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Immunomodulation under the lens of real-time in vivo imaging

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Abstract

Modulation of cells and molecules of the immune system not only represents a major opportunity to treat a variety of diseases including infections, cancer, autoimmune and inflammatory disorders but could also help understand the intricacies of immune responses. A detailed mechanistic understanding of how a specific immune intervention may provide clinical benefit is essential for the rational design of efficient immunomodulators. Visualizing the impact of immunomodulation in real-time and in vivo has emerged as an important approach to achieve this goal. In this review, we aim to illustrate how multiphoton intravital imaging has helped clarify the mode of action of immunomodulatory strategies such as antibodies or cell therapies. We also discuss how optogenetics combined with imaging will further help manipulate and precisely understand immunomodulatory pathways. Combined with other single-cell technologies, in vivo dynamic imaging has therefore a major potential for guiding preclinical development of immunomodulatory drugs.

Capturing the consequences of immunomodulation with *in vivo* multi-photon microscopy

The immune system is composed of an extensive network of specialized cells distributed throughout the body, ready to detect and eliminate intruding microbes or transformed cells and that can infiltrate tissues in case of sterile inflammation (e.g. myocardial infarction, stroke)[1]. The initiation and orchestration of immune responses is highly regulated in time and space, typically involving the sequential recruitment and activity of distinct cellular subsets. Over the years, strategies to modulate these stages have emerged with the aim to treat patients with a variety of diseases such as infections, cancer, autoimmune and inflammatory disorders. Thus, understanding the spatiotemporal components of immunomodulation is of critical importance and requires cells to be scrutinized *in vivo* within their physiological environment. To this aim, multi-photon microscopy is a technique of choice as it provides extensive information on single-cell dynamics *in vivo* (e.g. migration pattern or cellular interactions) and can thus be particularly resourceful to understand the immediate or longer-term consequences of immunomodulators. Importantly, intravital microscopy (IVM) may reveal unexpected cell behaviors or cellular crosstalk otherwise difficult to grasp in a classical “hypothesis-driven” approach.

Two-photon microscopy uses high power pulsed laser and infrared wavelengths providing good tissue penetration, intrinsic optical sectioning, and reduced toxicity compared to other microscopy techniques [2]–[4]. Tracking of cells requires their fluorescent labelling and various approaches to meet this need exist. For instance, many fluorescent-reporter mice are available to track particular immune subsets such as T cells [5],[6], dendritic cells (DC) [7], macrophages [8],[9], natural killer (NK) cells [10],[11] or neutrophils[12],[13]. To add a level of complexity to our understanding, biosensors (e.g. cell death sensors, calcium sensors, cytokine reporters, phagocytosis reporter etc.) allow cell behavior and functional outcome to be linked [14]–[17]. Moreover, adoptive transfer of fluorescent cells is another strategy to recapitulate some immune responses and is typically used to study cell therapies *in vivo* (e.g. tumor-specific T cells or CAR T cells) [18]–[20]. Alternatively, injecting a monoclonal fluorescent antibody (Ab) targeting a specific cell marker can stain a population of interest within minutes *in vivo*, allowing to quickly evaluate its behavior [14],[21]. However, potential caveats of this method are limited diffusion of Abs in some organs/tissues and possible artifacts generated by unspecific Ab bindings or Fc receptors engagement. The use of fluorescent Fab fragments can somehow limit some of these issues [22]. Additionally, important features of tissue architecture can be visualized through imaging of collagen fibers by second harmonic generation or by staining vasculature through intravenous (i.v.) injection of dyes or antibodies [23]. In particular, visualizing blood vessels can provide information on the ins and outs of trafficking cells. One major advantage of IVM is the possibility to image the exact same location within a tissue before and immediately after the injection of an immunomodulator allowing the early timepoints to be characterized (**Figure 1**). Altogether, IVM is a powerful technique to

track multiple cell populations and cell functions in real time before (at steady state) during and after immunomodulation. Such approach should help unravel new mechanisms of action and suggest means to optimize therapeutic efficacy.

