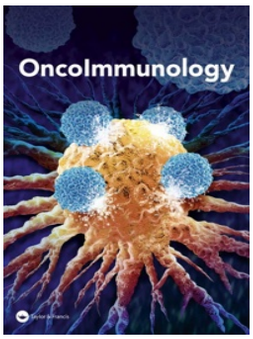

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Estrogen-related differences in antitumor immunity and gut microbiome contribute to sexual dimorphism of colorectal cancer

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ABSTRACT

Colorectal cancer (CRC) is a multifaceted disease whose development and progression varies depending on tumor location, age of patients, infiltration of immune cells within cancer lesions, and the tumor microenvironment. These pathophysiological characteristics are additionally influenced by sex-related differences. The gut microbiome plays a role in initiation and progression of CRC, and shapes anti-tumor immune responses but how responsiveness of the immune system to the intestinal microbiota may contribute to sexual dimorphism of CRC is largely unknown. We studied survival, tumor-infiltrating immune cell populations and tumor-associated microbiome of a cohort of $n = 184$ male and female CRC patients through high-dimensional single-cell flow cytometry and 16S rRNA gene sequencing. We functionally tested the immune system-microbiome interactions in in-vivo and in-vitro models of the disease. High-dimensional single-cell flow cytometry showed that female patients are enriched by tumor-infiltrating invariant Natural Killer T (iNKT) cells but depleted by cytotoxic T lymphocytes. The enrichment of oral pathobionts and a reduction of β-glucuronidase activity are distinctive traits characterizing the gut microbiome of female patients affected by CRC. Functional assays using a collection of human primary iNKT cell lines demonstrated that the gut microbiota of female patients functionally impairs iNKT cell anti-tumor functions interfering with the granzyme-perforin cytotoxic pathway. Our results highlight a sex-dependent functional relationship between the gut microbiome, estrogen metabolism, and the decline of cytotoxic T cell responses, contributing to the sexual dimorphism observed in CRC patients with relevant implications for precision medicine and the design of targeted therapeutic approaches addressing sex bias in cancer.

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Colorectal cancer; gender medicine; gut microbiome; iNKT; sexual dimorphism; tumor immunology

Background

Colorectal cancer (CRC) is one of the most common causes of cancer morbidity both in males and females. CRC cannot be considered merely as a type of disease; its pathogenesis depends, for example, on the anatomical location of the tumor, patients' age, and it is characterized by sex- and gender-related differences.[1] Sex is primarily defined as a biological attribute determined by factors such as sex hormone production, chromosomal makeup, and the presence of reproductive organs. Gender, on the other hand, refers to an individual's self-identified identity, which may or may not align with their biological sex. Gender identity can involve aspects such as social roles, behaviors, and personal expression. It's important to note that gender may be influenced by factors such as hormone therapy, which can impact an individual's clinical history and health outcomes.[2] Overall, sexual dimorphism exists at multiple levels in CRC. Females, compared to males, have a lower risk to develop CRC[1] and a higher survival probability[3] although incidence and mortality in patients over 65 years old are higher in females than males.[4–6] Female patients have a higher prevalence of right-sided colon cancer that is often characterized by high level of microsatellite instability (MSI). This form of CRC is associated with a 20% increased risk of death compared to cancer occurring on the left side.[4] Hormonal factors may contribute to the incidence and mortality of CRC. Estrogen, for instance, play a protective role against CRC,[7] as evidenced by the higher survival in young female patients with CRC and the significant reduction in colorectal cancer risk and mortality among postmenopausal women who underwent hormone replacement therapy.[3,8–10] The influence of sex hormones on CRC pathophysiology may be associated with

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their ability to regulate the development and function of the immune system, thereby shaping both innate and adaptive immunity and leading to clear consequences on antitumor responses. Generally, CRC can be classified by the frequency and type of tumor-infiltrating immune cells.[11,12] Patients with high infiltration of immune cells have a lower risk of disease recurrence.[11] Nevertheless, the activation state of tumor-infiltrating immune cells and their production of effector molecules dictate the anti- vs pro-tumor immune responses and patient survival.[12,13] Estrogen's influence on cytotoxic $CD8^+$T cell functions in CRC indicate a more robust adaptive inflammatory T cell response in female than male patients.[14] However, estrogens have been implicated also in regulating neutrophil chemotaxis and proliferation[15] and elevated levels of tumor-associated neutrophils (TAN) are an independent factor that contribute to the poor prognosis of CRC patients.[16] We recently demonstrated that invariant Natural Killer T (iNKT) cells can contribute to the remodeling of the tumor microenvironment (TME) by recruiting TANs in the early phases of tumor development, thereby sculpting the CRC progression trajectory with negative outcomes in terms of patients' overall survival.[17] iNKT cells are a subset of tissue resident T cells with innate-like functions involved in tumor immune surveillance[18] endowed with killing capabilities toward CRC cells *in vitro*.[19] However, iNKT cell role in cancer biology is still controversial, possibly as a consequence of the negative impact of the tumor-associated microbiome on their functions.

The gut microbiome is an oncogenic driver of CRC.[20] Some tumor-associated bacterial species can promote carcinogenesis *via* the production of proinflammatory toxins and stimulating immunosuppressive responses.[21] Dysbiosis gains further importance considering the sexual dimorphism of CRC as the gut microbiota composition changes between male and female subjects.[22] Moreover, the gut microbiome affects the bioavailability of sex hormones.[23] During phase II metabolism, estrogens are glucuronidated in the liver; upon entry into the gastrointestinal tract, they are exposed to microbiota-secreted β-glucuronidase that deconjugate the sugar moiety reactivating the parent compound. This allows unconjugated estrogens to be reabsorbed in the blood stream through the enterohepatic recirculation[24] suggesting a role for the estrobolome *i.e.*, the bacterial gene repertoire affecting estrogen metabolism,[25] in CRC pathophysiology.

Here, by taking advantage of a cohort of human CRC patients, murine models of colon carcinogenesis and *in vitro* functional assays, we report sex-dependent differences in antitumor immunity and the tumor-associated microbiome associating with distinct dynamics in CRC pathophysiology.

Methods

Human samples

Tumor samples were collected with informed consent from patients ($n = 184$) diagnosed with colorectal adenocarcinoma between October 2017 and April 2023 undergoing surgical resection at IRCCS Policlinico Ospedale Maggiore, Milan, Italy, as approved by the Institutional Review Board (Milan, Area B) with permission number 566_2015.

Isolation of tumor-infiltrating cells

Tumor samples were taken transversally to collect both marginal and core tumor zone. Human lamina propria mononuclear cells (LPMCs) were isolated as previously described.[26] Briefly, the dissected intestinal mucosa was freed of mucus and epithelial cells in sequential steps with DTT (0.1 mmol/l) and EDTA (1 mmol/l) (Sigma-Aldrich) and then digested with collagenase D (400 U/ml) (Worthington Biochemical Corporation) for 5 h at 37°C in agitation. LPMCs were then separated with a Percoll gradient.

Mice

C57BL/6 mice were housed and bred at the European Institute of Oncology (IEO) animal facility (Milan, Italy) in SPF conditions. Sample size was chosen based on previous experience. No sample exclusion criteria were applied. No method of randomization was used during group allocation, and investigators were not blinded. Age-matched male and female mice were used for experiments. Animal experimentation was approved by the Italian Ministry of Health (Auth. 10/21 and Auth. 1217/20) and by the animal welfare committee (OPBA) of the European Institute of Oncology (IEO), Italy.

