanoma, as well as their potential effects on HLA class II antigen positive tumors of different histology,[79] their ubiquitous expression and potential toxicity make their direct therapeutic targets seem irrelevant.

NK cells can kill various types of cancer cells and cancer stem cells, while keeping normal cells intact. Different subgroups of NK cells have been identified: some immune checkpoint

molecules are expressed on NK cells and are associated with the course of melanoma and treatment response to different therapies.[80] NK cells and T cells may share a depleted epigenetic program,[81] which is associated with the expression of immune checkpoint PD-1.[82] Javed et al[83] found that liver NK cells share a common ecological niche with intrahepatic uveal melanoma micrometastasis, suggesting the important role of NK cells in the control or progression of melanoma. Recent research results have shown that the frequency of NK cells is directly related to the overall survival rate of melanoma patients treated with ipilimumab.[84] Based on the findings of this study, we can better understand how these inhibitory pathways are differentially regulated in NK cells and T cells, which will help design immunotherapy to activate innate and adaptive cytotoxic lymphocytes in melanoma patients.

Finally, we found CD66b on CD66b++ myeloid cells, CD3 on CD4+ T cells, CD4RA on terminally differentiated CD4+ T cells, CD11b on Granulocyte MDSCs were the protective factors in melanoma. These conclusions are consistent with previous experiments on melanoma mice,[85] where tumor-infiltrating lymphocytes not only exhibited T cell function and CTL population activity, but also showed enhanced tumor recruiting CD11b+ cell activity after treatment with anti-PD-L1 monoclonal antibodies. The newly developed carbohydrate molecule BG34-200 can regulate tumor associated myeloid cells by targeting the cell surface receptor CD11b, disrupting the function of tumor associated bone marrow cells and inhibiting the occurrence of melanoma.[86] In addition, an immunohistochemical analysis of inflammatory cell infiltration (CD66b neutrophils and CD163 macrophages) found no correlation between consumption of epidermis and increased inflammatory response (CD163 macrophages or CD66b neutrophils), supporting the noninflammatory nature of proliferation drive. Bønnelykke Behrndtz et al[87] defined ulceration in primary melanoma as epidermal loss, evidence of host response (neutrophil infiltration or fibrin deposition), as well as thinning, disappearance, or reactive proliferation of the surrounding epidermis. These findings provide ample evidence for the significant impact of dynamic changes in the immune cell population on the immune response process during melanoma occurrence. At the end of the study, we also found that 5 immune cell phenotypes were expressed through the genus Erysipelatoclostrium. Genus Prevotella7 and Genus Blautia play a mediating role, providing further evidence for future exploration of the mechanisms of mutual influence between GM, immune cells, and melanoma.

However, this study is subject to several limitations. Firstly, the use of the GWAS program for the analyzed cohort introduces constraints such as a lack of quality checks for diagnostic accuracy and inadequate control for age or other patient factors.[88] We intend to expand this study and conduct further research in the future. Additionally, it is important to consider the applicability of our findings to specific ethnic groups as the entire study population consists of individuals with European ancestry. Changes in lifestyle, host metabolism, and resident transgenes are observed globally, and differences in genetic variant distribution among different ethnic or racial groups may lead to population stratification that could impact our findings.[89] Therefore, caution should be exercised when generalizing our results to other ethnic or racial groups; future studies should include more diverse populations. Lastly, while MR assumes a linear relationship between exposure and outcome, it is possible that the actual relationship is more complex involving nonlinear relationships and interactions with environmental and genetic factors. For instance, certain genetic variants may have a greater effect on outcomes at higher or lower levels of exposure or the effect of exposure on outcomes may be mediated or modulated by other factors. Henceforth, future MR studies should account for these complexities in their analyses.

5. Conclusions

Our research findings indicate that specific GM taxa and immune cells may exert influence on the initiation of melanoma skin cancer, highlighting 5 immune cell phenotypes as potential mediators in this association. This study provides new insights into the pathogenesis of melanoma skin cancer, and regulating GM may be a potential approach for tumor prevention and treatment. Similarly, research on specific immune cells may also help develop clinical biomarkers for the prevention, screening, and monitoring of changes in the course of melanoma skin cancer.

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