Removal of pathological cells using therapeutic antibodies

Using monoclonal antibodies (mAb) to induce the elimination of malignant or autoreactive cells has proven to be a very effective strategy for patients with cancer or autoimmune disorders [24]. A classical example is the clinical use of anti-CD20 mAbs (rituximab, obinutuzumab) for treating B cell malignancies (lymphomas, leukemia) or removing self-reactive B cells (for example in the context of multiple sclerosis or rheumatoid arthritis) [24],[25]. Such depleting antibodies are designed to trigger cell elimination by either activating the complement pathway[26],[27] (**figure 2**) or triggering antibody-dependent cell cytotoxicity (ADCC) or antibody-dependent cell phagocytosis (ADCP) [24],[28]. ADCC and ADCP occur following antibody Fc-recognition by effector cells such as NK, macrophages or neutrophils. *In vitro* studies have shown that certain anti-CD20mAb (e.g. obinutuzumab) may be capable of inducing direct cell death, a mechanism known as antibody dependent cell death (ADCD) [29],[30]. Yet, the respective contribution of these mode of action (MOA) in distinct anatomical sites remains to be ascertained as they are likely influenced by the type, numbers and phenotype of available immune effectors. Using IVM, depletion of circulating healthy and malignant B cells was visualized in the liver within minutes of anti-CD20 mAb injection as Kupffer cell arrested and engulfed targets through ADCP [31]. It was highly efficient as nearly 60% of the circulating B cell compartment was depleted within 2 hours. Kupffer-cell mediated ADCP was also reported in a melanoma and colon tumor mouse model using TA99 (anti-gp75 mAb) or Cetuximab (anti-hEGFR mAb) therapies respectively [32]. These additional models suggest that Kupffer-mediated ADCP might be a common MOA for mAbs depleting circulating cell targets. To boost effector functions and thus therapeutic efficacy, glycomodifications can be applied to the Fc-portion of the mAb [33]. IVM of two glycoengineered mouse and human anti-CD20 mAbs revealed their superior abilities to trigger Kupffer cell-mediated ADCP compared to their wild-type counterpart [34]. Additionally, Fc modifications may also favor alternative MOA *in vivo*. By performing IVM of the liver after injection of a glycosylated anti-CXCR3 mAb, Kupffer cells were seen inducing liver iNKT cell apoptosis following nibbling of large cellular fragments, a phenomenon abrogated in Fc-deficient mice or when using a non-glycosylated anti-CXCR3 mAb [35]. Beyond the elimination of circulating targets, ADCP has also been identified in the bone marrow of two independent mouse tumor models using anti-CD20 or anti-CD52 mAbs. In this instance, ADCP was largely limited in time and space (**Figure 2**) with only a small fraction of tumor B cells being eliminated after antibody

injection [14],[36]. This bottleneck was attributed to the largely sessile phenotype of bone-marrow associated macrophages and their reduced presence in tumor-bearing animals [14],[36]. Importantly, this limitation could be circumvented by chemotherapy that triggered macrophage recruitment and enhanced ADCP following anti-CD52 mAb therapy [36]. Macrophages seemed to also undergo a phase of hyporesponsiveness as phagocytosis events were no longer detected a few hours following antibody injection despite high levels of circulating antibody [14]. This additional limitation may be the consequence of FcRs downregulation following target engulfment [37]. Finally, therapeutic activity in tumor-bearing mice was also limited at later time points possibly due to antibody consumption by tumor cells [14].

Altogether, mAbs designed to induce Fc-dependent cell elimination may face different bottlenecks depending on anatomical features, effector density and responsiveness, antibody diffusion and consumption. By documenting limitations in antibody-mediated mechanisms, IVM can guide the rational design of future therapeutical strategies and their optimal use in clinical settings.

Elimination of suppressive and deleterious immune cells

Targeting suppressive immune cells such as regulatory T cells (Tregs), neutrophils or immune-suppressive macrophages is an attractive strategy to boost immune reactions typically in the context of established tumors. Several studies have visualized the impact of Treg depletion in various mouse models and have identified distinct mechanisms of downregulation of immune responses *in vivo*. In this respect, IVM of lymph nodes (LNs) showed that Tregs could directly interact with DCs or with DC:T cell clusters in a cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-dependent manner to dampen T cell priming [38],[39]. Similarly, the elimination of Tregs boosted T cell priming by increasing contact duration between DC and T cells [40],[41]. Imaging showed that Tregs could also physically impede cytotoxic T lymphocytes (CTLs) infiltration in the tumor by forming a ring-like structure around malignant B16 melanoma cells [42]. As a consequence, Tregs elimination with chemotherapy, improved CTLs infiltration and increased interactions with tumor cells [42].

