Obesity exacerbates colitis-associated cancer via IL-6-regulated macrophage polarisation and CCL-20/CCR-6-mediated lymphocyte recruitment

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Colorectal cancer (CRC) is one of the most lethal cancers worldwide in which the vast majority of cases exhibit little genetic risk but are associated with a sedentary lifestyle and obesity. Although the mechanisms underlyingCRC and colitis-associated colorectal cancer (CAC) remain unclear, we hypothesised that obesity-induced inflammation predisposes to CAC development. Here, we show that diet-induced obesity accelerates chemically-induced CAC in mice via increased inflammation and immune cell recruitment. Obesity-induced interleukin-6 (IL-6) shifts macrophage polarisation towards tumour-promoting macrophages that produce the chemokine CC-chemokine-ligand-20 (CCL-20) in the CAC microenvironment. CCL-20 promotes CAC progression by recruiting CC-chemokine-receptor-6 (CCR-6)-expressing B cells and γδ T cells via chemotaxis. Compromised cell recruitment as well as inhibition of B and γδ T cells protects against CAC progression. Collectively, our data reveal a function for IL-6 in the CAC microenvironment via lymphocyte recruitment through the CCL-20/CCR-6 axis, thereby implicating a potential therapeutic intervention for human patients.
The current obesity epidemic not only accounts for the increased incidence of classical comorbidities such as type 2 diabetes mellitus, but also predisposes to the development of certain cancers—primarily those that require an inflammatory tumour microenvironment (TME). One cancer type that is strongly associated with obesity is colorectal cancer (CRC)

Obesity-induced alterations in microbiota composition and stem cell modulation have been demonstrated to promote CRC development,

Here we investigate the role of obesity-induced IL-6 during development and progression of CAC in mice. We demonstrate that macrophage-specific IL-6Rα inactivation strongly ameliorates CAC in obesity. This is owing to a reduction of the chemoattractant CC-chemokine-ligand-20 (CCL-20) derived from M2 macrophages, which in turn facilitates recruitment of B cells and γδ T cells into the TME in a CC-chemokine-receptor-6 (CCR-6) dependent manner. Thus, we identify IL-6R signalling in macrophages as an important mediator of colon carcinogenesis during obesity.

Results

Diet-induced obesity increases CAC development. In a first experiment, we aimed at elucidating whether diet-induced obesity affects colon inflammation and CAC. To model obesity-induced CAC in mice, we exposed cohorts of C57BL/6 mice to either normal chow (NCD) or high-fat diet (HFD) feeding from weaning on. As expected, 8-week-old HFD-fed animals exhibited increased body weight and body fat content with increased serum insulin and leptin levels as well as impaired glucose homeostasis (Supplementary Fig. 1a-e).

Next, we investigated expression of inflammatory cytokines as well as markers for immune cells in the colon tissue of these mice. This analysis revealed the elevated expression of inflammatory cytokines Il6, Tnf, Il1β and Il10 in the colon of obese mice compared with lean controls (Fig. 1a). Examination of immune cell markers in the obese colon revealed increases in T cells (Cd3), regulatory T cells (Tregs) (forkhead-box-protein P3 (Foxp3)), B cells (Cd19) but similar expression of the common macrophage marker epidermal growth factor-like module-containing mucin-like hormone receptor-like 1 (Emr1) (Fig. 1b).

Thus, HFD-feeding accelerates inflammation and lymphocytes in the colon of mice.

Next, we subjected cohorts of NCD- and HFD-fed mice to the established colitis-associated adenoma protocol induced by azoxymethane (AOM) injection directly followed by three repetitive cycles of 2.5% dextrane sodium sulphate (DSS) in the drinking water over 7 days (Supplementary Fig. 1f)30. Strikingly, obese mice either died spontaneously or had to be killed during the first DSS cycle, as these animals lost more than 20% of their initial body weight owing to symptoms associated with exaggerated colitis (Supplementary Fig. 1g, h). As the standard CAC protocol turned out to be highly lethal for obese mice, we adjusted the conditions accordingly to ensure survival of all cohorts throughout the treatment period. Therefore, we injected 8-week-old mice with AOM (day 1) and supplemented the drinking water with 1.5% DSS from day 5 for 5 days. At day 10 of the 1.5% AOM/DSS protocol the animals received normal water until the end of the experiment at day 62 (non-tumour, tumour) (Fig. 1c). To examine the colitis phase animals were killed with 10 weeks of age at day 13 (colitic) and as control with 8 weeks of age at day 0 (non-colitic) of the 1.5% AOM/DSS protocol.

Notably, lean mice that developed colitis exhibited an adapted colonic microenvironment at day 13 that resembled the inflammatory state of non-colitic HFD-fed mice (Fig. 1a, b, d, e). Strikingly, HFD in colitic mice increased Il6 expression and to a lower extent Tnf, Il1β and Il10 (Fig. 1d). Although colitic mice, independent of diet, had similar presence of T cells and B cells in colons compared with non-colitic HFD-fed colons, expression of the Treg transcription factor FoxP3 was increased suggesting a shift in T-cell composition (Fig. 1b, e). Macrophages increased only in tendency in obese colitic colons (Fig. 1e). Thus, HFD feeding mimics the inflammatory phenotype found in colitis.