Animal experiments

7-8 weeks old mice were injected intraperitoneally with azoxymethane (AOM, Merck) dissolved in isotonic saline solution at a concentration of 10 mg/kg body weight. After 7 days, mice were given 1% (w/v) dextran sodium sulfate (DSS MW 40 kD; TdB Consultancy) in their drinking water for 7 days followed by 14 days of recovery. The cycles were repeated for a total of 2/3 DSS cycles, and mice sacrificed at day 49 and 70.

Murine colonoscopy

Colonoscopy was performed using the Coloview system (TP100 Karl Storz, Germany). Tumor endoscopic score has been quantified as previously described.[27] During the endoscopic procedure mice were anesthetized with 3% isoflurane.

Murine cells isolation

Single-cell suspensions were prepared from the colon of C57BL/6 mice as previously described.[28] Briefly, cells were isolated via incubation with 5 mm EDTA at 37°C for 30 min, followed by mechanical disruption with GentleMACS (Miltenyi Biotec). After filtration with 100-μm and 70-μm nylon strainers (BD), the LPMC were counted and stained for immunophenotyping.

ELISA assay

Detection of murine estrogens in serum was performed using the Mouse Estrogen ELISA Kit (Abcam) according to manufacturers' instructions.

iNKT cell cytotoxicity assay

The human iNKT cell lines used in this study were generated as previously described.[29] All cells were maintained in a humidified incubator with 95% air, 5% CO_2 at 37°C. iNKT cell cytotoxicity toward the human CRC cell line RKO (American Type Culture Collection, ATCC) was performed as previously described.[19]

iNKT cell conditioning by human tumor microbiota

Monocyte derived dendritic cells (moDCs) were pulsed with heat-inactivated gut microbiota of female and male CRC patients as well as healthy controls and co-cultured with iNKT cells (2×10^5 cells) in a 2:1:10 iNKT:moDC:microbiota ratio in RPMI-1640 supplemented with 10%FBS, Pen/Strep. After 24 h, iNKT cell activation status was estimated by extracellular or intracellular staining. Microbiome samples from the intestinal mucus scraped from patients' tumor lesions were resuspended 1:10 (w/v) in PBS and filtered through a 0.75 µm filter to remove large debris; microbiota cell density was quantified by qPCR using the primers F_Bact 1369: CGGTGAATACGTTCCCGG and R_Prok1492: TACGGCTACCTTGTTACGACTT.[30] Briefly, 1 ml of resuspended samples have been used for total gDNA extraction using the FastDNA™ SPIN Kit for Feces. For quantification of bacterial load, we constructed a seven-point standard curve consisting in 10-log-fold serial dilutions (from 10^3 to 10^9) of gDNA extracted from a pure culture of *E. coli* at known concentration to convert the threshold cycle (Ct) values into the average estimates of target bacterial genomes present in 1 ml of sample. Amplification specificity of target gene was checked by melting curve analysis. Upon quantification, resuspended samples were adjusted to the concentration of 2×10^7 cell·mL^{-1} and heat-killed at 95°C for 10 minutes before being stored at −80°C until use in downstream experimentation. For the experiments with exposure to estradiol, iNKT cells were preincubated (24 h) with 17β-Estradiol (E2) (Tocris Bioscience, Bio-Techne) at the concentration of 100 nM in RPMI w/o phenol red. Directly after the preincubation, iNKT cells were transferred to new plates and cultured in RPMI w/o phenol red added with E2 (100 nM) to perform the tumor microbiome iNKT conditioning experiments described above. The ERβ-selective antagonist PHTPP (Tocris Bioscience, Bio-Techne) was added at an equimolar concentration of E2.

Flow cytometry

Cells were stained with labeled antibodies diluted in PBS with 1% heat-inactivated fetal bovine serum (FBS) for 20 min at 4°C. iNKT cells were stained and identified using human or mouse CD1d:PBS57 Tetramer (NIH Tetramer core facility) diluted in PBS with 1% heat-inactivated FBS for 30 min at 4°C. For intracellular cytokine labeling cells were incubated for 3 h at 37°C in RPMI-1640 + 10% FBS with PMA (50 ng/ml, Merck), Ionomycin (1 µg/ml, Merck) and Brefeldin A (10 µg/ml, Merck). Before intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm (BD). Samples were analyzed with a FACSCelesta flow cytometer (BD Biosciences, Franklin Lakes NJ, USA) and the FlowJo software (Version 10.8, TreeStar, Ashland, OR, USA). For the multidimensional analysis using t-SNE visualization and Phenograph clustering,[31] FCS files were quality checked for live, singlets and antibody agglomerates and normalized to avoid batch effects. Data were cleaned for antibodies aggregates by checking each parameter in a bimodal plot. Gate on singlets, on viable lymphocytes and subsequently on $CD3^+$cells were applied. $CD3^+$ populations were down-sampled to 5000 events per sample using the DownSample plugin (Version 3.3.1) of FlowJo to create uniform population sizes. Down-sampled populations were exported as FCS files with applied compensation correction. Files were then uploaded to RStudio environment (Version 3.5.3) using the flowCore package (Version 1.38.2). Data were transformed using logicleTransform() function present in the flowCore package. To equalize the contribution of each marker they were interrogated for their density distribution using the densityplot() function of the flowViz package (Version 1.36.2). Each marker was normalized using the Per-channel normalization based on landmark registration using the gaussNorm() function present in the package flowStats (Version 3.30.0). Peak.density, peak.distance and number of peaks were chosen according to each marker expression. Normalized files were analyzed using the cytofkit package through the cytofkit_GUI interface. For data visualization we used the t-Distributed Stochastic Neighbor Embedding (t-SNE) method, while for clustering we used the Phenograph algorithm. t-SNE plots were visualized on the cytofkitShinyAPP with the following parameters: perplexity = 50, iterations = 1000, seed = 42, k = 50. FCS for each cluster were generated and re-imported in FlowJo to be manually analyzed for the determination of the integrated MFI. The iMFI of different markers was scaled from 0 to 1 and used to identify Phenograph clusters.[31]

16S rRNA gene sequencing and data analysis

Intestinal mucus scraped from human tumor lesions and murine feces were stored at −80°C until DNA extraction. DNA extraction, 16S rRNA gene amplification, purification, library preparation and pair-end sequencing on the Illumina MiSeq platform were performed as previously described.[28] Reads were pre-processed using the MICCA pipeline (v.1.7.2) (https://micca.readthedocs.io/en/latest/#).[32] Forward and reverse primers trimming and quality filtering were performed using micca trim and micca filter, respectively. Filtered sequences were denoised using the UNOISE[33] algorithm implemented in micca otu to determine true biological sequences at the single nucleotide resolution by generating amplicon sequence variants (ASV). Bacterial ASVs were taxonomically classified using micca classify and the Ribosomal Database Project (RDP) Classifier v2.13.[34] Multiple sequence alignment (MSA) of 16S rRNA gene sequences was performed using the Nearest Alignment Space Termination (NAST) algorithm[35] implemented in micca msa. Phylogenetic trees were inferred using micca tree.[36] Sampling heterogeneity was reduced rarefying samples at the depth of the less abundant sample using micca tablerare. Alpha (within-sample richness) and beta-diversity (between-sample dissimilarity) estimates were computed using the

phyloseq R package.[37] Permutational multivariate analysis of variance (PERMANOVA) test was performed using the adonis2 function in the vegan R package with 999 permutations. ASVs differential abundance testing was carried out using the R package DESeq2[38] using the non-rarefied data.[39] Spearman's correlations were tested using the psych R package.[40] Prediction of functional metagenomic content was inferred by using Tax4Fun2[41] and the reference curated databases from Kyoto Encyclopedia of Genes and Genomes (KEGG)[42] implemented within the MicrobiomeAnalyst pipeline.[43] Metabolic pathway maps were visualized using iPATH 3.[44]

Statistical analysis

Statistical tests were conducted using Prism (Version 9.5.1, GraphPad) and R (version 4.3.1). Spearman's correlation coefficient was used for the analysis of correlations. Kaplan-Meier analysis was carried out using the R packages survival (version 3-2-11) and survminer (version 0.4.9).