Macrophages are also endowed with immunomodulatory functions and represents an interesting cell population to target and tune immune responses. IVM has helped assess their roles and the consequences of their elimination. For example, IVM of a mammary mouse tumor showed that macrophages could directly interact with CTLs thus mechanically restraining them from infiltrating the tumor mass [43]. As a result, depletion of macrophage following pexidartinib, a colony stimulating factor 1 receptor (CSF1R) inhibitor, improved CTLs number and infiltration [43].

Neutrophils are rapidly recruited to tissues and represent another first-line cell candidate to target to modulate immune responses. For example, in a mouse model of leishmania infection, neutrophils were visualized engulfing *Leishmania major* (L.m.) parasites, which remained viable and

which in turn promoted infection. In this instance, neutrophils depletion slowed down Leishmania infection [44]. An extensive review on the importance of IVM in dissecting how neutrophils contribute to modulate immune responses has been recently published elsewhere [45].

Altogether, direct visualization of these cell subsets *in vivo* has helped understand how they exert their immunomodulatory functions providing additional clues into their therapeutic activities.

Modulation of T cell activity with immune checkpoint inhibitors

Sustained stimulation of T cells during viral infection or cancer often results in immune exhaustion [46]. This phenomenon is classically characterized by the loss of functional capacities and the upregulation of negative regulatory molecules such as Programmed cell death protein 1 (PD-1) or CTLA-4. Immune checkpoints blockade (ICB) used to treat cancer patients include monoclonal antibodies that target PD-1 (nivolumab, pembrolizumab, cemiplimab), PD-L1 (atezolizumab, avelumab, durvalumab) or CTLA-4 (Ipilimumab) molecules with the strategy to unleash T cell cytotoxic functions. [46]. Yet, what factors contribute to their efficacy and how they finely modulate the dynamics of immune responses *in vivo* remains unclear.

Several imaging studies have highlighted their possible role in modulating T cell motility. In an infectious model of lymphocytic choriomeningitis virus (LCMV), virus-specific T cells that had arrested in the splenic red pulp after chronic exposure to viral antigen, regained motility upon PD-1 and PD-L1 treatment [47]. This phenotype correlated with increased T cell function and IFN- γ production [47]. Similarly, in B16 melanoma and 4T1 breast tumor models, intratumoral tumor-specific T cells increased their velocity upon anti-CTLA-4 mAb injection [48],[49]. In other contexts, ICB appeared to stabilize interactions between T cells and their targets. Indeed, in a mouse model of allogeneic hematopoietic stem cell transplantation, anti-PD-1 mAb treatment favored the graft versus leukemia effect in lymph nodes by promoting stable interactions between T cells and their targets [50]. These effects are consistent with the idea that PD-1-PD-L1 interactions inhibit TCR induced stop signal [51]. These different studies suggest that ICB can have multiple impact on T cell dynamics highlighting the need to dissect their MOA in specific settings. The impact of anti-PD1 has also been visualized by IVM in a colon carcinoma model using cytokine reporter mice [52]. These imaging experiments revealed the induction of IFN- γ and IL-12 at the tumor site reflecting the importance of cellular crosstalks between tumor infiltrating lymphocytes (TILs) and DCs essential for therapeutic activity [52]. Finally, IVM can help pinpoint *in vivo* bottlenecks of anti-PD-1 therapy. For example, Fc-mediated uptake of anti-PD-1 mAb by neighboring tumor-associated macrophages was found to limit the effect of antibody therapy on TILs in mice bearing MC38 tumors [52]. Notably, blocking FcRIIB improved anti-tumor T cell responses. Importantly, removing glycans present on the anti-PD1 mAb Fc portion also improved antibody activity

[53]. Altogether, these imaging observations may contribute to the rational design of future therapeutics. New checkpoint inhibitors targeting other inhibitory proteins (e.g. Tim 3) and/or other immune cells have gradually emerged [54] and should benefit from future IVM studies to detail their MOA *in vivo*. For instance, anti-CD47mAbs which prevent interactions between SIRP α -expressing macrophages and CD47-expressing tumor cell are thought to unleash the break on phagocytosis but characterizing the precise *in vivo* impact of this strategy remains to be performed [55]–[57].

