

1    **Anti-Metastatic Defense by CD8<sup>+</sup> T Cells**

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13   **Highlights**

- 14   • CD8<sup>+</sup> T cells prevent metastasis in most steps of the metastatic cascade.  
15   • The interaction between DTCs and CD8<sup>+</sup> T cells in the metastatic organ  
16   determines whether DTCs are eliminated, progress to make metastatic lesions or  
17   become dormant.  
18   • CD8<sup>+</sup> T cell-derived cytokines can induce metastatic dormancy in DTCs.  
19   • CD8<sup>+</sup> T cells play a major role in tumor cell clonal evolution by exerting selective  
20   pressure on tumor cells during metastatic progression.

21

22   **Keywords:** Metastasis, CD8<sup>+</sup> T cells, metastatic dormancy, immunoediting.

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26

27 **Abstract**

28 Metastasis is an intricate process whereby tumor cells migrate from the primary tumor,  
29 survive in the circulation, seed distal organs and proliferate to create metastatic foci.  
30 CD8<sup>+</sup> T cells can detect and eliminate tumor cells. Research of CD8<sup>+</sup> T cell-dependent  
31 anti-tumor immunity has classically focused on their role in the primary tumor. There is  
32 increasing evidence, however, that CD8<sup>+</sup> T cells have unique anti-metastatic functions in  
33 various steps of the metastatic cascade. Here, we review the mechanisms whereby CD8<sup>+</sup>  
34 T cells control metastatic lesions. We discuss their role in each step of metastasis,  
35 metastatic dormancy and metastatic clonal evolution as well as the consequent clinical  
36 repercussions.

37

38 **The metastatic cascade and anti-tumor CD8<sup>+</sup> T cell immunity**

39 Most morbidity and mortality caused by solid tumors is due to metastasis. To metastasize,  
40 tumor cells must leave the primary tumor, **intravasate (see glossary)** into the circulation,  
41 survive as **circulating tumor cells (CTCs)**, extravasate into the target organ, adapt to  
42 a new environment as **disseminated tumor cells (DTCs)** and proliferate to create a  
43 macro-metastatic lesion[1]. The ability of DTCs to initiate new cancer lesions in distal  
44 organs resembles the capacity of **cancer stem cells** to initiate tumors[2, 3]. Indeed,  
45 multiple genes involved in metastatic spread, such as SOX-2 or SOX-9, are also  
46 associated with stemness[4, 5] and metastatic lesions often have similar histological  
47 features to the primary tumor in most human cancers. During the process of metastasis,  
48 tumor cells must evade the attack of different immune populations such as NK cells[6, 7]  
49 and T cells[8].

50 T cells can recognize tumor cells if the latter express **neoantigens** or **ectopic antigens**.  
51 The interaction between tumor cells and T cells is illustrated by the cancer immunity  
52 cycle[9]. Here, tumor cells release antigens that are taken up by antigen-presenting cells  
53 (APCs), such as dendritic cells (DCs). APCs process these antigens, mature, and migrate  
54 to the draining lymph nodes where they cross-present antigens to T cells. This process  
55 requires 3 signals: (i) Recognition of the antigen-derived peptide::major histocompatibility  
56 class I (MHC-I) complex by the T cell receptor (TCR), (ii) expression of co-stimulatory

57 molecules and receptors by the DC and CD8<sup>+</sup> T cell, respectively, and (iii) expression of  
58 activating cytokines by the DC[9]. Upon activation, CD8<sup>+</sup> T cells clonally expand,  
59 upregulate activation markers such as immune checkpoints and effector molecules, and  
60 migrate into the tumor. There, they recognize tumor cells that express the cognate  
61 antigen-derived peptide::MHC-I complex and kill them by effector molecules such as  
62 perforin, granzyme or by cytokines such as IFN-γ or TNF-α. Dying tumor cells will release  
63 antigens, thus fueling the cycle[9]. While research of CD8<sup>+</sup> T cell-dependent anti-tumor  
64 immunity has classically focused on the primary tumor, recent advances underscore a  
65 unique role for CD8<sup>+</sup> T cells in every step of the metastatic cascade. Because the primary  
66 tumor plays an essential role in priming of anti-tumor CD8<sup>+</sup> T cells, the role of CD8<sup>+</sup> T  
67 cells in metastasis should not be studied in models that omit T cell priming by the primary  
68 tumor, such as intravenous injection of tumor cells (Box 1).

69

## 70 **CD8<sup>+</sup> T cells in the early steps of metastasis**

71 The cancer immunity cycle is not equally effective in every tumor because a key step of  
72 this cycle, T cell priming, does not always spontaneously occur. Indeed, tumor-reactive  
73 CD8<sup>+</sup> T cells cannot be detected in a significant proportion of cancer patients[10]. In such  
74 situations, CD8<sup>+</sup> T cells are unlikely to play a role in restricting metastasis. To successfully  
75 metastasize, tumor cells must evade spontaneous or therapy-induced CD8<sup>+</sup> T cell attack  
76 during every step of the metastatic process (Figure 1).