Results

Decline of cytotoxic T cell responses correlates with adverse disease outcomes in female patients over 65 years old

Several studies reported a substantial survival advantage of female patients under 65 years of age.[3–6] This may in part be explained by the strong survival benefit provided by sex hormones. To investigate whether age significantly impacts survival rates among female patients, we analyzed a cohort from The Cancer Genome Atlas (TCGA) colon adenocarcinoma (COAD) dataset ($n = 414$; females = 198, males = 216). Our analysis revealed opposing relationships between age and survival in males and females. Pearson correlation analysis showed a negative correlation between age and years of survival in female patients and a positive correlation in male patients (Supplementary Figure S1A-B). Moreover, ANCOVA confirmed that both the intercept and slope of the age-survival relationship differed significantly between genders ($p < 0.001$), with a significant interaction between age and gender. These findings suggest that age impacts survival outcomes differently in males and females, indicating a gender-specific effect of age on survival. Despite these differences, no significant difference in the 10-year overall survival (OS) probability was observed between sexes in the TCGA cohort (Supplementary Figure S1C). Next, we compared the OS rates between female and male patients under 65 years of age and those over 65. OS was significantly higher in women under 65 years of age compared to those over 65 ($p = 0.047$) (Supplementary Figure S1D), while no significant difference in OS probability was observed within male patients ($p = 0.13$) (Supplementary Figure S1E). These results suggest that aging plays a more significant role in influencing survival outcomes among female compared to male patients. Next, we analyzed a cohort of $n = 184$ patients (female = 87, male = 97) enrolled at the Policlinico Hospital Milan between 2017 and 2023 to gain deeper insights into sex-related differences in survival, immunological landscape, and tumor-associated microbiota of CRC (Figure 1(a)). The clinical characteristics of CRC patients from our cohort are detailed in Table 1. On overall, female patients showed a trend decrease in 5-year survival rate compared to men ($p = 0.062$) and no difference in event-free survival ($p = 0.15$). However, if stratified according to age, female patients over 65 years had lower 5-year and event-free survival rates compared to their age-matched male counterparts ($p = 0.05$) (Figure 1(b-c)) while we did not observe differences in younger patients (<65 years old). The unsupervised multidimensional immunophenotyping of freshly isolated tumor-infiltrating T cells (TILs) by FACS showed a different distribution of T cell populations between male and female patients (Figure 1(d)). Tumor-infiltrating T cells belonging to clusters of T helper (C14 and C20) and cytotoxic T cells (C9) expressing IFNγ (C5) were less frequent in female than male patients (Figure 1(e)). Conversely, clusters of T helper cells expressing TNFα (C13), Ki67 and PD-1 (C24) as well as a cluster of iNKT cells positive for PD-1 were more frequent in female patients (Figure 1(e)). We further confirmed that age and the immune system are important variables when considering sex-bias in CRC by manual gating analysis of FACS data (Supplementary Table S1). Indeed, we observed that CRC lesions from females were significantly infiltrated by iNKT and MAIT cells, another innate-like population of tissue resident lymphocytes, but depleted by conventional cytotoxic cells compared to age-matched male patients older than 65 years, as measured by reduced frequency of Natural Killer (NK) cells, conventional $CD4^+$T-helper (Th), cytotoxic $CD8^+$T lymphocytes (CTL) and iNKT cells expressing IFNγ and GzmA (Figure 1(f)). We found no differences in TILs expressing the co-inhibitory receptors PD-1, CTLA4, TIGIT, and TIM-3 (Supplementary Table S1); however, there was a trend toward an increased frequency of tumor-infiltrating PD-$L1^+$ neutrophils and dendritic cells in male patients (Supplementary Table S1 , Supplementary Figure S2A). Younger female patients were enriched by antigen-presenting cells expressing the non-polymorphic MHC-Ib molecule CD1d, which is involved in the presentation of lipid antigens to iNKT cells (Supplementary Table S1 , Supplementary Figure 2B). Thus, the decline of cytotoxic T cell responses in female patients may, in part, explain their reduced survival compared to male patients over 65 years old.

The tumor-associated microbiome of female patients is enriched in oncomicrobes correlating with immunosuppressive responses

The gut microbiome is an oncogenic driver of CRC[20,21] and its composition changes according to sex.[45] Thus, we characterized the tumor-associated microbiota from patients ($n = 35$, female = 17, male = 18) to unravel its implication in the sexual dimorphism of CRC. Albeit, we did not find significant shifts in the overall microbial community structure between male and female patients (Figure 2(a-b)), we observed a specific sex-biased enrichment of different oncomicrobes (Figure 2(c), Supplementary Table S2). Male patients were enriched in potentially genotoxic and immunogenic bacteria belonging to the genera *Bacteroides* and *Escherichia*.[21] Conversely, female patients were enriched by immunosuppressive pathobionts of