Immunomodulation by bispecific antibodies

Bispecific antibodies have been recently developed with the strategy to bring effectors and target cells into contact to promote cell elimination. In particular, BiTEs (bispecific T cell engager) consist of two linked single-chain variable fragments (scFvs), recognizing a T cell-specific CD3 subunit at one end and a tumor-associated antigen (TAA) at the other end. Blinatumomab, a bispecific T cell engager targeting CD3 and CD19 is now used for the treatment of B cell acute lymphoblastic leukemia and several clinical trials are ongoing to broaden up the use of bispecific antibodies to the treatment of solid tumors [58]. In a humanized mouse model of colon carcinoma, IVM showed that a bispecific antibody targeting the CEA antigen and the CD3 protein increased the effector to target ratio by crosslinking multiple T cells to a single tumor target [59]. Similarly, an anti CD3xCD20 T cell bispecific antibody increased stable synapses between human T cell and tumor cells [60]. In the PyMT mouse model of breast cancer, tracking of a fluorescent BiTE directed against epidermal growth factor receptor (EGFR)vIII revealed that BiTE anti-tumor activity was highly dependent on antibody dose and TAA expression level. In addition, CD103⁺DCs appeared necessary to maximize therapy efficacy by enhancing CTL expansion [61]. Thus far, only few studies have undertaken real time visualization of bispecific antibodies *in vivo* and future work will undoubtedly bring important insights on their MOA and possible limitations.

Immunomodulation by adoptive cell therapies

Adoptive cell therapy (ACT) represents an extreme form of immunomodulation whereby the density of effector cells is artificially increased for therapeutic purposes. T cell-based ACT includes transfer of TILs and T cells with engineered TCRs or CARs. IVM in combination with genetically-encoded fluorescent sensors to detect signaling and cell death have been particularly useful to quantify the efficiency of T cell-mediated killing in different settings.

In a model of solid tumor, CTL killing was relatively inefficient with an average of one target eliminated every 6 hr [15]. Reflecting this observation, tumor regression required a high density of intratumoral effector T cells [15]. By contrast, CAR T cells appeared more rapid at killing lymphoma B cells in the bone marrow, a process that took on average only 25 minutes [19],[18]. Yet, CAR T cells were heterogenous

in their capacity to induce cell death in part due to the differential capacity to recognize their targets as recorded by calcium (Ca^{2+}) signaling . In melanoma-bearing mice, CTL interactions with tumor cells could be prolonged when combined with an anti-CD137mAb resulting in a more efficient induction of tumor cell apoptosis [62].

Related to the efficiency of CTL killing is the capacity of tumor cells to resist CTL attack. *In vitro* studies had initially provided evidence that tumor cells were capable of avoiding CTL-mediated killing by rapidly repairing membrane damages [63]. Such sublethal hits result in a transient increase of intracellular Ca^{2+} due to pore formation and a rapid return to basal levels after repair. The use of tumor cells expressing a Ca^{2+} reporter confirmed that most CTL hits delivered *in vivo* were indeed sublethal [16]. Of note, sublethal hits by multiple T cells can have additive effects, favoring target cell apoptosis [64],[65]. Furthermore, CTL activity can be largely modulated by the environment, as shown in a mouse model of graft versus leukemia effect, where CTL interaction with targets was specifically inhibited in lymph nodes but not in the liver due to differential PD-L1/PD-L2 expression [50]. Beyond enhancing the rate of target cell killing by effectors, ACT can profoundly modulate the host immune response. For instance, IFN γ production by CAR T cells can drive the recruitment and activation of endogenous T and NK cells and sustain CAR T cells cytotoxicity [18]. Mechanisms of failure of ACT include the emergence of antigen-negative tumor variants. In this respect, dual expression of anti-CD19 and anti-CD123 CARs limited tumor relapse in a xenograft B-ALL tumor model and IVM revealed that CAR T cells could still eliminate CD19 negative tumor cells through CD123 recognition [66]. Another potential limitation of CAR T cells is the severe adverse events experienced by some patients including cytokine release syndrome and neurotoxicity [67]–[69]. Yet, mechanisms leading to neuronal dysfunction remain unclear. IVM was used to visualize brain capillaries and established that mice treated with CAR T cell therapy had disrupted blood flow in the brain due to plugged capillaries [70]. Mechanical obstruction of brain capillaries was associated with abnormal behavior providing a possible mechanistic basis for neurotoxicity [70].