### 77 *Local invasion*

78 Metastasis starts with invasion of adjacent tissues by the primary tumor. In this step,  
79 tumor cells face CD8<sup>+</sup> T cells that will try to eliminate them before they enter the  
80 circulation. To evade immune attack, tumor cells orchestrate the development of an  
81 immunosuppressive tumor microenvironment (TME)[11]. Tumor cells remodel the  
82 adjacent extracellular matrix aided by mesenchymal cells and myeloid cells. CD8<sup>+</sup> T cells  
83 can interfere with these populations, thus preventing tumor cell invasion. For example,  
84 extracellular vesicles secreted by CD8<sup>+</sup> T cells reduced local tumor invasion and  
85 metastasis by depletion of intratumoral mesenchymal cells[12]. However, it is still unclear  
86 whether CD8<sup>+</sup> T cells also directly interfere with the invasion capacity of tumor cells.

87 *Epithelial-to-mesenchymal transition and intravasation*

88 In some cases, carcinoma cells undergo an epithelial dedifferentiation, known as  
89 epithelial-to-mesenchymal transition (EMT), that can increase their metastatic  
90 potential[13]. EMT is not a binary process, and distinct intermediate states between  
91 epithelial and mesenchymal phenotypes exist[14]. EMT, however, seems not always  
92 required for metastatic colonization[15]. Indeed, in some cases, lack of EMT and  
93 presence of an epithelial phenotype favors metastasis formation[14]. Factors that inhibit  
94 CD8<sup>+</sup> T cell-mediated anti-tumor immunity, such as TGF-β, have been shown to promote  
95 EMT and increased metastasis *in vivo*[16]. Whether CD8<sup>+</sup> T cells directly regulate EMT,  
96 however, is unclear. A series of studies compared a breast cancer cell line derived from  
97 Her2/neu transgenic mice before and after undergoing *in vivo* immunoediting. In this  
98 model, *in vivo* CD8<sup>+</sup> T cell-mediated immunoediting was associated with increased EMT  
99 and stemness markers[17-19], however, this did not lead to increased primary tumor  
100 growth *in vivo*, and metastatic capacity was not evaluated[17]. T cell effector cytokines,  
101 such as IFN-γ and TNF-α, can induce EMT when incubated *in vitro* with breast[20],  
102 pancreatic[21] or prostate cancer[22] human cell lines. Nonetheless, whether *in vivo* T  
103 cell-derived IFN-γ and TNF-α can induce EMT and whether this leads to increased  
104 metastasis is unknown. The fact that immune surveillance promotes immune evasion may  
105 explain the apparent paradox that immune surveillance promotes EMT[14]. This  
106 hypothesis is supported by the fact that EMT results in upregulation of PD-L1 and  
107 downregulation of MHC-I, both of which render tumor cells resistant to CD8<sup>+</sup> T cells[23].  
108 Therefore, it is also possible that, rather than directly inducing EMT, CD8<sup>+</sup> T cells  
109 preferentially eliminate cells that did not undergo EMT. After undergoing EMT and  
110 intravasation, tumor cells will circulate until dissemination into distal organs.

111 *Survival as CTCs*

112 CTCs must survive in the circulation until they arrive at the target organ. Because CTCs  
113 lack the immunosuppressive environment of the primary tumor, they may be more  
114 susceptible to immune elimination. However, CTCs can increase their metastatic potential  
115 by forming clusters with neutrophils[24], platelets[25] or other CTCs[26]. Such clusters  
116 may allow CTCs to evade immune surveillance. For instance, CTCs bind to platelets,

117 which protect them from NK cytotoxicity[25]. Platelets also secrete TGF-  $\beta$ [16], which is a  
118 known inhibitor of anti-tumor T cell immunity[27]. While the capacity of NK to eliminate  
119 CTCs has been widely described[6, 7], the role of CD8 $^{+}$  T cells in this process is largely  
120 unknown. Although a correlation between the numbers of CTCs and the percentage of  
121 cytokine producing CD8 $^{+}$  T cells in the circulation[28], as well as between PD-L1  
122 expression by CTCs and worse outcome[29] suggest a role for CD8 $^{+}$  T cells in controlling  
123 CTCs, direct experimental evidence is missing.

124 *Extravasation into the target organ*

125 Before CTCs can extravasate, they need to arrest their motion, potentially becoming an  
126 easier target for CD8 $^{+}$  T cells. While the lead role of NK cells and patrolling monocytes in  
127 controlling CTCs in the capillaries is well established[6, 7, 30], the evidence for CD8 $^{+}$  T  
128 cell involvement in this step is scarce. A study found that CTCs trapped in the capillaries  
129 of the lungs can activate conventional resident DCs. Depletion of these DCs promoted  
130 metastasis, presumably due to compromised CD8 $^{+}$  T cell control[31]. Also, for this step  
131 in the metastatic cascade, further experiments are required to define a role for CD8 $^{+}$  T  
132 cell-mediated killing of extravasating cells.