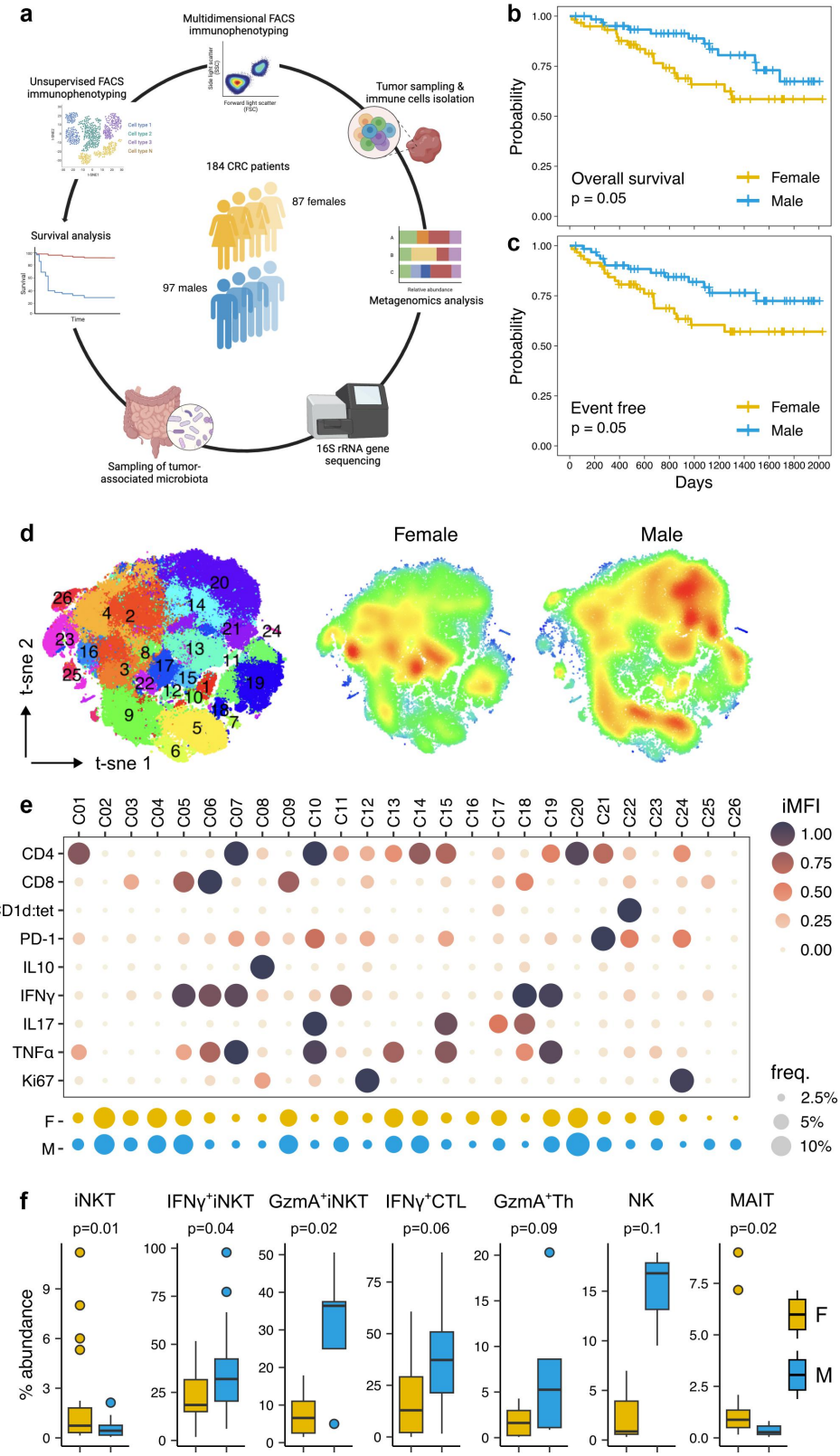

Figure 1. Decline of cytotoxic T cell responses in women vs men in CRC. a) overview of the human study design. b-c) Kaplan-Meier curves for b) overall and c) event-free survival in male vs female patients with >65 years old. d) t-sne map with density plot of tumor-infiltrating $CD3^{+}$T cells based on phenograph clustering in male and female patients. e) balloon plot of the scaled integrated MFI of phenograph clusters generated in D. f) frequency of significantly different tumor-infiltrating T cell populations in male and female patients with >65 years old as measured by manual gating analysis of FACS data.

Table 1. Characteristics of the patient cohort.

	F (*N* = 87)	M (*N* = 97)	Total (*N* = 184)	*p* value
****Age****				0.766
Mean (SD)	74.356 (13.353)	73.773 (13.137)	74.049 (13.206)	
Range	30.000 - 97.000	42.000 - 95.000	30.000 - 97.000	
****Age_at_Diagnosis****				0.734
Mean (SD)	71.069 (13.360)	70.412 (12.795)	70.723 (13.033)	
Range	27.000 - 92.000	41.000 - 93.000	27.000 - 93.000	
****Location_General****				0.053
Right colon	42 (48.3%)	32 (33.0%)	74 (40.2%)	
Left colon	44 (50.6%)	65 (67.0%)	109 (59.2%)	
Multiple	1 (1.1%)	0 (0.0%)	1 (0.5%)	
****CT_RT_Neoadj****				0.151
NO	83 (95.4%)	86 (88.7%)	169 (91.8%)	
YES	3 (3.4%)	10 (10.3%)	13 (7.1%)	
YES (RT only)	0 (0.0%)	1 (1.0%)	1 (0.5%)	
YES (CT only)	1 (1.1%)	0 (0.0%)	1 (0.5%)	
****Stage****				0.484
0	0 (0.0%)	3 (3.1%)	3 (1.6%)	
I	19 (21.8%)	19 (19.6%)	38 (20.7%)	
II	3 (3.4%)	4 (4.1%)	7 (3.8%)	
IIA	15 (17.2%)	25 (25.8%)	40 (21.7%)	
IIB	2 (2.3%)	2 (2.1%)	4 (2.2%)	
IIC	2 (2.3%)	2 (2.1%)	4 (2.2%)	
IIIA	3 (3.4%)	5 (5.2%)	8 (4.3%)	
IIIB	24 (27.6%)	21 (21.6%)	45 (24.5%)	
IIIC	7 (8.0%)	2 (2.1%)	9 (4.9%)	
IVA	9 (10.3%)	7 (7.2%)	16 (8.7%)	
IVB	1 (1.1%)	3 (3.1%)	4 (2.2%)	
IVC	2 (2.3%)	4 (4.1%)	6 (3.3%)	
****Surgery****				0.337
Anterior_Resection	18 (20.7%)	26 (26.8%)	44 (23.9%)	
Hemicolectomy	51 (58.6%)	58 (59.8%)	109 (59.2%)	
****Tumor_Residual****				0.48
Complete_Resection	83 (95.4%)	95 (97.9%)	178 (96.7%)	
Macroscopic_Residual	1 (1.1%)	0 (0.0%)	1 (0.5%)	
Microscopic_Residual	3 (3.4%)	2 (2.1%)	5 (2.7%)	
****Vascular_Invasion****				0.987
NO	61 (70.1%)	69 (71.1%)	130 (70.7%)	
YES	25 (28.7%)	27 (27.8%)	52 (28.3%)	
NA	1 (1.1%)	1 (1.0%)	2 (1.1%)	
****Lymphatic_Invasion****				0.991
NO	84 (96.6%)	94 (96.9%)	178 (96.7%)	
YES	2 (2.3%)	2 (2.1%)	4 (2.2%)	
NA	1 (1.1%)	1 (1.0%)	2 (1.1%)	
****Perineural_Invasion****				0.53
NO	66 (75.9%)	76 (78.4%)	142 (77.2%)	
YES	20 (23.0%)	18 (18.6%)	38 (20.7%)	
NA	1 (1.1%)	3 (3.1%)	4 (2.2%)	
****Budding****				0.945
NO	25 (28.7%)	28 (28.9%)	53 (28.8%)	
YES	60 (69.0%)	66 (68.0%)	126 (68.5%)	
NA	2 (2.3%)	3 (3.1%)	5 (2.7%)	
****MMR_status****				0.004
Deficient	23 (26.4%)	10 (10.3%)	33 (17.9%)	
Proficient	64 (73.6%)	87 (89.7%)	151 (82.1%)	

oral origin such as *Fusobacterium*, *Parvimonas*, *Anaerococcus* and *Alloprevotella*[46] (Figure 2(c)).