Colitis compromises the gut barrier function, reflected by rectal bleeding, by mounting immune responses against commensal
Fig. 1 Obesity enhances colonic inflammation and CAC tumour burden in C57BL/6 mice. a, b qPCR analysis of indicated gene expression in distal colons derived from 8-week-old non-colitic NCD (n = 5) and HFD-fed (n = 5) C57BL/6 mice; following results are presented relative to non-colitic NCD. c Scheme of the 1.5% AOM/DSS protocol of mice fed a NCD or a HFD from weaning (3 weeks of age) until the end of the experiment with 17 weeks of age. 8-week-old mice were injected with 10 mg/kg AOM i.p. at day 1 of the 1.5% AOM/DSS protocol. From day 5 on, mice received 1.5% DSS in the drinking water for 5 days. Mice were either killed with 8 weeks of age at day 0 (non-colitic), 10 weeks of age at day 13 for analysis of colitic conditions (colitic) or with 17 weeks of age at day 62 (non-tumour and tumour) for analysis of tumourigenesis. d, e qPCR analysis of indicated gene expression in distal colons of 10-week-old NCD (n = 5) and HFD-fed (n = 5) colitic C57BL/6 mice; results are presented relative to non-colitic NCD. f Normalised albumin concentration in faeces of NCD (n = 10) and HFD-fed (n = 10) C57BL/6 mice under non-colitic (day 0) and colitic (day 13) conditions. g Representative endoscopic pictures of 13-week-old NCD and HFD-fed C57BL/6 mice of the 1.5% AOM/DSS protocol. h Tumour number of 17-week-old NCD (n = 18) and HFD-fed (n = 13) C57BL/6 mice counted at day 62 of the 1.5% AOM/DSS protocol. AOM, azoxymethane; DSS, dextran sodium sulphate, NCD, normal chow diet; HFD, high-fat diet; CAC, colitis-associated colorectal cancer. centre line: median; box limits: 1st and 3rd quartiles; whisker: maximum to minimum, *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 two-tailed unpaired Student’s t-test a, b, h or two-way ANOVA followed by Fisher LSD d–f.
Factors such as bacterial lipopolysaccharides (LPS)\textsuperscript{30}. To examine gut barrier function and rectal bleeding, we measured the presence of blood-abundant albumin in faeces of mice\textsuperscript{31}. This analysis showed elevated immunoreactive albumin in the faeces of HFD-fed mice both under non-colitic and colitic conditions, indicating impaired gut barrier function (Fig. 1f). Further colonic investigation using endoscopy\textsuperscript{32} revealed elevated presence of dysplastic neoplasia in colitic HFD mice compared with NCD-fed mice (Fig. 1g). Importantly, macroscopic investigation of colons demonstrated an increased tumour burden in obese mice when compared with their lean littermates at day 62 (Fig. 1h and Supplementary Fig. 1i). Furthermore, colons of diet-induced obese mice were shortened, which is indicative of elevated inflammation and colitis (Supplementary Fig. 1j)\textsuperscript{30}. Moreover, we observed an exaggerated immune cell infiltration into tumours of obese mice as determined by histochemistry using F4/80 and CD3 antibodies to identify macrophages and T cells (Supplementary Fig. 1k, l). Taken together, diet-induced obesity exaggerated colonic inflammation, impaired gut barrier function and enhanced CAC development. Furthermore, HFD-feeding per se induced colonic inflammation, and colitis in lean mice increased inflammation to levels of non-colitic HFD-fed mice. Importantly, of the investigated cytokines, only IL-6 was upregulated in colitis of obese mice implicating that IL-6 might have a role in obesity-induced CAC development.

**IL-6Ra deficiency in myeloid cells reduces CAC development.** Next, we asked whether the obesity-induced elevation in IL-6 is driving CAC development. First, to identify the cell type that

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**Fig. 2** IL-6Ra deficiency in myeloid cells reduces CAC. AOM/DSS-induced tumour numbers in colons of a NCD-fed (n = 9–16) and b HFD-fed (n = 7–20) 17-week-old Il6r\textsuperscript{fl/fl}, Il6r\textsuperscript{αα25}, Il6ra\textsuperscript{KO}, Il6ra\textsuperscript{αα25-KO}, Il6ra\textsuperscript{myl-KO} mice at day 62 of the 1.5% AOM/DSS protocol. Tumour numbers counted at day 62 of 17-week-old c NCD (n = 6–7) or d HFD (n = 6–7)-fed C57BL/6 mice injected with either PBS, 500 µg anti-IL-6 antibody or 150 µg sGP130Fc at day 3 of the 1.5% AOM/DSS protocol. e Representative immunofluorescent staining of macrophages using F4/80 antibody (red) counterstained by Hoechst 33422 (blue) in colons of 17-week-old HFD-fed Il6r\textsuperscript{fl/fl} and Il6r\textsuperscript{αα25} mice at day 62 of the 1.5% AOM/DSS protocol. f, g qPCR analysis of indicated gene expression in tumours (n = 6–7) of 17-week-old NCD or HFD-fed Il6r\textsuperscript{fl/fl}, Il6r\textsuperscript{αα25} and Il6ra\textsuperscript{myl-KO} mice at day 62 of the 1.5% AOM/DSS protocol, results are presented relative to non-colitic distal colon of Il6r\textsuperscript{fl/fl} NCD at day 0. h Relative cell number (%) of CD11c\textsuperscript{+} / CD11b\textsuperscript{+} dendritic cells, CD11c\textsuperscript{+} /CD11b\textsuperscript{+} macrophages and CD11c\textsuperscript{−} /CD11b\textsuperscript{+} lymphocytes from lymphocyte gate SSC/FSC in colitic colons of NCD-fed Il6r\textsuperscript{fl/fl} and Il6ra\textsuperscript{αα25} mice at day 13 of the 1.5% AOM/DSS protocol. i Representative FACS plots of CD11c and CD11b expression in immune cells from a NCD, azoxymethane; DSS, dextran sulphate; NCD, normal chow diet; HFD, high-fat diet; CAC, colitis-associated colorectal cancer. Centre line: median; box limits: 1st and 3rd quartiles; whisker: maximum to minimum; *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 two-tailed unpaired Student’s t-test, h, one-way a–d or two-way ANOVA followed by Fisher LSD f, g. Scale bar, 100 µm. 