133

134 **Control of disseminated tumor cells in the metastatic organ**

135 Once CTCs have seeded the target organ, they will face CD8 $^{+}$  T cell attack with three  
136 possible outcomes: **Metastatic dormancy**, complete elimination of DTCs by CD8 $^{+}$  T  
137 cells, or immune evasion and consequently, metastatic lesions (Figure 2, Key Figure).

138 *Metastatic dormancy*

139 While in some cases metastasis is clinically apparent already at diagnosis, in others it  
140 appears years after resection of the primary tumor[32]. This phenomenon, known as  
141 metastatic dormancy, is particularly prevalent in certain cancers such as ER $^{+}$  breast  
142 cancer[33] and melanoma[34]. Indeed, patients who underwent breast cancer resection  
143 have increased mortality even 20 years after surgery[35]. Moreover, early-stage  
144 melanomas can have metastatic relapses decades after resection[36]. Such late  
145 metastatic relapses require awaking of dormant DTCs[37] and depend on tumor-cell-

146 intrinsic[38] and -extrinsic[37] mechanisms including the immune response. Observations  
147 in immunosuppressed organ transplant recipients, who developed tumors of donor origin  
148 after receiving organs from patients who were apparently cured of cancer decades  
149 ago[39], underscore the potency of the immune system to protect against metastasis.  
150 Metastatic dormancy differs from immune equilibrium[40]: Whereas metastatic dormancy  
151 is characterized by the presence of non-cycling DTCs, a balance between proliferation  
152 and apoptosis keeps the number of DTCs constant in immune equilibrium.

153 Different immune populations have been associated with metastatic dormancy including  
154 CD8<sup>+</sup>[41] and CD4<sup>+</sup> T cells[42] as well as NK cells[43]. Recently, CD39<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T  
155 cells were identified as the essential immune population mediating metastatic dormancy  
156 in a preclinical breast cancer model[44]. Despite the expression of so-called **exhaustion**  
157 **markers**, CD39<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells were enriched in effector molecules and had a  
158 **tissue-resident** memory signature (Box 2). Using depletion and adoptive transfer  
159 experiments, CD39<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells were unequivocally shown to be essential for  
160 metastatic dormancy. In a cohort of breast cancer patients, the frequency of tumor-  
161 infiltrating CD39<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> but not total CD8<sup>+</sup> T cells correlated with delayed metastatic  
162 relapse after resection (disease-free survival)[44]. In the same study, T cell-derived IFN-  
163  $\gamma$  and TNF- $\alpha$  were identified as essential mediators of metastatic dormancy. This is  
164 consistent with the previously described capacity of these cytokines to induce  
165 senescence in tumor cells through activation of p16INK4a and downstream Rb protein  
166 hypophosphorylation leading to G1/G0 permanent growth arrest[45]. The involvement of  
167 T cell-derived effector cytokines in metastatic dormancy contrasts with their possible  
168 ability to drive EMT[20-22] as mentioned above. Because T cell-dependent promotion of  
169 EMT was linked to a cancer stem cell phenotype[19], it is possible that IFN- $\gamma$  or TNF- $\alpha$   
170 restrict the proliferation and activates stemness pathways in cancer cells, which support  
171 tumor cells in hostile environments such as the blood (EMT) or in distal organs (metastatic  
172 dormancy). While induction of cell cycle arrest may be the main mechanism underlying  
173 metastatic dormancy, involvement of cytotoxicity cannot be excluded. For example, CD8<sup>+</sup>  
174 T cells may mediate dormancy by killing most of DTCs and inducing cell-cycle arrest in  
175 those that escaped[46]. Furthermore, in a mouse model of spontaneous uveal melanoma,

176 DTCs migrated to the lungs early, where they showed limited proliferation and caused  
177 late metastasis. Depletion of CD8<sup>+</sup> T cells promoted proliferation of DTCs and the  
178 appearance of macro-metastasis[41]. Depletion of CD8<sup>+</sup> T cells also led to the awaking  
179 of dormant lung metastasis in a model of fibrosarcoma[47].

180 How dormant DTCs evade CD8<sup>+</sup> T cell-mediated killing is an aspect of special interest.  
181 DTCs seed the metastatic organs as small clusters or as single cells[26, 44, 48], making  
182 the orchestration of an immunosuppressive TME in which CD8<sup>+</sup> T cells lose function  
183 unlikely. Instead, DTCs preferentially seed perivascular niches[49], which are rich in  
184 immunosuppressive molecules[50, 51], and may benefit from systemic  
185 immunosuppression generated by the primary tumor[52]. Loss of MHC-I by DTCs in the  
186 liver was proposed as an evasion mechanism in a model of pancreatic cancer[48]. In this  
187 study, suppression of the epithelial phenotype and downregulation of MHC-I resulted from  
188 an endoplasmic reticulum stress response. This mechanism mimics the process of EMT  
189 described above, where tumor cells lose their epithelial phenotype and downregulate  
190 MHC-I[23]. Indeed, MHC-I downregulation is a common immune evasion mechanism in  
191 patients treated with **immune checkpoint inhibitors**[53]. Although downregulation of  
192 MHC-I could result in increased sensitivity to NK cells[23], the evidence that NK cells  
193 control solid tumors or DTCs is scarce[7].