Given the impact of enterohepatic recirculation on estrogen half-life and systemic levels in males and postmenopausal female subjects,[47] we hypothesized that sex-related differences in the tumor-associated microbiome could be associated with reduced microbial β-glucuronidase activity, leading to a diminished availability of active estrogens. We performed a functional metagenomic prediction analysis of the microbiome revealing a significant gene content reduction of microbial β-glucuronidases (KEGG orthology K01195) in tumor lesions from female compared to male patients (Figure 2(d)). Moreover, we observed the enrichment of metabolic pathways related to amino acid and lipid metabolism in females and metabolism of simple sugars like galactose, mannose, and fructose in males (Supplementary Figure S3). We did not find significant differences in the tumor-associated microbiome between younger and older female patients, nor in the gene content of microbial β-glucuronidases (Supplementary Figure S4A-D). These data suggest that aging, overall, does not significantly impact the composition of the tumor-associated microbiome in female patients. Next, we evaluated whether the tumor-associated microbiome contributes to functionally polarize specific immune responses that align with sexual dimorphism in CRC. The correlation analysis of the most abundant bacterial taxa (mean abundance > 0.25%) with the frequency of tumor-infiltrating immune cells (Supplementary Table S3) revealed distinct clusters of bacteria showing positive

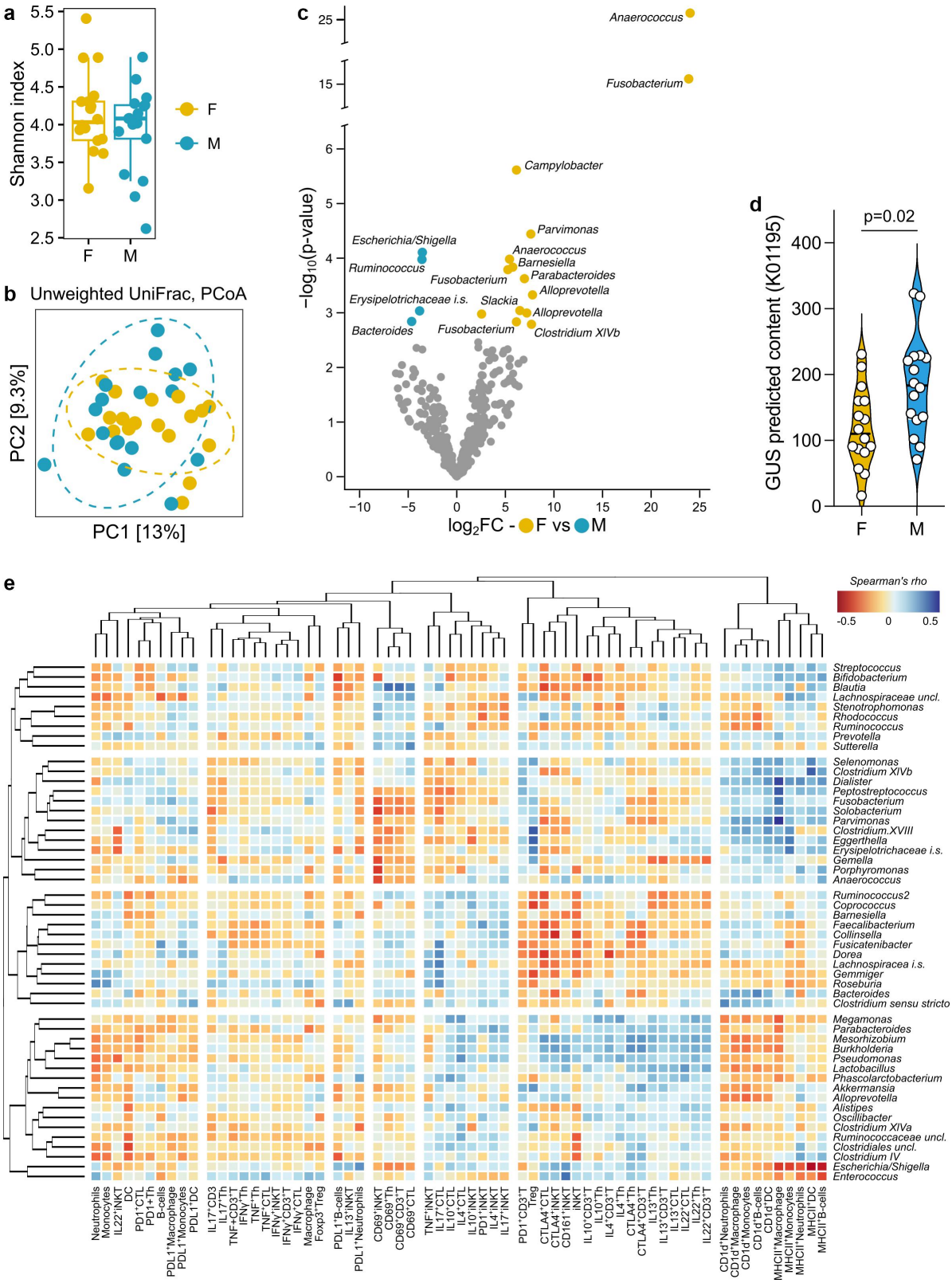

Figure 2. Tumor-associated immune-microbiota interactions in CRC patients. A) microbiota α-diversity as measured by Shannon index in female and male CRC patients. B) PCoA of microbial β-diversity as measured by unweighted UniFrac distance. C) volcano plot showing the significantly enriched bacterial amplicon sequence variants (ASVs) (fdr-corrected $p < 0.05$) by DEseq2 analysis. The names of significantly enriched bacterial ASVs classified up to the genus level are reported. D) predicted metagenomic gene content of β-glucuronidase (GUS) inferred by Tax4Fun2 analysis. E) Heatmap of Spearman's rho correlations between the relative abundance of the most abundant (mean relative abundance > 0.25%) tumor-associated bacterial taxa with the frequency of tumor-infiltrating immune cells analyzed by FACS in male and female CRC patients. CTL, $CD8^+$ cytotoxic T lymphocytes; Th, $CD4^+$ T helper cells; DC, dendritic cells.

or negative correlations with antigen presenting cells (APC) expressing MHCII and CD1d (Figure 2(e)). Interestingly, these bacterial clusters also exhibited corresponding positive or negative correlations with type 17 and type 2 immune responses and with T cells expressing the immune checkpoint CTLA-4, aligning inversely with the correlations observed for APCs (Figure 2(e)). A cluster of oral pathogens, including *Fusobacterium, Porphyromonas, Peptostreptococcus*, and *Parvimonas*, exhibited a positive correlation with the frequency of APCs, T cells expressing PD-1, and T-regulatory (Treg) cells. Conversely, this bacterial cluster showed a negative correlation with activated/tissue-resident $CD69^+$T cells (Figure 2E). Altogether, these data suggest that sex-based differences in the tumor-associated microbiome and its metabolism may influence diverse immune responses potentially contributing to sexual dimorphism of CRC.