While T cells have been the focus of most ACT, other immune cells may represent an interesting alternative. Intra-lymphatic adoptive transfer of CCR7-transduced regulatory macrophages (CCR7⁺Mregs) could successfully migrate to the T cell zone of the lymph node where they reduced the time of interaction between antigen-specific T cells and DCs and attenuated delayed-type hypersensitization responses [71]. Other immune cells such as $\gamma\delta$ T cells, NK cells and macrophages are attractive candidates for CAR-based strategies and *in vivo* imaging approaches are expected to provide valuable information into their respective activities.

Immunomodulation by cytokines

Interfering with cytokine signaling represents an important immunomodulation strategy. However, IVM analysis of such immunomodulatory approaches remains challenging as altering cytokine networks may

not have an immediate impact on immune cell dynamics or may have visible consequences only after a long period of time. Nevertheless, injection of chemokines can have rapid effects on immune cell behaviors as noted with injection of CCR7 ligands that promoted intranodal T cell motility [72]. In a few instances, cytokines were seen rapidly altering cell behaviors. For example, liver-associated NKT cells arrested in liver sinusoids upon injection of IL12 and IL18 [73].

However, it is possible to dissect the effects of cytokine signaling by IVM more readily using appropriate fluorescent biosensors. For example, the use of an IFN- γ -responsive reporter or that of a STAT1-GFP probe have revealed the long-range diffusion of IFN- γ within the tumor microenvironment [74],[75]. Thus, longitudinal IVM through imaging windows combined with functional reporters may provide opportunities to dissect cytokine-based immunomodulation.

Optogenetics for immunomodulation with spatiotemporal control

Strategies to modulate immune responses using magnetic field, temperature or light have recently emerged. In particular, optogenetics use light sensitive protein domains to manipulate cell functions with exquisite spatiotemporal control (**figure 3**) [76]. The use of optogenetics in the context of IVM may prove to be particularly useful for fundamental analysis of immunomodulatory pathways and may even find clinical applications [77]. Optogenetic control of calcium signals has been achieved *in vivo* and shown to promote T cell arrest in LN [78]. In this respect, manipulation of Ca²⁺ signals with light in either DCs or T cells has been exploited to increase anti-tumor CTL responses [79],[80]. Optogenetics also provide opportunities to better understand and control immune cell migration. For example, a strategy to control protein secretion with light have highlighted how neutrophils respond to chemokine-producing cells *in vivo* [81]. Furthermore, local photoactivation of CTLs expressing a light-sensitive CXCR4 promoted their migration *in vitro* and could be used to direct them towards B16 melanoma tumors *in vivo* [82]. The capacity to elicit a specific cellular function *in vivo* and visualize its outcome at the single cell level represents a unique opportunity to dissect complex immune networks.

Limitations and future improvement

With few exceptions, *in vivo* imaging has been largely limited to rodent models and the mechanisms of action of certain therapies may differ in humans. Indeed, some anatomical or gene expression differences persist between mice and humans, with rodent models also lacking genetic diversity. One way to partially circumvent some of these limitations may lie in the combination of IVM with humanized mouse models, although this is often at great cost. Moreover, the rather clean environment of mice husbandry is unlikely to reflect the constant exposure of humans to infectious threats and is likely impacting the immune responses to immunomodulators [83]. Finally, experimental procedures associated with IVM are not devoid of limitations. For example, access to certain organs such as the

lungs or the heart can be challenging and requires extensive technical expertise [84]–[86]. In addition, access to a variety of fluorescent reporter mice is often needed to identify the cellular players implicated in immunomodulation. Nevertheless, the constant development of fluorescent biosensors, optogenetic actuators and animal/diseases models (zebrafish, mouse, humanized mouse) should fuel the use of IVM for understanding the ever-growing panel of immunomodulatory treatments.