194 Dormant DTCs can awake and progress to macro-metastases, but it is still unclear what  
195 drives this process. Although there is compelling evidence that adaptive immunity induces  
196 metastatic dormancy[41, 44, 48], it is unknown whether continued immunosurveillance is  
197 needed for maintenance of dormancy. Dependence on constant immunosurveillance was  
198 observed in some studies where CD8<sup>+</sup> T cell depletion resulted in awaking of dormant  
199 DTCs and macrometastasis[47, 48]. Consequently, awaking is only possible when  
200 dormant cells evade immune surveillance. On the one hand, DTCs can acquire resistance  
201 to CD8<sup>+</sup> T cell-mediated induction of senescence[46], however, it is unclear whether this  
202 happens during dormancy. Alternatively, the tumor-specific CD8<sup>+</sup> T cell response might  
203 wane over time, especially in the setting of minimal residual disease. Indeed, the memory  
204 response of tissue-resident T cells is unstable in the absence of antigen stimulation[54,  
205 55]. On the other hand, DTC can awake due to activation of cellular proliferation  
206 programs. Particularly, inflammatory events such as cigarette smoke-derived LPS[56] or

207 surgery[57] seem to wake dormant tumor cells. Therefore, both inflammation and immune  
208 evasion may contribute to the awaking of dormant DTCs. However, whether constant  
209 immunosurveillance is always needed or whether strong proliferative signals can  
210 overcome immune control is still unknown.

211 The involvement of CD8<sup>+</sup> T cells in metastatic dormancy has direct clinical implications.  
212 PD-1 blockade reduced the number of dormant DTCs in the lungs in a preclinical  
213 model[44], presumably by increasing CD8<sup>+</sup> T cell activity. This finding may explain the  
214 observation that stage IV melanoma patients treated with PD-1 blockade had  
215 exceptionally durable responses[58]. Along these lines, the analysis of metastasis-  
216 specific readouts, such as metastasis free survival, in immunotherapy clinical trials could  
217 potentially improve our understanding on the influence of CD8<sup>+</sup> T cell stimulation in  
218 metastasis and metastatic dormancy. Furthermore, many cancer patients receive  
219 corticosteroids to treat cancer- and chemotherapy-related complications. Corticosteroids  
220 are known inhibitors of anti-tumor CD8<sup>+</sup> T cell responses[59, 60], and could contribute to  
221 metastatic relapses even by non-immune related mechanisms[61]. More studies are  
222 needed to understand the effect of corticosteroids and other cancer treatments in  
223 metastatic dormancy.

224 *Immune-mediated elimination or immune evasion*

225 The interaction between CD8<sup>+</sup> T cells and DTCs in the metastatic organ does not always  
226 lead to dormancy, and other scenarios exist (Figure 2).

227 There are a few studies showing that CD8<sup>+</sup> T cells eliminate all DTCs, thus preventing  
228 metastasis. This was seen in the EMT6 model of breast cancer, where the primary tumor  
229 primed CD8<sup>+</sup> T cells that killed all DTCs in the lungs by induction of apoptosis[62]. Another  
230 study in a spontaneous model of breast cancer showed that exposure of mice to an oral  
231 carcinogen increased the immunogenicity of the primary tumor by increasing its  
232 mutational burden and expression of CCL21 in tumor cells. This led to a stronger CD8<sup>+</sup> T  
233 cell response that eliminated DTCs in the lungs[63].

234 Alternatively, DTCs can evade immune surveillance and form metastatic lesions[27]. This  
235 can be due to resistance to CD8<sup>+</sup> T cell-mediated growth arrest induction or killing.  
236 Resistance to IFN- $\gamma$  and TNF- $\alpha$ , which mediate growth arrest in metastatic dormancy[44],

237 is common in cancer[64, 65]. Additional mechanisms, such as secretion of TGF- $\beta$ , can  
238 diminish T cell-mediated immune surveillance and thus promote development of  
239 macrometastasis[27]. Finally, any parameter that compromises efficient T cell priming by  
240 the primary tumor[10, 44] precludes CD8 $^{+}$  T cell-mediated control of metastasis.

241

## 242 **Modulation of systemic CD8 $^{+}$ T cell immunity in metastatic disease**

243 Despite systemic, protective immunity, some DTCs will eventually produce clinically  
244 apparent metastatic lesions. A locally compromised immune defense in the metastatic  
245 organ may be essential for metastatic outbreaks.