Estrogen-related differences in immunity and the microbiome composition promote sexual dimorphism of CRC in the AOM/DSS mouse model

To better understand sex-biases influencing the developmental pathway of CRC, we compared tumor burden and immunophenotypes between female and male animals in the AOM/DSS colon cancer model at different time-point of tumorigenesis (Figure 3(a)). In this animal model, we observed sexual dimorphism in CRC tumorigenesis, with male animals exhibiting a worse endoscopic score and a higher tumor count compared to females at an early timepoint (T1) of tumorigenesis. (Figure 3(b-d)). However, the disparity in tumor burden significantly diminished at a later timepoint (T2) (Figure 3(c-d)), suggesting that aging in female mice may influence CRC progression more than in males. Immunophenotyping of tumor-infiltrating cells revealed a reduction of conventional $CD4^+$ helper and $CD8^+$ cytotoxic T cells at T2 compared to T1 in female mice (Figure 3(e-g)). In contrast, $CD8^+$T cells increased over time in male mice, suggesting a progressive decline in antitumor immunity in females, as observed in CRC patients. Furthermore, we observed a consistently higher frequency of iNKT cells in tumor-bearing female mice at both T1 and T2 (Figure 3(h-i)), mirroring the observations in human patients. As alterations in gut microbiome composition can impact CRC progression,[21] we performed a metagenomic analysis of female tumor-bearing C57BL/6 mice and sex and age-matched control mice (CTRL) at both timepoints of tumorigenesis. The gut microbiome of AOM/DSS mice exhibited a reduction of microbial α-diversity compared to controls ($p = 0.132$; AOM vs CTRL, T1; $p = 0.027$; AOM vs CTRL, T2) (Figure 3(j)) and significant changes of the microbial community structure as the disease progressed, as measured by β-diversity (unweighted UniFrac, $R^2 = 0.19$, F-stat = 2.11, $p = 0.021$; AOM/DSS, T1 vs T2) (Figure 3(k)) and DESeq2 analysis (Figure 3(i), Supplementary Table S4). The gut microbiome of tumor-bearing female mice at T1 demonstrated a greater similarity with tumor-free control animals (CTRL) than AOM/DSS mice at T2 (Figure 3(k)). Notably, tumor-bearing animals showed a greater reduction in β-glucuronidase activity (KEGG orthology K01195) at T2 compared to T1 and CTRL animals, as measured by the analysis of predicted microbiome functions (Figure 3(m)). The analysis of serum estradiol (E2) showed that its level decreases over time and is significantly lower in female tumor-bearing mice compared to age-matched controls (Figure 3(n)). In tumor-bearing mice, estradiol levels correlated positively with $CD4^+$T cell infiltration (Figure 3(o)) and negatively with iNKT cells (Figure 3(q)) while frequency of $CD8^+$T cells was unaffected by estradiol levels (Figure 3(p)). Taken together, these findings suggest an intertwined relationship among the gut microbiome, estrogen metabolism, and antitumor immune responses.

The gut microbiota affects iNKT cell cytotoxic functions in a disease and sex dependent manner

iNKT cells are potentially cytotoxic but their infiltration correlates with negative outcomes in CRC due to the detrimental effects of CRC-associated microbes on their phenotype.[17,48] Since we found iNKT cells significantly enriched in CRC lesions of female patients (Figure 1(f)), we hypothesized that these cells may be directly involved in the sexual dimorphism of CRC. To test this hypothesis, we induced tumorigenesis using AOM/DSS in iNKT cell-deficient mice (*Traj18*$^{-/-}$ mice). We observed no difference in tumor burden between female and male *Traj18*$^{-/-}$ mice at both T1 and T2 (Figure 4(a-b)), providing the first evidence that iNKT cells contribute to the sexual dimorphism of CRC. Next, we asked whether the antitumor functions of iNKT cells could be impaired by the tumor-associated microbiome in a sex-dependent manner. Thus, we performed a series of experiments involving the priming of intestinal and circulating human iNKT cell lines[19,29] with monocyte-derived dendritic cells (moDC) loaded with the tumor microbiota from female and male CRC patients (Figure 4(c), Supplementary Figure S5A-B). We observed that the microbiome from female patients significantly reduced the iNKT cell-mediated killing of CRC cells compared to both male patients and healthy controls (Figure 4(d)). We observed also a reduced release of granzyme (Figure 4(e)) and perforin (Figure 4(f)) upon stimulation with the microbiome from female patients, in agreement with our previous demonstration that iNKT cells depend on the perforin – granzyme pathway for proper elimination of colon cancer cells.[19] Finally, we assessed whether exposure to estrogens could affect iNKT cell functions and provide resistance against the immunosuppressive signals induced by the female-derived CRC microbiome. Indeed, we observed that conditioning iNKT cells with the tumor microbiome from female patients significantly increased the frequency of these cells expressing the co-inhibitory receptors CTLA-4, PD-1, and TIGIT (Figure 4(g-j)). However, exposure to 17β-Estradiol (E2) notably reduced the expression of these co-inhibitory receptors, while increasing the frequency of $GMZA^+PFN^+$ iNKT cells (Figure 4(g-j)). Inhibition of E2 signaling using the ERβ-selective antagonist PHTPP reinstated the negative effects of the female-derived CRC microbiome on the expression of exhaustion and cytotoxic markers in iNKT cells, suggesting that estrogens armor iNKT cells against the immunosuppressive signals of oncomicrobes enriched in the tumor microbiome of female patients (Figure 4(g-j)). Altogether these data suggest that iNKT cells may play a role in the progression of CRC in female patients.