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responds to IL-6 we induced CAC in lean and obese mice with conditional ablation of the IL-6Ra in all cells (Il6raKO), in IECs (Il6raIEC-KO)27, in mature T cells (Il6raT-KO) or in the myeloid lineage19 (Il6raImm-KO) using the 1.5% AOM/DSS protocol (Fig. 1c). As a consequence of Cre recombinase expression, exons 2 and 3 of the Il6ra allele are excised to conditionally inactivate IL-6Ra in the respective cell type (Supplementary Fig. 2a-d). In line with a previous report employing IL-6 knockout mice, IL-6Ra deficiency protected against CAC development under lean and under obese conditions (Fig. 2a, b, Supplementary Fig. 2e–j)25. The cell type-specific inactivation of IL-6 signalling revealed that IEC-specific and T cell-specific inactivation of
IL-6Ra did not decrease CAC development. However, myeloid lineage-specific IL-6Ra deficiency protected against CAC (Fig. 2a, b, Supplementary Fig. 2c–j). Macrophages can provide IL-6 and sIL-6Ra in CAC, but IL-6 signalling intrinsic in macropohages might also have a role in CAC25. To investigate whether neutralising IL-6 or sIL-6Ra affects CAC, we injected anti-IL-6 antibody or the designer cytokine soluble GP130 (sGP130Fc) before the colitis phase at day 3 of the 1.5% AOM/DSS protocol in lean and obese C57BL/6 mice. C57BL/6 mice had similar body weight loss in colitis independent of anti-IL-6 and sGP130Fc treatment under NCD and HFD conditions (Supplementary Fig. 2k, l). Of note, tumour numbers were only reduced in tendency upon neutralisation with anti-IL-6 in lean and obese mice (Fig. 2c, d, Supplementary Fig. 2m–r). On the other hand, lean and obese mice with blockade of sIL-6Ra signalling had similar tumour numbers independent of neutralisation (Fig. 2c, d).

However, anti-IL-6 and sGP130Fc treatment in lean mice reduced affected colonic area (Supplementary Fig. 2m), which is in line with a previous report, demonstrating IL-6 deficiency reducing tumour numbers in CAC25. Of note, other IL-6-type cytokines such as CNTF that have not been blocked by antibody-mediated IL-6 depletion and blockade of sIL-6Ra via sGP130Fc might compensate in CAC development. Nevertheless, neutralising IL-6 and sIL-6Ra agents are less effective than genetic IL-6Ra ablation and in line with our previous experiments might even suggest macrophage-intrinsic IL-6 signalling functions impacting on CAC.

In this context, we have previously demonstrated that IL-6Ra deficiency in the myeloid compartment impedes M2 macrophage polarisation19. Furthermore, reports allocate M2-like macrophages a tumour-promoting function via angiogenic and immunomodulatory factors35. On the other hand, M1-like macrophages combat tumours via their proinflammatory and cytotoxic potential36. As we observed similar total macrophage numbers in tumour tissues independent of genotype (Fig. 2e, f), we next asked whether IL-6Ra deficiency might alter specific macrophage subsets in CAC. Thus, we examined gene expression to identify M1-inducible nitric oxide synthase (iNOS) and M2/arginase 1 (Arg1)-expressing macrophages on isolated tumour tissue derived from control, Il6ra KO and Il6ra myl-KO mice. This analysis revealed that IL-6Ra deficiency in macrophages largely prevented M2-like polarisation as evidenced by reduced Arg1 expression, whereas iNOS expression and thus M1 polarisation remained unaltered (Fig. 2g). Thus, a compromised M2 polarisation might hamper CAC in IL-6Ra-deficient mice. In line with this finding, fluorescence-activated cell sorting (FACS) analysis of immune cells revealed increases in CD11c+ M1 macrophages in colitis colons of Il6ra KO mice, whereas the lymphocytes were reduced in Il-6ra-deficient colons (Fig. 2h, i). These findings suggest that a shifted macrophage polarisation capacity towards the inflammatory M1 lineage is contributing to the reduced CAC development found in Il6ra KO and Il6ra myl-KO mice.

Altered macrophage composition might affect DSS-induced colitis, impairments of gut barrier function and proliferation of IECs by providing growth factors and cytokines. In line with this evidence, increased inflammation and IEC death in a three times DSS-mediated colitis of IL-6-deficient mice is a result of impaired IL-6-mediated signal transducer and activator of transcription 3 (Stat3) action providing survival capacities in normal and premalignant IECs25. However, colitis symptoms such as weight loss and faecal albumin were comparable between control and Il6ra KO mice under NCD and HFD conditions, which might be a consequence of the mild HFD-adapted CAC protocol applied here (Supplementary Fig. 3a–d). Moreover, even colitis-associated proliferation as examined via western blot analyses against the proliferation marker proliferating cell nuclear antigen (PCNA) was similarly detectable in control and IL-6Ra-deficient samples during colitis (Supplementary Fig. 3e, f). In contrast, IL-6Ra-deficient tumours had less PCNA immunoreactivity compared with control tumours, whereas tumours from HFD-fed control mice had increased PCNA levels indicative of increased proliferation compared with NCD-fed controls (Supplementary Fig. 3g, h). Furthermore, decreased proliferation of IL-6Ra-deficient tumours was confirmed via Ki67 staining (Supplementary Fig. 3i, j). Thus, whereas colitis and colitis-associated proliferation is not affected by IL-6Ra deficiency, tumours of Il6ra KO mice exhibited less PCNA and Ki67 reactivity. Hence, the altered macrophage composition in IL-6Ra-deficient mice is not affecting the colitis phase of CAC but impacts on later stages of tumourigenesis.