### 246 *Modulation of immunity by environmental cues*

247 Local conditions in the metastatic or dormant niche influence the anti-tumor T cell  
248 response. Organ-specific particularities can lead to the development of different immune  
249 contexts in different metastatic lesions. This was illustrated in a case of an ovarian  
250 cancer patient whose metastases progressed differently during off-treatment periods.  
251 Analysis of the individual lesions showed abundant T cell infiltration and clonal expansion  
252 in regressing lesions but immune exclusion in progressing lesions[66]. Similarly, co-  
253 occurrence of different immune landscapes within the same patient was recently reported  
254 in a case of a melanoma patient treated with immunotherapy[67] and in a cohort of  
255 colorectal carcinoma patients[68]. A recent study showed that lung metastases shared  
256 an immune signature that differed from the signature in other metastatic sites and,  
257 moreover, was independent of the origin of the primary tumor[69]. Furthermore, non-small  
258 cell lung cancer (NSCLC)-derived brain metastases contained less T cells and more  
259 macrophages compared with primary tumors[70]. This finding, however, could also reflect  
260 divergent evolution of metastatic cells towards a more immunosuppressive phenotype.  
261 The importance of the immune microenvironment in the target organ may explain why  
262 immunotherapy seems to be more effective in lymph node metastasis than in other  
263 organs[71, 72]. The abovementioned examples illustrate that the metastatic niche may  
264 have organ-specific features. In case of the liver, the influence of the metastatic niche  
265 seems to have systemic components. For example, patients with liver metastases –  
266 independently of the type of primary cancer – respond worse to immunotherapy than

267 patients with metastases in other organs[73]. The effect seems to involve dampening of  
268 systemic anti-tumor immunity by regulatory T cells[74] or loss of circulating activated  
269 CD8<sup>+</sup> T cells[73]. Metastatic infiltration of some organs, however, can support systemic  
270 anti-tumor immunity. This was recently described in a mouse model of melanoma with  
271 brain metastasis where the presence of extracranial disease increased the traffic of CD8<sup>+</sup>  
272 T cells to the brain which led to increased response to immune checkpoint blockade[75].  
273 A wide array of local cues probably contributes to the heterogeneity of local and systemic  
274 anti-tumor immunity. For instance, individual organs contain different numbers and  
275 subtypes of tissue-resident T cells[54, 55] and myeloid cells[76], which influence  
276 protective anti-tumor immunity[77]. Also, metabolites[78, 79], microbiota and biophysical  
277 conditions[80] vary among different tissues and all influence T cell function.  
278 Consequently, mechanisms underlying immune evasion by DTCs presumably vary  
279 depending on organ-specific conditions.

280 *Modulation of immunity by tumor-cell-intrinsic factors*

281 Tumor-cell-intrinsic features, such as expression of neoantigens, immune checkpoint  
282 molecules, cytokines or MHC-I, can influence the TME and therefore, spontaneous as  
283 well as therapy-induced anti-tumor immunity[77, 81]. In addition, tumor-cell-derived  
284 factors often influence systemic immunity. Analysis of the immune infiltrate of various  
285 organs in multiple mouse cancer models showed that even localized tumors can alter the  
286 immune environment of distal organs leading to systemic immunosuppression[82]. The  
287 changes induced by the primary tumor included a re-organization of the T cell  
288 compartment and a recruitment of myeloid cells to the peripheral tissues. The latter can  
289 promote metastatic seeding of CTCs by creating a pre-metastatic niche[52].

290 The occurrence of cancer-related systemic immune suppression could be explained by  
291 the capacity of many tumors to secrete exosomes that are rich in PD-L1, inhibit CD8<sup>+</sup> T  
292 cells and are associated with reduced response to PD-1 blockade[83]. Systemic  
293 immunosuppression may restrict the defense to bacterial and viral infections[82], and  
294 indeed, sepsis is a common ultimate cause of death in cancer patients[84]. Furthermore,  
295 systemic immune suppression is particularly deleterious in the context of  
296 immunotherapy[85]. The observation that systemic immune suppression is reverted after

297 primary tumor resection[82] may have important consequences for the treatment of  
298 cancer patients, since it is unclear whether immunotherapy is more effective when given  
299 before (neo-adjuvant) or after (adjuvant) primary tumor resection. Nonetheless, while  
300 adjuvant therapy is not affected by primary tumor-mediated immunosuppression, neo-  
301 adjuvant therapy seems more effective in mouse models[86] and expands more tumor-  
302 resident T cell clones in cancer patients[87]. The possible superiority of neo-adjuvant  
303 immunotherapy is still unclear, but factors like the more abundant presence of antigen or  
304 the lack of post-surgical immunosuppression could potentially explain this.

305

### 306 **Metastatic clonal evolution during systemic immune pressure**

307 A tumor is composed of heterogenous tumor cells that continuously evolve due to  
308 accumulation of mutations and selective pressure. Intratumor heterogeneity explains why  
309 resistant tumor cells emerge upon treatment[88] and why metastasized cancers often  
310 differ from the primary lesion[68].