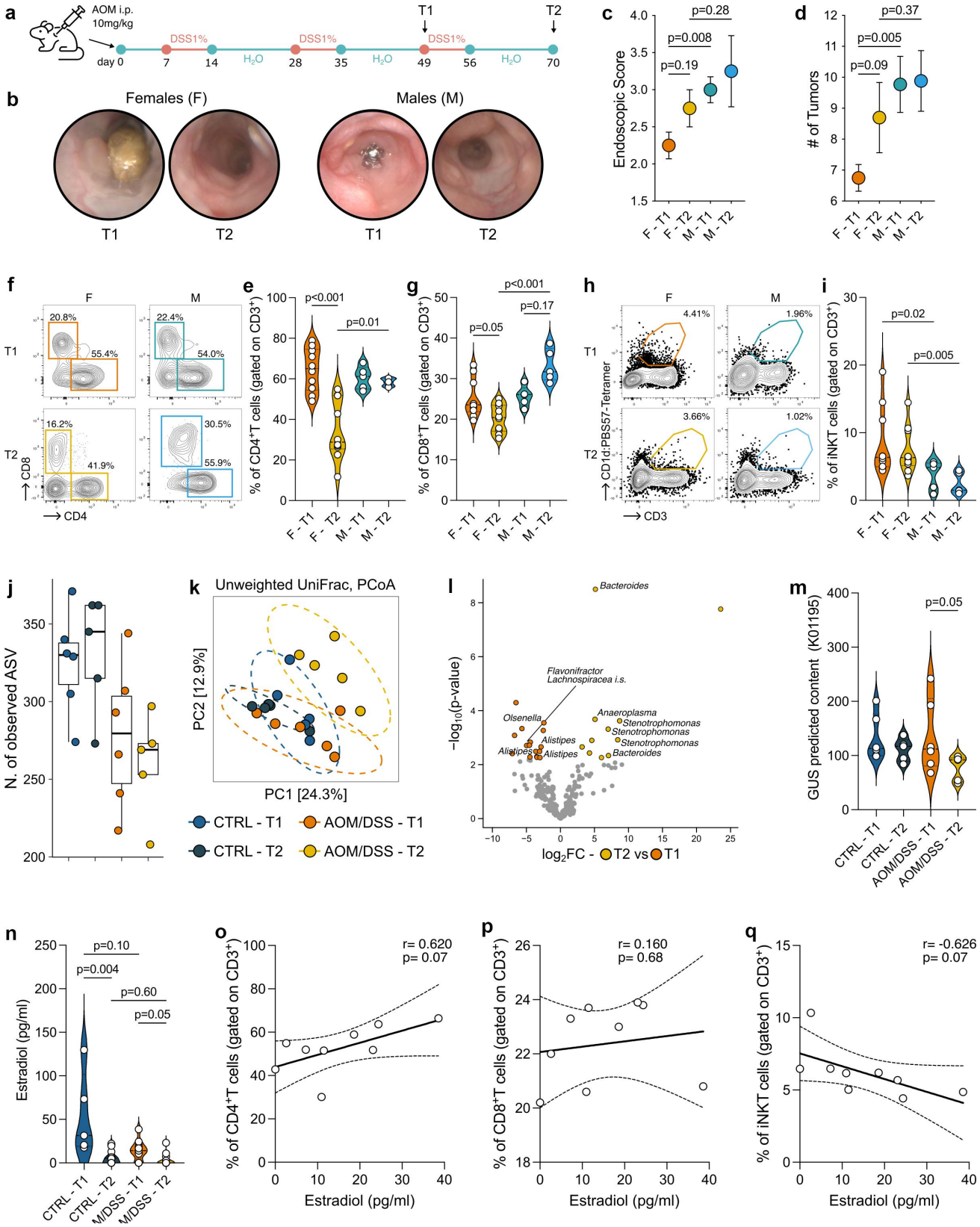

Figure 3. Age-related decline of estrogen levels and antitumor immunity correlates with reduced protection from colorectal tumorigenesis in female tumor-bearing mice. a) schematic representation for the AOM/DSS experimental plan. b-c) tumor endoscopic score and representative endoscopic pictures; d) number of tumors from male (M) and female (F) AOM/DSS treated C57BL/6 animals at early (T1) and late (T2) timepoints from the induction of tumorigenesis. e-g) frequency of tumor-infiltrating f) $CD4^+$ and g) $CD8^+$ T cells in the AOM/DSS model with representative plots. h-i) frequency of tumor-infiltrating iNKT cells with representative plots. j) observed number of ASVs in female AOM/DSS treated C57BL/6 animals at early (T1) and late (T2) timepoints from the induction of tumorigenesis and age-matched control mice (CTRL). k) PCoA of microbial β-diversity as measured by unweighted UniFrac distance. l) volcano plot showing the significantly enriched bacterial amplicon sequence variants (ASVs) (fdr-corrected $p < 0.05$) by the DEseq2 analysis. The names of significantly enriched bacterial ASVs classified up to the genus level are reported. m) predicted metagenomic gene content of β-glucuronidase (GUS) inferred by Tax4Fun2 analysis. n) measurement of serum estrogen by quantitative ELISA. O-Q) correlation of serum estrogen with tumor-infiltrating o) $CD4^+$T, P) $CD8^+$T and Q) iNKT cells. Data points, $n = 5$-10 from two pooled independent experiments representative of three.

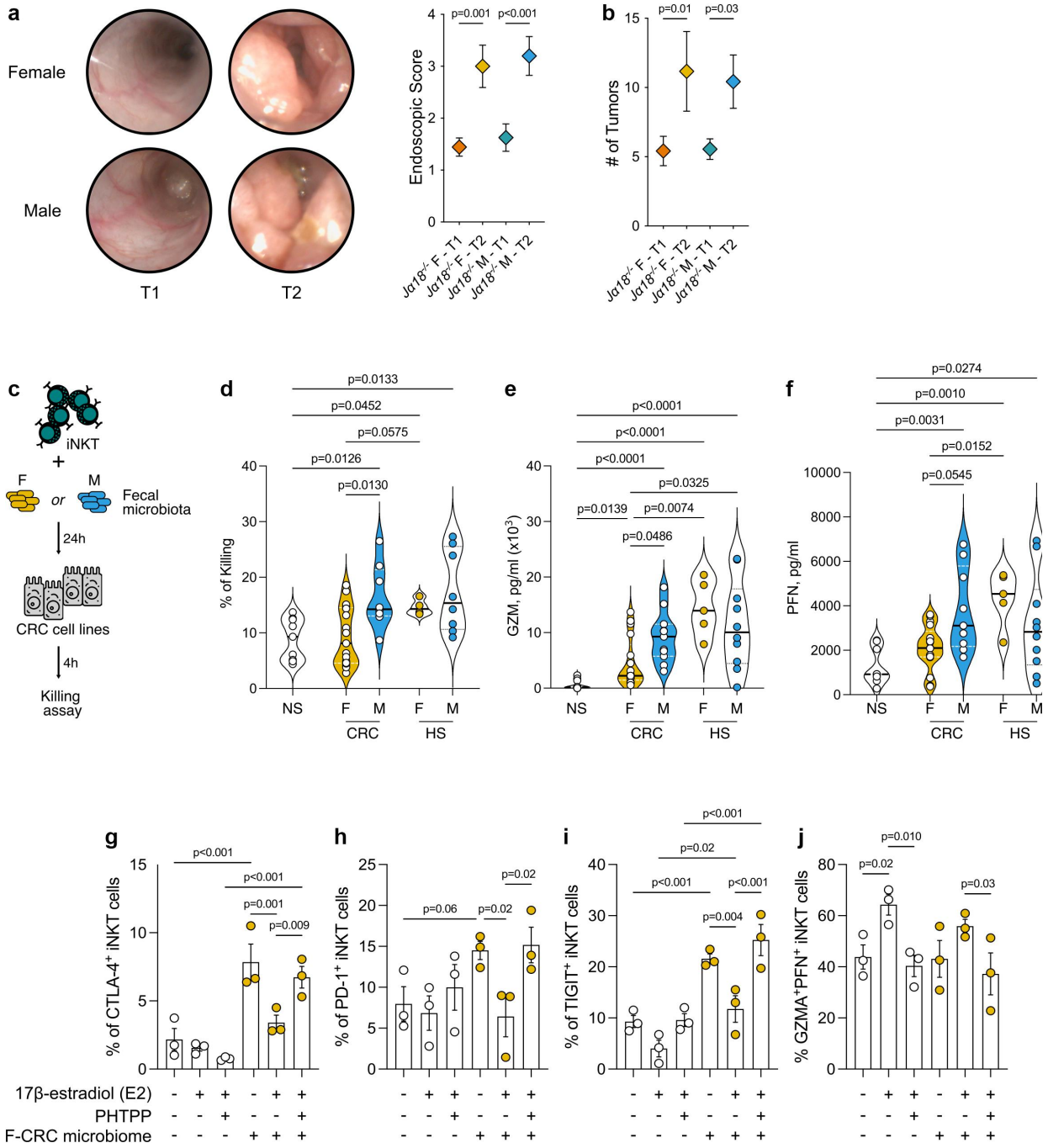

Figure 4. The tumor microbiota of female CRC patients impairs iNKT cell cytotoxicity. a) tumor endoscopic score and representative endoscopic pictures; b) number of tumors from male (M) and female (F) AOM/DSS treated *Traj18*$^{-/-}$ mice at early (T1) and late (T2) timepoints from the induction of tumorigenesis. c) schematic representation of the experimental plan. d) percentage of killed tumor cells by iNKT cells conditioned by the tumor microbiota of female (F) and male (M) CRC patients and the fecal microbiota of healthy subjects (HS). e) granzyme B (GzmB) and f) perforin (PFN) concentration in the culture supernatant of iNKT cells conditioned by the microbiota of female (F) and male (M) CRC patients and healthy subjects (HS). G-J) frequency of g) CTLA-4^{+}, h) PD-1^{+}, i) TIGIT^{+} and j) GZMA^{+}PFN^{+} iNKT cells conditioned by the tumor microbiota of female CRC patients. NS, non-stimulated. Data are representative of at least three independent experiments.