IL-6Ra deficiency attenuates M2-mediated CCL-20 expression. To gain further insights into the molecular mechanisms that promote CAC via obesity-induced IL-6, we compared global gene expression in obese control vs IL-6Ra-deficient tumour samples by microarray. Although examination of upregulated genes failed
to clearly identify pathways, a detailed analysis of downregulated genes revealed that the TME has been altered in the absence of IL-6 signalling (Fig. 3a, Supplementary Table 1). Gene Ontology analyses revealed that cell recruitment was impaired in knockout tumours compared with controls. Here, important chemokine receptors such as Ccl20 and Ccl5 were downregulated in the microarray of IL-6Ra-deficient tumours (Fig. 3b). Ccl20 and its unique receptor Ccr6 were among the 30 genes that were downregulated in the absence of IL-6Ra deficiency in CAC, expression levels of Ccl20 and of its unique receptor Ccr6 were reduced in knockout tumours (Fig. 3c). Consistently, Ccl20 and Ccr6 were elevated in non-colitic obese

![Image of a network diagram showing gene interactions related to IL-6 signalling](image)

**Figure 3**

**a** Network diagram of gene interactions related to IL-6 signalling. **b** Box plot showing relative expression of Il6r to Il6ra in non-colitic and colitic tissues. **c** Representative images of FoxP3/Hoechst staining in non-colitic and colitic tissues. **d** and **e** Representative images of γδTCR/Hoechst and IgM/Hoechst staining in non-colitic and colitic tissues. **f** Bar graph showing total cell number in CD45+alive, F4/80+, and F4/80- cells in non-colitic and colitic tissues. **g** Bar graph showing total cell number in CD19+, γδTCR+, and βTCR+ cells in non-colitic and colitic tissues. **h** Bar graph showing total cell number in CD19+, γδTCR+, and βTCR+ cells in non-colitic and colitic tissues.
macrophages in numerous types of tumours, which causes the recruitment of CCR-6-expressing lymphocytes. To this end, we Magnetic-activated cell sorting (MACS)-separated macrophages and IECs from colitic colons derived from control, Il6rKO and Il6rmyl-KO mice and subjected them to qPCR to examine Ccl20 expression. This analysis revealed increased Ccl20 expression in control macrophages isolated from colitic colons when compared with IL-6Ra-deficient macrophages (Fig. 3h), whereas IL-6Ra inactivation via LysM-Cre had a tendency to reduced Ccl20 expression (Fig. 3h). However, also control IEC samples exhibited increased Ccl20 expression in colitis either via IEC-derived Ccl20 expression or potentially via containing macrophages in the samples (Supplementary Fig. 4a). From our previous experiments that demonstrate IL-6 signalling in macrophages as crucial mediator in CAC, we speculated that intestinal macrophages are a main source of CCL-20 in CAC. Therefore, IL-6 signalling in macrophages might either directly control CCL-20 expression on the transcriptional level or indirectly via its ability to polarise towards M2 macrophages. To test these hypotheses, we generated bone marrow-derived macrophages (BMDM) from control C57BL/6 mice and polarised them towards M1 via LPS/interferon-γ (IFNγ) treatment or to M2 via IL-4/IL-6 treatment. Examination of CCL-20 in the supernatant revealed that IL-6 stimulation did not directly induce CCL-20 expression in both M1 and M2 (Fig. 3i). However, LPS stimulation mimicking invading commensals increased CCL-20 in M2 compared to LPS-stimulated M1 macrophages (Fig. 3i). This experiment revealed that IL-6 indirectly controls CCL-20 expression in macrophages via its ability to polarise towards M2-type macrophages.

To confirm that IL-6Ra-deficient macrophages fail to produce CCL-20, we subjected control and Il6raKO BMDM to M1 and M2 protocols and stimulated them with IL-6 and LPS, respectively. Consistent with our previous finding, IL-6Ra-deficient M2 had a blunted ability to differentiate into M2 macrophages substan-
tiated by a compromised upregulation of M2 markers Arg1 and Il4r upon IL-6 stimulation (Supplementary Fig. 4b, c). Note, M1 differentiation was similar between control and IL-6Ra-deficient macrophages as revealed by iNos expression (Supple-
mental Fig. 4d). Whereas IL-6 stimulation of M1 and M2 showed undeterminable Ccl20 expression, LPS-stimulated control M2 increased Ccl20 expression compared with the blunted response in IL-6Ra deficiency (Fig. 3j, k). Furthermore, control M2 secreted more CCL-20 compared with IL-6Ra-deficient BMDM in the M2 protocol under LPS-stimulated conditions (Fig. 3i). Thus, CCL-20 expression in macrophages is not directly controlled via IL-6-induced transcriptional regulation but instead, IL-6 polarises macrophages in the TME towards M2 capable to produce CCL-20 upon exposure to commensal antigens such as LPS.

Collectively, these experiments suggest that CCL-20 is expressed by M2-type macrophages in colitis and that the inability of IL-6Ra-deficient macrophages to polarise towards M2-type impedes on CCL-20 expression and presumably on recruitment of CCR-6-expressing cells. Thus, the CCL-20/CCR-6 axis might recruit cell types that promote CAC development.

**IL-6Ra-deficient tumours have reduced lymphocyte quantity.** CCR-6, the unique receptor for CCL-20, is mainly expressed on T and B lymphocytes and CCR-6 binding to CCL-20 causes chemotraction of these cells towards the CCL-20 source. In line with this fact, investigation of gene expression data from the microarray demonstrated that lymphocyte-specific genes, such as Cd19, Cd3d, Cd79a, B cell activating factor receptor (Baff-R), Burton’s tyrosine kinase (BTK) and paired box protein 5 (Pax5) for B cells and Cd3e, Cd3b, Cd4 and Il2ra for T cells, were downregulated in IL-6Ra-deficient tumours (Figs. 3a and 4a, Supplementary Table 1). Furthermore, Gene Ontology analysis revealed a reduced quantity of B cells and T cells in CAC in the absence of IL-6Ra signalling (Fig. 4a). To further validate these findings, we performed qPCR and immunohistochemistry of tumours to detect Treg, αβ T cells, γδ T cells and B cells (Fig. 4b–e). Indeed, the qPCR experiments confirmed the reduction of these lymphocyte subsets in IL-6Ra-deficient tumours when compared with controls (Fig. 4b). Notably, these data are in line with our previous observation of reduced lymphocyte counts in IL-6Ra-deficient mice in colitis (Fig. 2h, i). To quantify CCR-6⁺ lymphocyte recruitment in 1.5% AOM/DSS-induced colitis, we examined total cell numbers from non-colitic and colitic control as well as IL-6Ra-deficient colons by FACS (Fig. 4f–h). Although consistent to our previous data, similar macrophage numbers were observed in control and IL-6Ra-deficient colons, IL-6Ra deficiency largely attenuated lymphocyte recruitment in colitis (Fig. 4f). Using specific antibodies in FACS to detect CD19-expressing B cells and γδ T cells, receptor-expressing T cells revealed that these lymphocyte subsets were reduced in the colitis colons of knockout animals (Fig. 4g).