311 CD8<sup>+</sup> T cells are a major driving force for competition between different tumor cell  
312 clones[88], which differ in immunogenicity for various reasons[67, 89]. CD8<sup>+</sup> T cells  
313 preferentially eliminate the more immunogenic clones, thus selecting for immune-evasive  
314 clones[40]. This process is known as **immunoediting**[90] and is especially relevant  
315 during metastatic progression, as underlined by a study that analyzed the immune  
316 infiltrate and clonality of different metastatic lesions in colorectal cancer patients[68].  
317 Another study in NSCLC showed that loss of MHC-I, which was identified as a sign of  
318 immunoediting in early-stage primary tumors, was further enriched in brain  
319 metastasis[89]. Although some tumor cell intrinsic features, such as EMT[14, 23] or  
320 activation of β-catenin signaling[91, 92], have been associated with both immune evasion  
321 and metastasis, it is unknown whether CD8<sup>+</sup> T cells preferentially control tumor cells that  
322 lack certain metastatic traits. The dynamic competition between the adaptive immune  
323 response and different tumor populations determines which tumor cell subclones will  
324 become prevalent in space and time over the course of metastatic disease[67].

325 Intratumor heterogeneity aids the tumor in coping with attacks by different classes of  
326 therapies including immunotherapy. For example, immune recognition and control of

327 mouse tumors composed of mixes of clones inversely correlated with the number of  
328 clones and genetic diversity of the tumor[93]. Along the same lines, a high degree of  
329 intratumor heterogeneity was associated with poor CD8<sup>+</sup> and CD4<sup>+</sup> T cell infiltration,  
330 increased infiltration of regulatory T cells and decreased survival in a cohort of breast  
331 cancer patients[94].

332 In addition, heterogeneity with respect to the immune infiltrate is observed between  
333 different lesions in the same patient but also even within the same lesion[68, 95]. Regional  
334 differences in tumor cell mutations seem an important driver of this immune  
335 heterogeneity. For example, a study of NSCLC patients described a correlation between  
336 the regional presence of CD8<sup>+</sup> T cell clones and mutations[95]. Thus, intratumor  
337 heterogeneity may result in a miscellaneous distribution of antigens and a heterogenous  
338 distribution of antigen-specific CD8<sup>+</sup> T cells. This can be especially relevant to the first  
339 steps of metastasis, since some studies suggest that metastatic tumor cell clones tend to  
340 originate from the center of the primary tumor[96], which means that the clonal anatomy  
341 of the primary tumor could potentially shield metastasizing populations from immune  
342 surveillance.

343

#### 344 **Concluding remarks and future perspectives**

345 Recent advances in the fields of metastasis research and tumor immunology have  
346 emphasized the specific anti-metastatic functions of CD8<sup>+</sup> T cells that only now we are  
347 starting to understand. It seems plausible that priming of CD8<sup>+</sup> T cells by the primary  
348 tumor is important for preventing the early steps of metastasis (Figure 1). Whether and to  
349 which extent CD8<sup>+</sup> T cells can restrict CTCs, however, is largely unknown. After  
350 dissemination to the metastatic organ, DTCs presumably face immunosurveillance  
351 mediated by CD8<sup>+</sup> T cells primed by the primary tumor. Depending on many tumor-cell-  
352 intrinsic as well as -extrinsic variables – some of which are still unknown – DTCs are  
353 eliminated, driven into cell-cycle arrest (dormancy) or evade immune attack to form a  
354 metastatic lesion (Figure 2). Especially processes related to metastatic dormancy are  
355 enigmatic, thus, their understanding requires further investigation. The research

356 questions, which seem most relevant to our opinion, are discussed in the Outstanding  
357 Questions Box.

358 A better understanding of the anti-metastatic functions of CD8<sup>+</sup> T cells will potentially lead  
359 to the design of immunotherapies to better target or even prevent metastatic disease.

360

361 **Figure 1. CD8<sup>+</sup> T cells control early steps of metastasis.** Anti-metastatic CD8<sup>+</sup> T cell  
362 responses are most likely primed by the primary tumor. Ineffective priming promotes  
363 immune escape and metastasis. In contrast, appropriately primed CD8<sup>+</sup> T cells can  
364 prevent invasion and extravasation of tumor cells in the circulation by direct elimination  
365 and depletion of mesenchymal cells[12]. Invading tumor cells can undergo EMT which  
366 promotes immune evasion[23]. CTCs are mainly controlled by NK cells in the circulation,  
367 it is unclear whether CD8<sup>+</sup> T cells also control CTCs. CTCs bind to platelets[16, 25], other  
368 tumor cells[26] and neutrophils[24] to increase their metastatic potential and to escape  
369 immuno surveillance. NK cells and patrolling monocytes are the main controllers of  
370 extravasating CTCs. Extravasating tumor cells can also activate DCs, which prime CD8<sup>+</sup>  
371 T cells that could potentially eliminate extravasating cells[31]. Created with  
372 BioRender.com”.