Discussion

The mortality rate for CRC is higher in men than in women in the general population,[1] creating a misconception that CRC is not a primary health concern for women. Contrary to this belief, CRC is the second cause for cancer death in women and, among individuals aged 65 and older, females exhibit lower survival rates than males, as observed in our study cohort (Figure 1(b-c)) and in previous studies.[4–6] A possible explanation for the survival pattern observed in female patients after the age of 65 could be linked to reduced estrogen exposure starting from the perimenopausal period.[3,10,49] The use of hormonal replacement therapy markedly drops among women after the age of 65,[50] which corresponds with gender-specific trends observed in our and other studies.[3,10,49] This reflects a major limitation of our human study cohort, as we lack specific indicators of hormonal status, such as information on menopausal status, use of hormonal replacement therapy and oral contraceptives.

Despite the established carcinogenic role of sex hormones in many tissues, estrogen exerts protective effects in the colon.[10,51] This could be explained by the role of sex hormones in shaping immune responses, and possibly as a consequence of their interaction with the gut microbiome. Indeed, by deconjugating estrogens, the gut microbiome can affect their bioavailability locally and systemically.[25] Dysbiosis *i.e.*, alteration in the microbiome composition, can disrupt this process, potentially reducing estrogen levels and diminishing their protective role in colorectal carcinogenesis. In this study, we observed sex-specific alterations in the tumor-associated microbiome in CRC patients, with a notable reduction in microbial β-glucuronidase functions in female patients (Figure 2(c-d)). This reduction has important functional consequences as estrogens regulate the expression of cytotoxic effector molecules, such as IFNγ and granzyme A, in $CD8^{+}T$[14] and iNKT cells.[52] The decreased expression of these effector molecules can result in a decline of intratumor cytotoxic T cell responses, as shown by the immunophenotyping of older female patients compared to age-matched male patients (Figure 1(f)). Additionally, in the AOM/DSS animal model of CRC, a decline in β-glucuronidase activity and circulating estrogens was observed as the disease progressed in female mice, accompanied by alterations in the composition of the gut microbiota (Figure 3(j-n)). Consistent with our findings, a recent study using this model showed that intestinal deletion of the estrogen receptor ERβ exacerbates the severity of CRC and affects gut microbiome composition.[53] Moreover, ovariectomy increases the tumor burden in the AOM/DSS CRC model, but 17β-estradiol (E2) supplementation mitigates this effect.[54] This indicates that endogenous estrogens protect against colon cancer in the AOM/DSS model, with exogenous E2 showing similar protection in ovariectomized mice,[54] potentially through NF-κB and Nrf2 signaling pathways.[55–57] Additionally, E2 supplementation in male mice also provides protection against AOM/DSS-induced tumorigenesis[58] promoting beneficial changes to the microbiome that could attenuate CRC development.[53,58] How sensing of estrogens may influence the gut microbiome has not been demonstrated but it is plausible to speculate that the immune system may be involved in this process. Indeed, there is a bidirectional crosstalk between the gut microbiota and the immune system that is important in health and disease.[59] When correlating the gut microbiome with tumor-infiltrating immune cell populations, we observed that females, compared to males, are enriched by oncomicrobes aligning with an immunosuppressive response (Figure 2(e)). Among the microbial taxa enriched in female patients, it is worth to mention *Fusobacterium*. The periodontal pathobiont *Fusobacterium nucleatum* is considered a risk factor for CRC. It has the potential to stimulate the proliferation of cancer cells, reshape the immune microenvironment, and contribute to metastasis and chemoresistance during the initiation and progression of CRC.[60] Moreover, *F. nucleatum* skews iNKT cell functions toward protumor immunosuppressive responses[17] rather than promoting antitumor cytotoxic immunity in CRC.[19] Notably, iNKT cells and *Fusobacterium* were enriched in female patients and when stimulated with a microbiome derived from these patients, iNKT cells lost their cytotoxic properties (Figure 4(d)). The enrichment of iNKT cells in older female patients aligns with a previous study on a cohort of Korean subjects, which found that the frequency of circulating NKT cells is higher in women than men and significantly increases with age.[61] Moreover, estrogens selectively enhance a Th1-like phenotype in iNKT cells, leading to gender differences in immune responses. Indeed, activated iNKT cells in female mice produce high levels of IFNγ, an effect reduced by ovariectomy but unaltered by orchidectomy, providing the first evidence that estrogens influence iNKT cells leading to gender dimorphism in their cytokine production profile.[52] Here, we showed that iNKT cells play an important role also in the sexual dimorphism of CRC since mice lacking iNKT cells showed no differences in tumor burden upon AOM/DSS-induced tumorigenesis (Figure 4(a-b)). Estrogens protected iNKT cells from immunosuppressive signals induced by the female-derived tumor microbiome on these cells by reducing the expression of co-inhibitory receptors and maintaining their cytotoxic functions (Figure 4(g-j)).

In this study, we primarily focused on biological sex, specifically examining how sex-biased factors such as hormones, immune cell infiltration, and the microbiome affect colorectal cancer outcomes. However, we recognize that sex and gender are distinct concepts, with gender representing a social construct that may also influence health outcomes. Gender roles, particularly in older populations, may contribute to disparities between men and women in health outcomes. Previous research has shown that older women are more likely than older men to report poor self-assessed health, partly due to their older age and lower socioeconomic status during their working lives. This may help explain the survival differences observed between older female and male patients in the TCGA and Italian cohorts. It is plausible that, due to traditional gender roles in Italian households,[62] older women may experience greater stress from lower levels of support. As primary caregivers, they may not receive the same spousal or social support that elderly men with cancer do; this disparity in support could influence survival rates. A recent population-based study of an Italian cohort with CRC confirmed a survival advantage for older men compared to women.[63] Interestingly, this pattern shifts when functional disabilities are considered. When controlling for factors such as age, class, and income, older women with functional impairments paradoxically report better health than older men.[64] This paradox highlights the complex relationship between gender, health perceptions, and outcomes, and warrants further investigation.

Although this study did not explore gender-based factors in depth, we recognize their potential significance and suggest that future research should consider both biological sex and gender roles when examining cancer outcomes in aging populations. In conclusion, our study emphasizes how the complexity of CRC is influenced by sex-biased factors such as hormones, immune cell infiltration, and the microbiome. By uncovering sex-specific mechanisms involving the interaction between the gut microbiome and

cytotoxic T cell responses, our findings pave the way to design targeted therapeutic approaches in precision medicine that address sex bias in CRC with the aim to advance the efficacy of cancer treatments and improve patient outcomes.

Ethics approval and consent to participate

The study was approved by the Institutional Review Board (Milan, Area B) with permission number 566_2015. Informed consent was obtained and accepted by all patients before enrollment.

Animal experimentation was approved by the Italian Ministry of Health (Auth. 10/21 and Auth. 1217/20) and by the animal welfare committee (OPBA) of the European Institute of Oncology (IEO), Italy.

Consent for publication

All authors have read and approved the final version of this manuscript.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Authors contribution

FS conceived the study. FF, FS, and GL designed the experiments. GL, ADB, FP, CA, BC, EC and AB performed the experiments. FF and FS supervised the experiments. FC, MV and LB contributed with reagents and resources. GL performed multidimensional FACS data analysis. FS performed 16S rRNA gene sequencing and statistical data analyses. FS wrote the manuscript. All authors reviewed and critically edited the manuscript. All authors contributed to the article and approved the submitted version.

Data availability statement

16S rRNA gene sequencing data are available in the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under accession numbers PRJEB56178 and PRJEB72057.

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