Furthermore, co-staining with CCR-6 antibody revealed that the majority of B cells and γδ T cells in colitis express CCR-6, whereas only a minority of αβ T cells express the CCR-6 receptor (Fig. 4h) when compared with their total numbers (Fig. 4g). Taken together, these findings clearly demonstrate the reduced

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**Fig. 4 IL-6Ra deficiency compromises CCR-6-expressing lymphocyte recruitment in CAC.** a IPA analysis diagram of quantity of downregulated (green) and upregulated (red) lymphocyte-specific genes in tumours derived from 17-week-old HFD-fed Il6ra⁺ or Il6raKO mice at day 62 of the 1.5% AOM/DSS protocol. Upper number represent respective p-value, lower number fold change, b qPCR analysis of indicated gene expression in tumours of 17-week-old Il6ra⁺ versus Il6raKO (n = 6–12) mice at day 62 after the 1.5% AOM/DSS protocol, results are presented relative to NCD Il6ra⁺ non-colitic colons. Representative immunofluorescent stainings of c Foxp3 (red), d γTcR (red) and e IgM (green) expressing cells in controls from 17-week-old HFD-fed Il6ra⁺ and Il6raKO mice at day 62 of the 1.5% AOM/DSS protocol counterstained with Hoescht 33342. f Total number of CD45⁺, F4/80⁺ and F4/80⁻ cells isolated from non-colitic and colitic colonies at day 0 or day 13 of the 1.5% AOM/DSS protocol in 8- and 10-week-old NCD-fed Il6ra⁺ (n = 4–5) versus Il6raKO (n = 4) mice. g Total number of CD19⁺, γTcR⁺ and jTcR⁺ cells isolated from non-colitic and colitic colonies at day 0 or day 13 of the 1.5% AOM/DSS protocol in 8 and 10-week-old NCD-fed Il6ra⁺ (n = 4–5) versus Il6raKO (n = 4) mice. h Total number of CD19⁺ CCR-6⁺, γTcR⁺ CCCR-6 and jTcR⁺ CCCR-6 cell isolated from non-colitic and colitic colonies at day 0 or day 13 of the 1.5% AOM/DSS protocol in 8- and 10-week-old NCD fed Il6ra⁺ (n = 4–5) versus Il6raKO (n = 4) mice. AOM, azoxymethane; DSS, dextran sodium sulphate; NCD, normal chow diet; HFD, high-fat diet; IPA, Ingenuity pathway analysis; CAC, colitis-associated colorectal cancer. Centre line: median; box limits: 1st and 3rd quartiles; whisker: maximum to minimum, *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 two-tailed unpaired Student’s t-test, one-way ANOVA followed by Fisher LSD. f-h. Scale bar, 100 μm c, e, 200 μm d.
presence of CCR-6-expressing lymphocytes in IL-6Rα-deficient CAC and imply an impaired chemoattractance of these cells into the CAC TME of IL-6Rα-deficient animals.

**CCR-6⁺ lymphocytes promote CAC development.** To directly examine whether the CCL-20/CCR-6 axis is necessary for lymphocyte recruitment during colitis, we investigated CCR-6-deficient mice (Ccr6KO) in our CAC model. During the colitis phase, CCR-6-deficient mice exhibited reduced weight loss (Fig. 5a). Importantly, colons from colitic control Ccr6WT mice had increased Ccr6 expression at day 13 compared with non-colitic controls, whereas consistently Ccr6 expression was absent in Ccr6KO mice (Fig. 5b). Colitic colons derived from CCR-6-deficient animals failed to develop CAC, both under lean and obese conditions (Fig. 5e, f, Supplementary Fig. 5a-c). Therefore, we conclude that CCL-20 released in the colitis phase recruits CCR-6-expressing lymphocytes that

![Figure 5](https://example.com/figure5.png)

**Fig. 5** CCR-6⁺ lymphocytes promote CAC. a Body weight loss in % of NCD-fed Ccr6WT (n = 10) and Ccr6KO (n = 11) mice at day 5 to day 13 of the 1.5% AOM/DSS protocol. b-d qPCR analysis of indicated gene expression in non-colitic and colitic colons of 8- and 10-week-old NCD-fed control Ccr6WT and Ccr6KO at day 0 and day 13 of the 1.5% AOM/DSS protocol (n = 5), results are presented relative to non-colitic Ccr6WT colons. e Tumour number of 17-week-old NCD (n = 17-13) and HFD (n = 9-12)-fed control Ccr6WT and Ccr6KO mice counted at day 62 of the 1.5% AOM/DSS protocol. f H&E staining of colons from NCD-fed 17-week-old Ccr6WT and Ccr6KO mice at day 62 of the 1.5% AOM/DSS protocol. AOM, azoxymethane; DSS, dextran sodium sulphate; NCD, normal chow diet; HFD, high-fat diet; CAC, colitis-associated colorectal cancer. Data are represented as mean ± SEM or centre line: median; box limits: 1st and 3rd quartiles; whisker: maximum to minimum, *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 two-way ANOVA followed by Fisher LSD a-e. Scale bar, 50 μm.
Next, we aimed at investigating which CCR-6+ Recruitment of B cells via CCL-20/CCR-6 promotes CAC.