373

374 **Figure 2. CD8<sup>+</sup> T cell-mediated control of DTCs.** Once DTCs arrive to the target organ,  
375 they face CD8<sup>+</sup> T cell attack. The interaction between DTCs and CD8<sup>+</sup> T cells can lead to  
376 3 different outcomes: (i) immune evasion by DTCs leading to proliferation, expression of  
377 the proliferation marker Ki67, and formation of macrometastasis[27], (ii) immune  
378 elimination of all DTCs by CD8<sup>+</sup> T cell effector molecules such as granzyme B (GzmB),  
379 perforin (PRF), IFN-γ and TNF-α[62, 63] and (iii) induction of metastatic dormancy by PD-  
380 1<sup>+</sup> CD39<sup>+</sup> CD8<sup>+</sup> T cell mediated IFN-γ and TNF-α[44]. Dormant DTCs can awake and  
381 cause macrometastasis. Alternatively, stimulation of CD8<sup>+</sup> T cells with PD-1 blockade can  
382 reduce the number of dormant DTCs[44]. Created with BioRender.com”.

383

384 **BOX 1: Models to study metastasis and anti-tumor immunity**

385 Metastasis is a challenging process to reproduce in a model, and different approaches  
386 have been used to obtain mouse models that recapitulate human disease. Many mouse  
387 models are used to study (parts of) the metastatic cascade. Although most models are  
388 suitable to study some aspects of metastasis, most models are insufficient in one way or  
389 another.

390 Quantification and visualization of metastasis require tagging cell lines with fluorescent or  
391 bioluminescent proteins (e.g. luciferase, mCherry), because counting nodules is only  
392 possible for overt macro-metastasis. Dependent on the tag and genetic background of  
393 the mouse, such modified cell lines may be more immunogenic than the parental line and  
394 therefore, display different metastatic behavior[57].

395 Below we discuss three fundamentally different models for studying metastasis: (i)  
396 **Experimental metastasis**, which results from introducing tumor cells in the blood stream,  
397 and (ii) spontaneous metastasis, which is seeded from a primary tumor and (iii)  
398 genetically engineered mouse models (GEMM)

399 *Experimental metastasis*

400 Tumor cells can be introduced into the blood stream to seed metastasis in different  
401 organs. For instance, injection in the tail vein leads to lung metastasis[6, 16, 24, 25, 44]  
402 whereas intraportal or splenic injections cause liver metastasis[48]. The advantages of  
403 this approach are the high penetration (usually 100%) and the synchronized kinetics of  
404 metastasis development. A major disadvantage, which is too often ignored, is the  
405 absence of a primary tumor. First, there are no metastases without a primary tumor in  
406 clinical situation. Second, a primary tumor influences many variables including priming of  
407 adaptive immune defense, as well as systemic effects including immunosuppression and  
408 dysregulated hematopoiesis[82].

409 *Spontaneous metastasis*

410 In these models, tumor cells are injected **orthotopically** or subcutaneously to create a  
411 primary tumor that spontaneously metastasizes. This approach allows T cell priming by  
412 the primary tumor and permits a more holistic approach to study metastasis[82, 86]. It  
413 appears that only selected cell lines spontaneously metastasize in a useful proportion of

414 mice. The underlying reason is not understood in most cases and presumably is  
415 multifactorial. Because of this, most metastasis research uses only a few mouse tumor  
416 cell lines, which is concerning. Furthermore, the primary tumor results from a single  
417 injection of thousands to millions of tumor cells. Therefore, such models have some  
418 disadvantages: Besides the risk that injection of a large number of tumor cells may induce  
419 inflammation, cancer usually starts from a single transformed cell. Finally, using patient-  
420 derived xenografts or human cell lines only allows studying features unrelated to immune  
421 defense.

422 **GEMM**

423 Mice can be genetically engineered to develop different kinds of malignancies. GEMM  
424 avoid the inflammation created by tumor injection and allow the generation of  
425 spontaneous orthotopic cancers in any organ[15, 27, 41]. Tumors generated in GEMM  
426 can be preceded by pre-neoplastic lesions which better recapitulate human disease[27].  
427 Moreover, GEMM are generally designed to express the same mutations as the human  
428 cancer they model[15, 27]. This is of special importance since cancer specific mutations  
429 have important consequences for tumor-specific immunity[27]. Despite similarities to  
430 human cancer, most GEMM develop multiple primary tumors[41], which differs from the  
431 human situation. A challenge, however, is that the timing and incidence of metastasis in  
432 GEMM oftentimes are heterogeneous, and genetic modification of tumor cells such as  
433 expression of fluorescent proteins, requires backcrossing.

434 *Models of metastatic dormancy*

435 Metastatic dormancy has been described in experimental metastasis[44, 48], orthotopic  
436 models with spontaneous metastasis[44, 47] and GEMM[41]. However, there are only a  
437 few models of metastatic dormancy in immunocompetent mice. While the aspects  
438 discussed for metastasis also apply to metastatic dormancy, we are only starting to  
439 understand the influence of anti-tumor immune response in this phenomenon, and further  
440 studies should clarify the influence of each model.