General conclusion

With the development of multiple approaches to modulate immune responses to treat a variety of diseases, it remains important to clarify the mode of action of these therapies with single cell resolution. IVM offers such possibility and has proven to be a key approach to unravel new mechanisms of immunomodulation. In particular, comparing the behavior of immune cells in the same location before or after immune intervention can be particularly instructive for therapies with rapid effects.

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Data availability statement:

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Conflict of interest:

The authors declare no commercial or financial conflict of interest.

Figure legends

Figure 1. IVM offers a unique opportunity to visualize the immediate effects of immunomodulators in real time

(A) Intravital microscopy (IVM) allows the exact same location to be imaged before and immediately after the injection of an immunomodulatory drug. This is particularly useful to characterize the early effects of an immune intervention on cell behaviors. DC is for dendritic cells. (B) An example of images obtained by IVM of the bone marrow of a mouse with an established B cell tumor, before and the first few minutes following injection of an anti-CD20mAb. Macrophages, stained with an anti-F4/80 antibody

are shown in green. Live tumor cells appear magenta before turning blue as a result of phagocytosis by macrophages. White arrowheads highlight tumor cells being engulfed and turning blue.

Figure 2. Insights into the mode of action of depleting antibodies using intravital imaging

(A) Multiple modes of actions have been reported *in vitro* for eliminating cells following antibody treatment and IVM can be used to dissect their relative contribution *in vivo* (B) As an example, the mode of action of anti-CD20mAb has been characterized in different anatomical sites and some limitations have been identified thanks to the use of IVM. In particular, tumor B cell elimination by anti-CD20mAb is restricted in time and space in the bone marrow. ADCP, antibody-dependent cell phagocytosis, ADCC antibody-dependent cell cytotoxicity, ADCD antibody-dependent cell death

Figure 3. Optogenetic control of cellular functions to study immunomodulation through IVM

The use of optogenetics in the context of IVM can refine our fundamental understanding of several immunomodulatory pathways. For example, optogenetic control can activate chemokine receptor signaling to promote cell adhesion to blood vessels (top panel), or trigger cytokine/chemokine secretion in turn favoring cell activation and recruitment (middle panel), or stimulate calcium signalling to promote cell arrest and improved cell cytotoxicity (bottom panel).