Promote CAC tumourigenesis. However, which CCR-6-expressing lymphocyte population, that is recruited to the colon in colitis promotes CAC, still has to be investigated. B cells, γδ T cells and Tregs are known to express CCR-6.

Recruitment of B cells via CCL-20/CCR-6 promotes CAC. Next, we aimed at investigating which CCR-6+ cell type contributes to CAC development. A prominent CCR-6-expressing lymphocyte population comprises mature B cells and B cells exert macrophage-polarising functions in colitis. In particular, regulatory B1b-like B cells in the colon (CD5+, IgM+, CD19high, B220low) provide IL-10 to polarise macrophages towards M2-like phenotype and such B cells are induced by gut microbiota-driven IL-1β and IL-6 production. Presumably, these B cells sense and control commensal bacteria during...
DSS-induced colonic damage\(^1\) and engaging innate toll like receptor (TLR)4 on B cells by LPS causes IL-6 production\(^9\). Thus, B cells can exert numerous activities on the CAC TME that are not directly committed to humoral immunity. To investigate the role of B cells and their recruitment via CCR-6/CCL-20 in CAC, we employed B cell-deicient mice. B cells rearrange their VDJ genes during B cell development and removal of J elements via gene targeting in mice results in B cell-deficient mice (JHT mice)\(^31\). We subjected B cell-deficient JHT mice to our CAC protocol and examined colitis parameters and CAC tumour development. Body weight loss in colitis and colitis-induced expression of Il6, Tnf, Il1\(\beta\) and Il10 were reduced in JHT mice (Fig. 6a, b). Furthermore, besides the lack of B cells, less immune cells such as T cells, Tregs and macrophages were present in colitis colons of JHT mice (Fig. 6c). This is in line with the finding that control mice developed dysplastic lesions, whereas the absence of B cells ameliorated colon pathology as revealed by endoscopy (Fig. 6d). Ultimately, control mice developed CAC,
whereas B cell deficiency largely protected against CAC (Fig. 6e, i). Investigation of gene expression revealed that JHT mice exhibited decreased colonic expression of Il6, Ifnβ and Il10, whereas Tnf was unaltered (Fig. 6g). Furthermore, not only B cells were absent in JHT colons but also gene expression markers for T cells and Tregs were reduced (Fig. 6h, i). Consistent with this fact was a reduced Ccr6 expression accompanied with a decreased Ccl20 expression in JHT colons, suggesting that B cells ultimately impact on the CAC TME (Fig. 6). Apparently, the decreased Ccl20 expression is in line with an impaired macrophage polarization to Arg1-expressing M2 as well as iNos-expressing M1-like in JHT colons (Fig. 6k). Collectively, these analyses reveal that during colitis CCL-20-recruited CCR-6+ B cells promote CAC. Contrariwise, we have also investigated JHT mice in the CAC protocol under obese conditions where B cell-deficient mice died either spontaneously or had to be killed owing to excessive colitis symptoms (Supplementary Fig. 5d, e). This discrepancy between diets in B cell-deficient mice hints to a more complex and dual role for B cells in colonic inflammation under different dietary exposures than hitherto assumed and even more strengthens our finding that CCR-6-expressing B cells affect CAC.

γδ T-cell inhibition ameliorates CAC. Although we have demonstrated a crucial function of CCR-6-expressing B cells in the CAC TME, also T cells such as γδ T cells express the CCR-6 receptor. γδ T cells can be separated into CCR-6- and NK1.1-expressing subtypes that fulfil T helper (Th) 17 and Th1 cell functions, respectively.13 Koeeneke et al. demonstrated that anti-γδ T-cell receptor (TCR) antibody injection in mice yielded inhibition of γδ T cells because they downregulate their γδTCR22. We aimed at addressing the function of γδ T cells and their chemoattraction via CCL-20 in our CAC model by injecting anti-γδTCR at day 3 of the 1.5% AOM/DSS protocol prior to the colitis phase of CAC into control and IL-6Rα-deficient animals. Anti-γδTCR injection led to downregulation of γδTCR as obvious from the appearance of CD3- αβ T cells and γδ T cells in the blood of these mice (Fig. 7a). Although most γδ T cells express CCR-6 during colitis in untreated mice, a proportion of γδ T cells in antibody-treated mice were negative for CCR-6 expression (Fig. 7b). Ultimately, lean control mice developed CAC, whereas γδTCR antibody injection drastically reduced tumour burden in control mice similar to IL-6Rα-deficient mice (Fig. 7c, Supplementary Fig. 6a-c). Notably, the reduced CAC burden in anti-γδTCR-treated control mice was largely recapitulated under obese conditions (Fig. 7d, Supplementary Fig. 6d-f). Analysis of tumour-derived gene expression revealed slightly reduced γδTCR upon anti-γδTCR antibody injection in lean mice (Fig. 7e). Our experiments are in line with previous reports assigning γδ T cells as crucial mediators in colonic inflammation by providing pathogenic IL-17.23 Thus, recruitment of CCR-6+ γδ T cells promotes CAC and inhibition of γδ T cells might be a reasonable therapeutically strategy against CAC.