441

442 **BOX 2: CD8<sup>+</sup> T cell memory in control of metastasis**

443 Memory CD8<sup>+</sup> T cells respond promptly upon antigen re-encounter and play an important  
444 role in protection against infections and metastasis[97-99]. In murine models of pancreatic  
445 and breast cancer, transient exposure to primary tumors generated a T cell memory  
446 response that induced metastatic dormancy when mice were re-challenged with  
447 intravenous tumor cells[44, 48]. Memory T CD8<sup>+</sup> responses were also detected in patients  
448 with metastatic melanoma who responded to immunotherapy. In this cohort, the same  
449 CD8<sup>+</sup> T cell clonotypes were detected as effector memory CD8<sup>+</sup> T cells ( $T_{EM}$ ) in the blood  
450 and tissue-resident memory CD8<sup>+</sup> T cells ( $T_{RM}$ ) in the tumor and skin affected with vitiligo  
451 even 9 years after treatment. These memory CD8<sup>+</sup> T cells expressed an *IFNG/TNFA*-high  
452 signature that correlated with survival[99].

453 Among the different subsets of memory CD8<sup>+</sup> T cells,  $T_{RM}$  have emerged as one of the  
454 most relevant types of T cells involved in metastasis control[97, 100].  $T_{RM}$  reside in non-  
455 lymphoid tissues and do not recirculate. They are characterized by the expression of  
456 CD69 and CD103, which contributes to its homing to peripheral tissues, and the lack of  
457 expression of lymph node homing receptors such as CD62L or CCR7[97, 98]. Their  
458 location predestines them for an immediate response in healthy or tumor tissues.  
459 Moreover,  $T_{RM}$  can propagate systemic anti-tumor immunity as described in a mouse  
460 model of melanoma where skin  $T_{RM}$  activated dendritic cells, which subsequently primed  
461 CD8<sup>+</sup> T cell systemically resulting in distal control of lung metastasis[100]. Indeed,  
462 abundance of  $T_{RM}$  correlates with improved survival in multiple human cancers such as  
463 triple negative breast cancer[77], melanoma[100] or NSCLC[101].

464 T cell memory is crucial not only for spontaneous or immune checkpoint-induced anti-  
465 tumor immunity but also for other cancer therapies such as cancer vaccines[102]. For  
466 example, in a cohort of HER2<sup>+</sup> breast cancer patients, vaccination against HER-2 induced  
467 expansion of memory CD8<sup>+</sup> T cells, which was associated with progression-free survival  
468 [103]. Expansion of memory CD8<sup>+</sup> T cells was also observed in melanoma patients that  
469 received a personalized neoepitope-derived mRNA vaccine[104]. These patients showed  
470 a decreased rate of metastasis after vaccination, which illustrates how memory T cell  
471 responses can lead to metastasis control[104].

472

473 **Outstanding questions**

- 474     • Can CD8<sup>+</sup> T cells control CTCs?
- 475     • How do dormant DTCs awake to create metastasis?
- 476     • How do CTCs and dormant DTCs evade immune surveillance?
- 477     • How can we stimulate CD8<sup>+</sup> T cells to eliminate dormant DTCs?
- 478     • Can local, immune-response-related cues in different organs explain the  
479       characteristic metastatic patterns that are observed in some cancer types?

480

481 **Glossary**

482 **Cancer stem cells:** Self-renewing, undifferentiated cancer cells which can give rise to  
483 differentiated cancer cells to create a heterogeneous tumor.

484 **Circulating tumor cells (CTCs):** Tumor cells that have left the primary tumor and entered  
485 the circulation.

486 **Disseminated tumor cells (DTCs):** Tumor cells that have settled in distant organs away  
487 from the primary tumor site after exiting the circulatory system.

488 **Ectopic antigens:** Proteins that are only expressed in some isolated anatomic locations,  
489 that become aberrantly expressed by tumor cells leading to immune recognition.

490 **Extravasation:** The process by which a tumor cell exits the circulation and reaches the  
491 metastatic organ.

492 **Exhaustion markers:** Markers that are upregulated during activation of T cells and that  
493 remain highly expressed on functionally impaired (exhausted) T cells. Such situation often  
494 occurs after persistent TCR stimulation.

495 **Experimental metastasis:** Injection of tumor cells in the circulation to generate  
496 metastatic lesions.

497 **Immune checkpoint inhibitors:** Antibodies that enhance anti-tumor immune response  
498 by blocking the interaction between inhibitory checkpoint receptors on immune cells and  
499 their ligands.

500 **Immunoediting:** Process by which the immune system eliminates immunogenic  
501 populations of tumor cells, leading to selection of less immunogenic populations.

502 **Intravasation:** The process by which a tumor cell enters the circulation.

503 **Memory T cells:** Antigen-specific T cells that persist after the antigen has been  
504 eliminated. Memory T cells rapidly proliferate and develop effector function upon re-  
505 exposure to the specific antigen, thus providing a fast protective response.

506 **Metastatic dormancy:** Process resulting from cell cycle arrest in DTCs for a prolonged  
507 period. Dormant DTCs can awake, start proliferating and produce metastasis.

508 **Neoantigen:** Mutated tumor-cell-specific protein that is absent in normal tissue

509 **Orthotopic:** Presence of a cell type or organ in a location in which it would be found  
510 under physiological circumstances.

511 **Tissue-resident immune cells:** Different immune populations that occupy certain  
512 tissues without recirculating.

513

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523

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