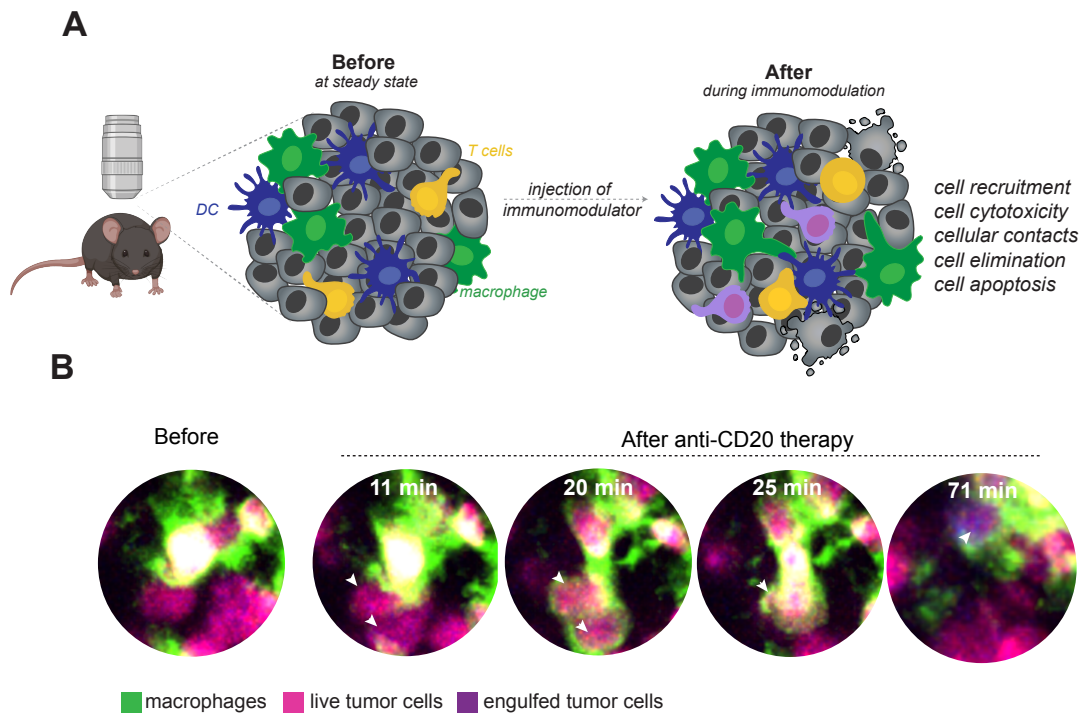


Figure 1

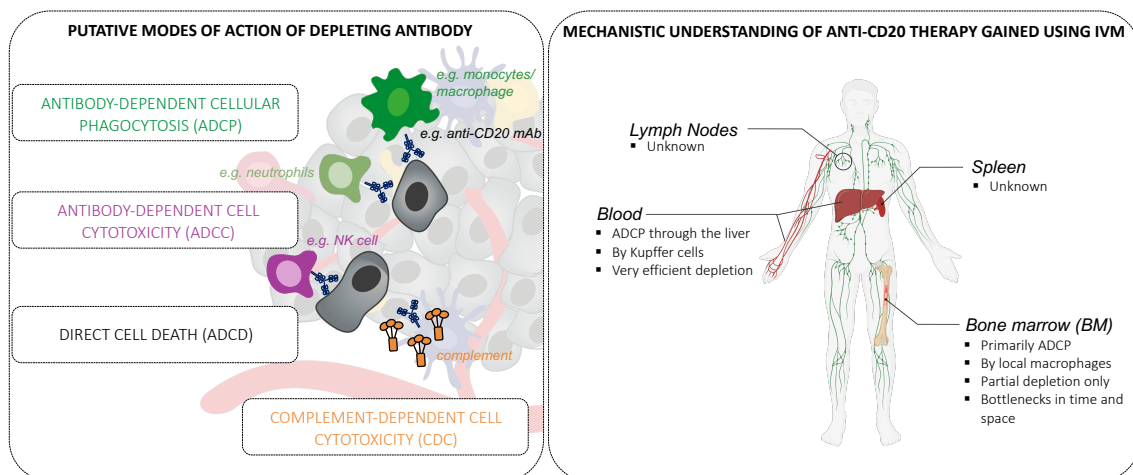


Figure 2

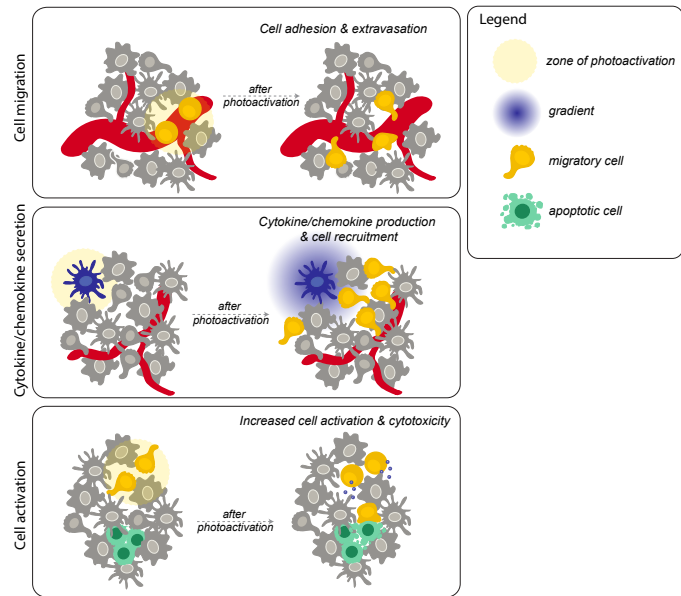


Figure 3

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