Treg depletion restores CAC in obese IL-6Ra-deficient mice. Another CCR-6-expressing T lymphocyte population that has been demonstrated to contribute to CAC are Treg cells, which control gut inflammation via their immunosuppressive functions.57 We have shown that IL-6 signalling in effector T cells is required to release them from Treg-mediated suppression using the well-established Ovalbumin (OVA) model.54 Suppression of effector function in IL-6Ra-deficient cells included a blunted Th1/Th17 cell polarisation and total absence of Th17 IL-17a response. However, the Th1 response could be restored in IL-6Ra-deficient T cells when Tregs were depleted by anti-CD25 antibody treatment. In order to examine whether Tregs also suppress effector functions in lean and obese IL-6Ra-deficient mice in our CAC model, anti-CD25 antibody was i.p. injected at day 3 of the 1.5% AOM/DSS protocol just prior to the colitis phase of CAC. This experimental procedure should provide only a transient Treg depletion during the colitis phase and should affect T effector cells to a lesser extent that upon activation upregulate CD25. Most likely, the single anti-CD25 antibody injection depletes Tregs only until anti-CD25 antibody is consumed/degraded where in the context of proceeding T-cell development these cells are able to repopulate the colon. Nevertheless, although in lean mice, anti-CD25 antibody-mediated transient depletion of Treg cells exhibited only minor effects, it restored CAC development in obese IL-6Ra-deficient animals, whereas obese control mice had unaltered CAC (Fig. 7f, Supplementary Fig. 6g-m). Detection of markers for αβ T cells and Treg cells in tumours of the obese cohorts revealed a reduction of these cells in IL-6Ra-deficient mice that was largely unaltered when mice received anti-CD25 treatment (Fig. 7g). However, despite the fact that also other cells express Ifnγ and Il17a, the examination of T-cell effector molecules revealed decreased Ifnγ and Il17a expression in obese IL-6Ra-deficient tumours that have been restored upon Treg depletion (Fig. 7h, i). Thus, Treg depletion in obese IL-6Ra-deficient mice restores CAC development via restoration of T-cell effector functions.

Collectively, these experiments reveal a crucial role for the recruitment of CCR-6-expressing lymphocytes into the colon during the colitis phase to promote CAC. Knockout and antibody-inhibition/depletion models demonstrate that recruitment of CCR-6-expressing B cells and γδ T cells promote CAC development whereas CCR-6+ Tregs exert an immunosuppressive role in CAC under obese conditions by interfering with T-cell effector functions. B cells and IL-6 synergise in the polarisation towards CCL-20-expressing macrophages causing a vicious circle of CCR-6+ lymphocyte recruitment in CAC (Fig. 8). Overall, our
experiments assign obesity-induced IL-6 an unappreciated role in the CAC TME by regulating macrophage polarisation and lymphocyte recruitment.

Discussion

The steadily increasing obesity epidemic and the simultaneous incidence of obesity-associated comorbidities such as colorectal cancer requires a detailed understanding of molecular and cellular mechanisms that are affected to develop potential novel therapies. Here, we show that obesity exacerbates AOM/DSS-induced CAC via increased expression of inflammatory cytokines. However, our experimental set up was unable to address whether in obesity preneoplastic lesions occur earlier than in lean mice. We demonstrate that obesity-induced impairment of gut barrier function recruits and activates immune cells that promote CAC. In the colitis phase of CAC, IL-6-polarised M2-like macrophages express the chemokine CCL-20 that recruits CCR-6-expressing lymphocytes, further promoting CAC progression. Our in vitro experiments suggest that intestinal M2 macrophages express CCL-20 in colitis via exposure to commensal antigens such as LPS and not as a consequence of direct IL-6-activated transcriptional traits. Though IL-6-type cytokine-activated STAT3 transcription is mainly regulated via inactivation and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)-controlled transcription55-58. Given that LPS-activated NF-kB-controlled gene expression in M1 macrophages has only minor effects on CCL-20 expression suggests extra transcription factors active in M2 macrophages that act in concert with LPS-induced NF-kB to increase CCL-20 expression. Hence, the substantially reduced Ccl20 expression in colonos of Il6raKO mice is a consequence of collapsed M2-type polarisation of IL-6Ra-deficient macrophages in CAC, thereby compromising CCR-6-expressing lymphocyte recruitment in colitis to promote tumourigenesis. CCR-6-expressing B cells, γδ T cells and Treg cells30,31 have either low or undetectable IL-6Ra expression underlying our observation that the protection against CAC in IL-6Ra-deficient mice is not a direct consequence of IL-6 action in these cells but is rather an indirect effect through compromised chemoattraction.

Nevertheless, these cells might still be able to receive IL-6 signalling in control mice via IL-6-transsignalling mechanisms that we have avoided by using IL-6Ra-deficient mice devoid of both membrane and soluble signalling capacities. Despite the crucial role of direct IL-6 function on the approval of Treg-mediated suppression of αβ T cells, we estimate that Tregs also inhibit other immune cells by a similar mechanism as T-cell-specific inactivation of IL-6 signalling using CD4-Cre is unable to prevent CAC development. In line with this assumption, γδ T cells in the colon were known to be suppressed via Tregs and γδ T cells are not affected by CD4-Cre-mediated recombination59. Although γδ T cells can promote CRC via allocation of IL-17 to transformed tumour cells60,61, they are also able to restrain αβ T-cell responses in cancer by providing inhibitory programmed cell death 1 ligand to αβ T cells62.

Collectively, our study assigns obesity-induced IL-6 as a modulator of the TME in CAC via macrophage polarisation and successive lymphocyte recruitment via the CCL-20/CCR-6 axis. In light of the steadily increasing obesity epidemic, novel treatment options to combat obesity-associated disorders are desperately required highlighting our study to interfere with inflammatory signalling and to inhibit corrupt cell types as important contribution to global health issues.

Methods

Animals. All animal procedures were in compliance with protocols approved by local government authorities (Bezirksregierung, Cologne, Germany Az. 8.87-50.10.31.08.279, 84-02.04.2014.A211 and Mainz, Germany G16-1-072) and were in accordance with NIH guidelines. The experimental cohorts of mice (mus musculus) were females (if not stated differently) that were housed in four different specific pathogen free animal facilities under similar conditions. Mice were housed in single ventilated cages (IVC, TypII long) in groups of three to five at 22–24 °C in a 12-hour–12-hour light-dark cycle (with lights on at 7:00 AM). The cages were implemented with bedding material (Lignocel SF14) and cotton nestlet (Plextr, NC1450). Mice were on C57BL/6 J (IWB; Harlan) or CD1 (Taconic Global, T.2018.R12; Harlan) containing 53.5% carbohydrates, 18.5% protein and 5.5% fat (12% of calories from fat) or, from 3 weeks of age, were fed a HFD (C1057; Altromin) containing 32.7% carbohydrates, 20% protein and 35.5% fat (55.2% of calories from fat). Food and water was available ad libitum. The conditional Il6ra mouse strain served as control mice (Il6raKO32). Mice bearing a complete (Il6raKO33, mythod-specific (Il6raKO34), intestinal epithelial-specific (Il6raKO35) and T-cell specific (Il6raKO36) knockout were generated and genotyped. For identifying the Il6raKO and the Il6raKO alleles PCR was performed with the following primers:

### Induction of CAC in AOM/DSS model and cell depletion

2.5% AOM/DSS protocol: 8-week-old mice fed a NCD or a HFD were i.p. injected with 10 mg/kg body weight AOM (A5486, Sigma-Aldrich) at day 1 of the 1.5% AOM/DSS protocol (Supplementary Fig. 1f). The animals were exposed to three repetitive cycles of 2.5% DSS (MW = 36,000–50,000, 0216011080, MP Biomedicals) in the drinking water for 7 days from day 1–7, day 22–28 and day 43–49 (DSS water was refreshed every second day) to induce colitis. Animals were killed with 8 weeks at day 0 for non-colitic control tissue or at day 13 for colitic analysis. At day 62 tumours were counted in a blinded fashion by number, size (<2 mm, >2 mm) and percentage of the affected colon length was measured from cecum to the anus. The distal part of the colon was used for non-colitic and colitic analysis. At day 62 tumours were counted in a blinded fashion by number, size (<2 mm, >2 mm) and percentage of the affected area of the distal colon. Tumour and non-tumour tissue from the distal colon was separated and used for tumour analysis.

To neutralise IL-6 and sIL-6R signalling, 8-week-old C57BL/6 animals were i.p. injected with 500 µg anti-IL-6 antibody (BE0046, BioXCell) or 150 µg sGP130Fc (provided by Christoph Garbers, Kiel University, Germany) at day 3 of the 1.5% AOM/DSS protocol, respectively. To deplete γδTCR-expressing and Treg cells, Il6raKO and Il6raKO mice were injected with 200 µg anti-γδ antibody (UC7-13D5, biocisence) or 1 mg anti-CD25 antibody (BE0013, BioXCell) at day 3 of the AOM/DSS protocol, respectively. Control animals were injected with the same volume of PBS.

Endoscopy. Non-invasive endoscopy (TRICAM endoscope Karl Storz) was performed under either i.p. injection of ketamine/Rompun or inhalation narcosis using isoflurane with 13-week-old animals.

Analysis of body composition. Fat mass was determined via nuclear magnetic resonance (NMR Analyser minispec mq7.5; Bruker Optik, Ettlingen, Germany) in 17-week-old mice.

Glucose-tolerance test. Glucose-tolerance tests were done with 7–8-week-old C57BL/6 mice. Mice were fasted overnight for 16 h and glucose concentrations in blood were measured after the fasting period. Then each animal received an i.p. injection of 20% glucose solution (10 ml/kg body weight) and glucose concentrations in venous blood were measured after 15, 30, 60 and 120 minutes. Glucose concentrations in whole venous blood were measured with an automatic glucose monitor (Bayer Contour; Bayer).
Analytical procedures. The concentrations of leptin and insulin in serum, CCL-20 in the supernatant of cultured BMDM and albumin in the faces were measured by enzyme linked immunosorbent assays, with mouse standards, according to many
ufacturer’s guidelines: Mouse Leptin ELISA (90030, Crystal Chem), Mouse Insulin ELISA (90080, Crystal Chem), Mouse CCL-20-MIP-3 alpha Quantikine ELISA Kit (MCC200, R&D Systems) and Mouse Albumin ELISA Quantification set (E90-134, Bethyl).

Generation of BMDMs. NCD-fed mice were killed by cervical dislocation and bone marrow was isolated from femurs and tibias. Bone marrow cells were plated in RPMI-1640 medium (supplemented with 10% fetal calf serum (FCS), 1% glutamine, 1% penicillin-streptomycin and 10–50 ng/ml macrophage colony-stimulating factor (130-101-706, Milteny)) and were allowed to differentiate for 7 days. At 24 h before all experiments, macrophage colony-stimulating factor was removed and cells were washed two times with sterile PBS. BMDMs were either stimulated 24 h with 20 ng/ml IFNγ (130-094-048, Miltenyi) and 100 ng/ml LPS (Escherichia coli strain O55:B5; Sigma) or 20 ng/ml IL-4 (130-097-757, Milteny) and 50 ng/ml IL-6 (130-096-685, Milteny) to obtain M1- and M2-polarised macrophages, respectively. Polarisated M1 and M2 were washed with PBS and then stimulated for 8 h or 24 h with 50 ng/ml IL-6 and 10 ng/ml LPS.

MACS sorting. Immune cells and IECs from the colon were isolated according to recent protocol14. IECs were removed from the upper phase after the percoll gradient, washed twice with PBS containing 2% FCS. To purify macrophages, the percoll fractions were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population. Subsequently, the isolated immune cells were used for magnetic separation according to the anti-F4/80+ cell population.
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Author contributions
C.M.W., P.J.A., A.L.O., P.A.-Q., M.C.V., M.-L.T., A.N. performed experiments and analysed data. A.W., C.G., S.T., J.M. and N.H. provided expertise and essential materials. C.M.W. and F.T.W. designed experiments and wrote the paper.

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